R.C. Sobti
Aastha Sobti *Editors*

Biomedical Translational Research

Technologies for Improving Healthcare

Springer

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We respectfully dedicate the book to
Late Mr S D Sobti and Late Mrs Sheela Sobti
for their blessings, even now from heaven
Parents of R C Sobti
Grand Parents of Aastha Sobti

Preface

Basic biomedical research aims to provide a comprehensive and detailed understanding of the mechanisms that underlie the development and normal functions of humans and other living organisms. Therefore, those insights into the pathological and pathophysiological mechanisms that cause disease are understood. There have been remarkable conceptual and technical advances in biological and biomedical sciences in the last few years and are continuing rapidly. The genome project and developments provide new language to understand the occurrence, mechanism, and prevention of disease. For this reason, organismal physiology has been a most significant challenge ahead in basic and clinical research. With new technologies, molecular mechanisms of many acquired and inheritable diseases are elucidated. Attempts are on to understand the integrated function of organs and organisms by using the combination of molecular, computational structural biology, and imaging technology.

Mysteries of the brain are being unravelled for the study of cells, organs, and patients. Though there has been an explosion of information in all these areas, it is difficult to collate all that for practical uses. There is, thus, a wide gap in knowledge and its applications. To mitigate the challenges faced by humans, this gap must be bridged. There is a dire need to have an effective dialogue between physicians and scientists. It will help to understand clinical medicine in a much practical way. The interaction of astute clinicians with patients may stimulate clinical investigations that may suggest novel mechanisms of disease. There is, in fact, a bidirectional flow of information from patients to the laboratory and back. It helps to accelerate understanding of human diseases, to develop new strategies for their prevention, diagnosis, and treatment. The route may pass through various experimentation and validation stages in lower and higher animal species and now on chips, cell-free systems, and bionomics. There can be no doubt that the frequency and intensity of interactions have tremendously increased now. The primary and clinical workforces linked by biomedical scientists are presently termed "translational" researchers. They are trained to be knowledgeable in the primary and clinical biomedical sciences and proficient in patient care.

The volume "Translational Biomedical Sciences" is a platform for clinical researchers, basic scientists, biomedical engineers, and computational biologists from various countries to express their experiences and futuristic thoughts in the form of chapters.

"Translational Biomedical Sciences" has been compiled in three volumes, i.e., it summarises emerging technologies for healthcare. **Volume II: From Disease Diagnosis to Treatment** discusses various aspects of biomedical research towards understanding the diseases' pathophysiology and improvement in diagnostic procedures and therapeutic tools. **Volume III: Drug Design and Discovery** focuses on biomedical research's fundamental role in developing new medicinal products.

This **Volume I** articulates the innovations and new technologies in biomedical sciences emphasising on genomic analysis, immunology, stem cell, tissue engineering, nanotechnology, computation and structural biology, and biomedical engineering. This book is a useful information source for clinical researchers, basic scientists, biomedical engineers, and computational biologists.

The editors are thankful to their family members Vipin, Aditi, Vineet, Aastha, Ankit, and Ira. We compliment the authors who have contributed scholarly chapters to this book. Special accolades to Dr. Sanjeev Puri for his help in preparing this volume.

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Chandigarh, India — R. C. Sobti
Lund, Sweden — Aastha Sobti

Contents

1 **Introduction to Emerging Technologies in Biomedical Sciences** 1
R. C. Sobti, Jagdish Rai, and Anand Prakash

2 **Bioprospecting: Boon or Curse** 23
Yusuf Akhter

3 **Growing Need for Interdisciplinary Biomedical Research** 33
Eshu Singhal Sinha, Prakash Kumar Sinha, and R. C. Sobti

4 **Interplay Between Theory and Experiment: A Future Approach for Biomedical Research** 41
Rolly Yadav, Anamika Shukla, and Devesh Kumar

5 **Past, Present, and Future Perspective of Biomedical Innovation in India** 69
Joseph L. Mathew and Thalakkotur Lazar Mathew

6 **Systems Biology Approaches to Study Disease Comorbidities** 97
Tammanna R. Sahrawat

7 **Bioinformatics: The Interactome of Multidisciplinary Approaches** 107
Veena Puri, Sanjeev Puri, and R. C. Sobti

8 **Role of Artificial Intelligence in Diagnosis of Infectious Diseases** . . . 115
Vandana Kaushal and Rama Gupta

9 **DNA Digital Data Storage: Breakthroughs in Biomedical Research** 135
Baljinder Singh

10 **Biomedical Applications Cloud-Based IoT** 141
Masood Ahmad, Rajeev Kumar, Alka Agrawal, and Raees Ahmad Khan

11 **Next-Generation Sequencing in Cancer Research** 155
Pushpinder Kaur

12 CRISPR: Genome Editing and Beyond 167
Aiana and Kashmir Singh

13 Omics in Tuberculosis Diagnosis: Today and Tomorrow 181
Indu Verma and Khushpreet Kaur

14 Advances in Proteomics Approaches and Chronic Kidney Disease 201
Rajpal Srivastav and Chanderdeep Tandon

15 3D Bioprinting of Tissues and Organs: A New Paradigm in Regenerative Medicine and Biomedical Engineering 215
Kanwaljit Chopra, Sandip V. Pawar, Manju Maurya, Tavish Gupta, and Jatinder Dhaliwal

16 Virtual Planning, Rapid Prototyping and 3D Printing in Orthopedic Surgery 239
Mandeep S. Dhillon, Siddhartha Sharma, and Prasoon Kumar

17 Tissue Chips: Contemporary Applications and Advancements 253
Taranjot Kaur, Jigyasa Sharma, and Seemha Rai

18 Flow Cytometry for Measuring Neutrophil Function 275
Paul Hutchinson

19 Flow Cytometer as Tool to Understand the Variation in Genome Size Among Medicinal Herbs and Nonedible Oil Yielding Trees of Northeast India 285
Supriyo Basak and Latha Rangan

20 Raman Spectroscopy Applications for Bacterial Detection: Potential and Challenges Towards Development of Field Deployable System 305
S. Sil, R. Mukherjee, N. S. Kumar, and S. Umapathy

21 Potential of Magnetic Resonance (MR) Methods in Clinical Cancer Research 339
Naranamangalam R. Jagannathan

22 Microfluidic Point-of-Care Diagnostic Devices: A Contemporary Relevance 361
Shristi Handa and Anupreet Kaur

23 Fluorescent Quantum Dots (QDs): A Theranostic Tool for Breast Cancer Management 371
Joga Singh, Jeslin Johnson, Gurpal Singh, Alka Bhatia, Ravi Pratap Barnwal, and Indu Pal Kaur

24 Molecular Mimicry: Unravelling the Role of Autoantibodies in Autoimmune Diseases and Infertility 395
Deepali Thaper and Vijay Prabha

25 Strategies of Vaccine Development 407
Meenakshi Sachdeva and Sunil K. Arora

26 Preclinical Models of Intimal Hyperplasia and Restenosis to Predict Clinical Events and Develop Novel Therapies 427
Finosh G. Thankam, Victoria E. D. Wilson, and Devendra K. Agrawal

27 Sphingolipids: A Roadmap from Biomarker Identification to Clinical Application 447
Priyanka Bhadwal and Navneet Agnihotri

28 Future of Gastrointestinal Endoscopy 473
Surinder Singh Rana

29 Beating Heart in a Box: The Future of Cardiac Transplant 483
Vidur Bansal, Anand Kumar Mishra, and Shyam Kumar Singh Thingnam

30 The Journey of Coronary Stent Technology 489
Manoj Kumar Rohit and Atit A Gawalkar

31 Materiovigilance in Clinical Surgery 497
Ram Samujh and Nitin J. Peters

About the Editors

R. C. Sobti is an Emeritus Professor and Indian National Science Academy (INSA) senior scientist at Punjab University. He is a former Vice Chancellor of the Panjab University, Chandigarh, and Babasaheb Bhimrao Ambedkar University (Central University), Lucknow. He started his career as a cytogeneticist and then moved on to molecular biology, including genomics, to understand the susceptibility and disease process of cancer, COPD, AIDS metabolic syndrome, and kidney diseases. He has also used stem cells and nanoparticles to understand the process of tissue organ development through a designed de-cellularisation protocol. He has published more than 300 research articles in the journals of international repute and has also published more than 40 books.

He is a Fellow of the Third World Academy of Sciences, National Academy of Sciences India, Indian National Science Academy, National Academy of Medical Sciences, National Academy of Agricultural Sciences, Canadian Academy of Cardiovascular Diseases, and few others. He was the General President of the Indian Science Congress for the 102nd session held at the University of Jammu in 2013. Dr. Sobti is the recipient of many prestigious awards like the INSA Young Scientist Medal, UGC Career Award, Punjab Rattan Award, JC Bose Oration and Sriram Oration Awards and the Life Time Achievement Awards of the Punjab Academy of Sciences, Zoological Society of India, and the Environment Academy of India.

Aastha Sobti BDS (PU), Master of Clinical Dentistry Oro and oro-maxillofacial surgery (UK), is working in the field of head and neck cancers using oral and maxillofacial surgery background by delving her intellect to it, in entirety aiding in the field to bring reforms that are required in the present multifarious surgical as well as research areas.

In her teaching and research career of 7 years she has contributed to the research field by publishing papers in reputed journals. She has been awarded a number of prizes and medals for her exceptional work. She has attended and presented papers in international conferences in Croatia, Brazil, Canada, USA, Japan, Switzerland and various other countries.

Introduction to Emerging Technologies in Biomedical Sciences

1

R. C. Sobti, Jagdish Rai, and Anand Prakash

Abstract

The emergence of newer technologies has revolutionized the biomedical sciences, which are in fact in the interface of various branches of science and engineering to find out ways to meet the challenges faced by humanity. It is now possible to make precise diagnosis and even detect the stage of diseases by using molecular biomarkers from noninvasive liquid biopsies. This is also facilitated by the use of **microRNA (miRNA) profiling**, the throughput novel sequencing and the **machine-learning approaches**, which help in determining marker DNA mutations. Certain nanoparticles such as the analysis of tissues by biophotonics and Raman spectroscopy augment the precision in disease diagnosis.

The synthesis of precise and stage specific structure-based and targeted drugs is possible by using in vivo and in vitro models coupled with molecular biology techniques and pharmacogenomics. This has further been facilitated by computational models (bioinformatic tools) for omics (genomic, transcriptomics, proteomics, and metabolomic) approaches. Accurate and better drug targets are provided by small interfering RNAs (SiRNAs), which are capable of sequence specific gene silencing.

It is now possible to overcome the treatment resistance by epigenetic and immune cell mechanisms and targeted therapy. The effectiveness of the drug can be detected by using organoid models (3D) of diseased (e.g., cancer) cell cultures from biopsies of primary patients. Genome editing such as CRISPR/Cas systems

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and RNA interference (RNAi) are playing an important role in the treatment of diseases by editing and regulating genome. Drug binding mechanisms and kinetics are understood by machine-learning algorithms. The absorption, distribution, metabolism, excretion, and toxicity as well as failure of drugs can be tracked through high-throughput screening technologies assisted computational methods and artificial intelligence. For this, in vivo models are also used. Drugs before administering to patients are tested in vitro in cell lines and patient-derived xenografts (PDXs). The immune-competent, cell line-derived xenografts (CDXs), PDXs, and genetically engineered mice (GEM) provide models for determining the effect of chemotherapeutic drugs, inhibitors, and immunotherapeutic agents. The impact of physiological variables such as oxygen, pH, and temperature that affect drug response can be determined by 3-D cell line bioreactors. The inhibitors of DNA methyltransferases (DNMT), histone deacetylases (HDACs), and anti-miRNAs can be used to target epigenetic mechanisms in different types of diseases. The epigenetic mechanisms can be modulated by certain dietary photochemicals. Nucleic acid-based therapy depends on the noncoding RNAs (ncRNAs). The immune therapy approaches use immune checkpoint inhibitors, T-cell, nonspecific immune therapies and vaccines against different diseases. G protein-coupled receptor targeting is an efficient approach to treat certain diseases. In the case of cancers, the immunopeptidomic approaches involving tumor-associated neo antigens are used for the development of anticancer vaccines and T-cell-based therapies. Emphasis on nutrition traffic to regulate the microenvironment and also differentiation and activation of immune cells is another approach in the management of diseases.

It is now evident that during the last couple of years, the biomedical sciences including engineering have tremendously increased the precision in disease detection for appropriate treatment and management.

Keywords

Advancements in biotechnology · Clinical trials and personalized treatment · Exosome-based immunotherapy · CRISPR/CAS · Trans-Magnetic Stimulation (TMS) · Cyprogs · Next-generation sequencing (NGS)

1.1 Introduction

Right from the earlier times, biological sciences with the support of chemical, physical, and mathematics have been trying to unravel the mysteries of life. The earlier breakthrough discoveries in biological sciences were made by people trained in techniques of other disciplines. Many of the Nobel prizes in chemistry are awarded for discoveries related to biological systems. Physicists have developed techniques that advanced the experimental science of every discipline. Mathematics is considered the mother of science, and nowadays, computational science and

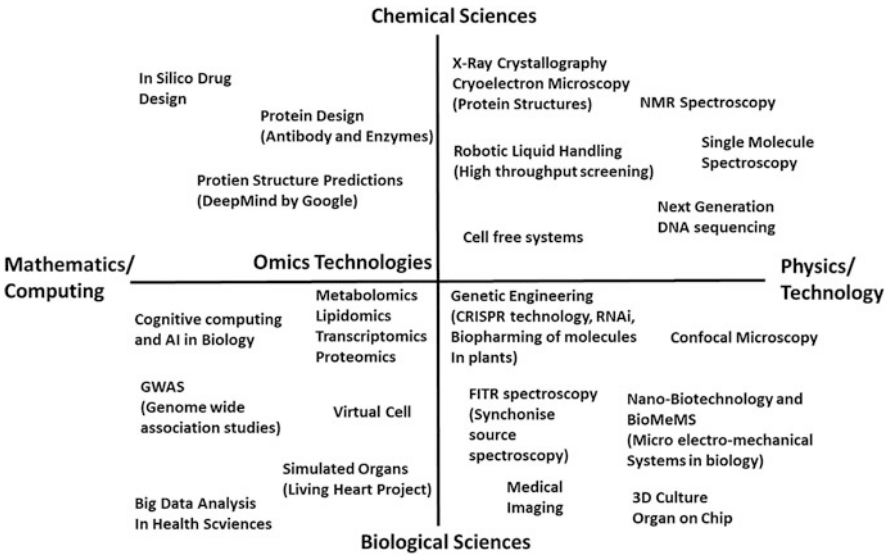

Fig. 1.1 Emerging techniques at the interface with other disciplines, for the study of biological systems at various levels of complexity

statistics are leading the measure developments in every sphere of science and business. The interrelations of various sciences leading to the emergence of new technologies have been depicted in Fig. 1.1.

The joining of engineering sciences to biological and medical sciences has brought biomedical sciences at revolutionary. Some of the emerging technologies in biomedical sciences have been tabulated below (Table 1.1).

1.2 Imaging Cells to Molecules in 3D

Visualizing structures is a solid proof by itself, as it is said: "Seeing is Believing." Microscopy was the primary technique in life sciences in its infancy and advanced microscopy or "imaging techniques" will remain a powerful tool to study biological systems. Today, fluorescence-based confocal microscopy is used to visualize the biological samples at molecular precision in 3D. It is possible to determine the location of molecules in subcellular organelles and detect other spatiotemporal changes in living systems. Electron microscopy enabled the visualization of biological structures at a resolution of 0.1 nm. Cryo-electron microscopy is emerging again as a promising tool for determining proteins' structure for which x-ray crystallography or NMR is not feasible (Zhang et al. 2012). Some proteins like membrane proteins are not easy to crystallize, and therefore, the structure determination through x-ray crystallography is not feasible. These membrane proteins are

Table 1.1 Emerging technologies in biomedical sciences

Technologies	Applications	Principle	Instrument infrastructure
Informatics and simulation	Data analysis, prediction of structure, function, and intervention design	Mathematics and statistics	Supercomputing facility, data
NGS	Genome sequence, gene expression	Optics, electronics, nanoscience	Optoelectronic fabrication
Genetic engineering (CRISPR, RNAi)	Crop improvement, therapeutics	Molecular biology and genetics	Reagents, oligonucleotide synthesis, and protein purification facility
Omics technologies transcriptomic, proteomics, metabolomics	System biology	Electrophoresis, separation of ions by their mass-to-charge ratio (m/z), oligonucleotide hybridization	Mass spectrometer robotic liquid handler
Spectroscopy	Molecular structure determination and chemical composition of mixtures or systems	Nuclear magnetic resonance, light diffraction, absorbance, optics	NMR spectrometer, cyclotron, synchrotron for a collimated light source
Imaging	Structure of molecules to cells and organs	Optics, electronics	Electron microscope, imaging facility
Bio-MEMS	Diagnosis, biology at micro and nanoscale details	Electronics, optics, fluidics, nanoscience	Cleanroom facilities and micro-/nano-fabrication facility for microfluidic systems with optical, electrical, and mechanical systems integrated

also not suitable for NMR-spectroscopy-based structure determination because they require high purity and protein concentration. The significant discovery of lipid bilayer structure of cell membrane was possible by cryo-electron microscopy.

Developments in low-cost and high-resolution medical imaging are emerging very fast. X-ray photography of bones, ultrasound imaging of internal organs, MRI of the brain, fluorescent tracer-dye-based angiography, etc., have enabled precise diagnosis of ailments. Combining artificial intelligence with very low-cost sensors like optical imaging and infrared-red imaging can also pick up signatures of diseases for accurate, but affordable diagnosis. FTIR-imaging can visualize the concentration of metabolites and macromolecules in cells of a tissue, using false-color rendering (Kumar et al. 2018). This technique is expected to be a cost-effective cytological diagnosis tool in the future. Foldscope developed by Manu Prakash at MIT is a low-cost ($1) cardboard-based educational microscope that may also find uses in point-of-care diagnosis (Cybulski et al. 2014).

1.3 Genetic Engineering with Precision

The discovery of RNAi (small RNA interference) by Andrew Fire and Craig C. Mello revolutionized reverse genetics studies, i.e., the effect of a gene on phenotype (Fire et al. 1998). Scientists can easily knock out a gene product from an organism using RNAi, whereas the earlier gene knockout methods were time-consuming and sometimes not feasible (Saurabh et al. 2014). As per central dogma, any gene transcribes as an mRNA, and the mRNA translates as a protein, which ultimately affects the organism's phenotype. RNAi's gene sequence to phenotype is interrupted by the degradation of specific mRNA, transcribed from the target gene. A piece of complementary small RNA hybridizes with the mRNA, and this unusual structure of double-stranded mRNA is recognized by protein machinery called RISC (RNA-induced silencing complex), which degrades it. Therefore, in principle, any gene can be silenced by adding a small RNA in the cell, which is complementary to the target gene.

Various techniques are used for changing the DNA of an organism starting from random mutagenesis to precise changes using CRISPR (clustered regularly interspaced short palindromic repeats) gene editing. In CRISPR gene editing, the nuclease cas9 is guided by a designed RNA sequence to cut the gene at the target complementary site and insert a new sequence (Cohen 2020). CRISPR (**c**lustered **r**egularly **i**nterspaced **s**hort **p**alindromic **r**epeats) is a modern, powerful genome-editing tool. The application of CRISPR/CAS was highlighted when a Chinese scientist used this technology that resulted in the birth of "CRISPR twins," although he was intensively criticized. CRISPR/CAS technique edge out other modern techniques like zinc-finger nucleases (ZFN) and transcription activator-like effector nucleases (TALEN) used in genome editing because CRISPR/CAS technique is more precise and efficient and it is incredibly customizable, which can edit several genes at once.

CRISPR is a short repetitive sequence present naturally in the prokaryotic cells where it plays a significant role in defense mechanism. The beauty of CRISPR relies on its simplicity. It is based on two components, i.e., a molecular scissor-Cas (**C**RISPR **as**sociated nuclease) that cuts the specific sequence, and the second one is the guide RNA (gRNA), which works as a GPS, driving the Cas to target a specific sequence. Although several Cas nucleases like Cas2, Cas5 are isolated from different bacterial strains, the most prominent is Cas9, isolated from *Streptococcus pyogenes* bacteria (Hsu et al. 2014). We know that if we talk about the gRNA, it is undoubtedly composed of two components of RNA: transactivating CRISPR RNA (tracrRNA) and CRISPR RNA (crRNA), where each component has its function. The crRNA is about 15–20 nucleotide base long customized sequence that exhibits the complementarity to the target DNA sequence while tracrRNA can work as a binding scaffold for the Cas nuclease. Cas is a nonspecific nuclease that can bind to any sequence other than the target sequence and might cleave it, so here, gRNA, as its name suggests, guides the Cas nuclease to target a specific DNA sequence inducing the double-strand nick. Here in CRISPR/Cas9 technique, two confusing names, gRNA and sgRNA, are alternatively used. sgRNA is the short abbreviation of **s**ingle

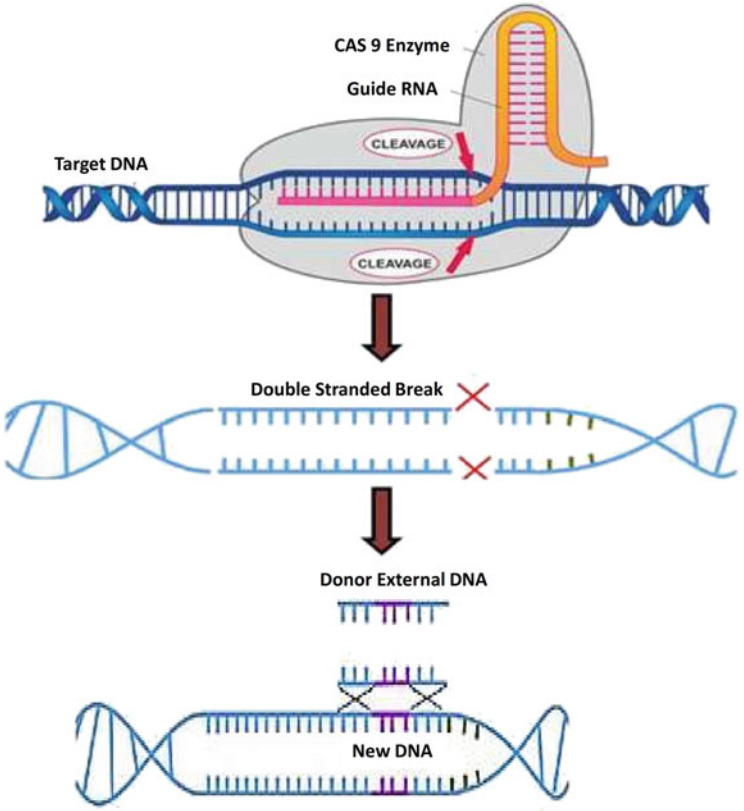

Fig. 1.2 Represents the sequential formation and function of the CRISPR/Cas9 system

guide RNA. The name of sgRNA is self-explanatory as it is a single RNA molecule exhibiting the properties of both the crRNA and tracrRNA.

Upon infection, the viral genome is inserted into the bacterial cell. Bacteria, on recognition of the viral genome, produce two short RNA sequences (gRNA: crRNA and tracrRNA) that form a complex with the Cas9 nuclease (CRISPR-associated protein 9) (Lee and Malykhina 2017). This complex is then directed to the viral genome, where Cas9 causes the double strand to break into viral DNA, thus rendering the virus disabled. Here a problem arises because if a similar target sequence is present in the bacterial DNA, there is always the possibility of destruction of bacterial DNA. To distinguish between self and foreign DNA molecules, there are PAM sequences on the viral genome. Cas9 has the property of binding only to those DNA molecules, which are immediately followed by the PAM (protospacer adjacent motif) (Fig. 1.2).

Enzymes of DNA recombination are also used to replace a target DNA piece in the genome. These precision genetic engineering techniques are precious for treatment of genetic diseases in humans (Cai et al. 2016). CRISPR technology can detect the DNA/RNA of a pathogen by binding to it through complementary guide RNA

and giving fluorescence signals while cleaving the target polynucleotide. It is also being explored to inactivate pathogens like COVID-19 by cleaving its genetic material (Konwarh 2020). CRISPR-cas9-based genome editing in stem cells opens up whole new possibilities of treatment and research models of diseases (Valenti et al. 2019).

Induced pluripotent stem (iPS) cells can be created from any differentiated cell by expressing only four factors Oct3/4, Sox2, c-Myc, and Klf4 (Takahashi and Yamanaka 2006). These combinations of techniques are being tried for genetic therapy of sickle cell anemia (Park and Bao 2021).

Genetic engineering techniques like site-directed mutagenesis are used to alter protein structure for a desirable property like thermostability. The genetic engineering techniques like gene domain shuffling, sequence homology-independent protein recombination (SHIPREC) method, and error-prone PCR together with rational design can yield novel protein with desired function (Lee 2005). The binding proteins called affibodies are designed on a small protein scaffold to replace antibodies in diagnosis kits. Other new chemical entities like oligonucleotides and heteropolymers are also used for molecular recognition by binding to target molecules for in vitro assays. These bioinspired synthetic molecules are used for detection and catalytic applications (Fodey et al. 2011). As the proteins due to their structural flexibility and functional-group diversity have very diverse types of functions, their engineering and in vitro evolution can materialize the concept of molecular machines for technological applications.

In a system biology approach, designed genetic networks and genes are constructed from component pieces available at a repository called iGEM (International Genetically Engineered Machine). This repository has DNA pieces available in a format that the components can be joined in any design because a convention is followed to provide restriction sites in these DNA elements. The repository has a wealthy catalog of promoters, reporters, regulatory elements, etc. These DNA components (also called BioBricks) are supplied to member labs at a nominal charge in the spirit of open science (Wang et al. 2021).

In an engineering approach to biological systems, the cell-free systems synthesize protein and metabolites using the necessary cellular machinery components rather than the live cell (Rollin et al. 2013). These necessary components for synthesis or other chemical reactions are extracted from cells using separation techniques such as ultracentrifugation. Using these cell-free systems, even non-coded (unnatural) amino acids can be incorporated into the proteins to expand the chemical repertoire of protein, which may require more stability or novel function in the industrial application of designed proteins (Noren et al. 1989).

1.4 Omics Technologies

Omics technologies like transcriptomics, proteomics, and metabolomics have, respectively, enabled the study of complete transcriptome, proteome, and genome of a cell or tissue. Advanced photolithography techniques have made it possible to

attach an oligonucleotide probe on a glass slide with a 1-μm precision. Therefore, millions of oligonucleotide probes can be packed on a glass slide, and all mRNA products from a tissue can be quantitatively detected at the same time, using only a small sample. In principle, this is a Southern blotting at a large scale miniaturized form. The mRNA is detected after converting it to DNA by reverse transcription and attaching a fluorophore. This technique has enabled a comprehensive study of the effect on transcription or gene expression in response to various drugs, other stimuli, or developmental plans. The embryonic development and cell differentiation are mainly due to spatiotemporal differences in gene expression caused by a signaling cascade.

Nevertheless, the difference in transcription is not the only difference between cells of different tissues. The translation process also has a regulatory role in gene expression through mRNA splicing, editing, and translational modification of proteins. Proteomics can comprehensively study the difference in the proteome of various tissues and disease conditions. The key technology in proteomics is a mass spectrometer that can determine the mass of many proteins simultaneously and with 1 Da precision. Traditionally, the proteins were identified by gel electrophoresis and western blotting, but various forms have not only decreased the amount of biological sample required but also decreased cost and time. Through mass spectroscopy, we can even detect post-translational modifications of a protein in a cell. Mass spectroscopy and various chromatographic separation techniques have also made it possible to study the complete metabolic, lipid, and carbohydrates profile, respectively, called as metabolomics, lipidomics, and glycomics. Integrating all this information of various molecules in cells through informatics has given rise to the discipline called system biology, which will comprehend the complexity of living systems and design intervention. The precision required in these experiments also mandates the robotic and microfluidic set ups.

1.5 Next-Generation Sequencing

A new era of advancement in biomedical sciences has evolved due to discovery of next-generation sequencing (NGS). Determining the nucleotide sequence (adenine, thymine, guanine, and cytosine) is the main objective of NGS. Earlier Sanger method and Maxam-Gilbert method were regularly employed in finding out the nucleotide base sequence. Both the methods require considerable time for determining sequence. Initially, the Sanger method was used to determine the whole human genome sequence started in 1990 and was completed in 2003; thus, it is about 13 years to complete and was very costly (3 billion USD). Thus, there was a great demand for newer technology to complete the sequence rapidly and at a reduced cost.

NGS (high-throughput sequencing) method can be used for sequencing DNA and RNA. This includes array-based sequencing, which uses Sanger's method that processes millions of reactions occurring in parallel. NGS involves the following three steps: library preparation, amplification, and sequencing. Initially, the DNA is

digested either enzymatically or mechanically (sonication) into small fragments followed by the attachment of short, double-stranded synthetic DNA called adaptor with an enzyme DNA Ligase. One end of the adaptor is cohesive, while the other is blunt or non-cohesive to provide an efficient ligation. However, the p wrong base pairing may cause problem. It is prevented by replacing 5′ end of the adapter by the 3′ end. It will not allow the formation of dimmers. It is followed by loading of libraries onto a flow cell and then application in the sequencer. The clusters of DNA fragments are amplified in a process called cluster generation, resulting in millions of copies of single-stranded DNA. In sequencing by synthesis (SBS), chemically modified nucleotides bind to the DNA template strand through natural complementarity. Each nucleotide contains a fluorescent tag and a reversible terminator that blocks the incorporation of the next base. The fluorescent signal indicates which nucleotide has been added, and the terminator is cleaved so the next base can bind. This type of method is called paired-end sequencing. After sequencing, the instrument software identifies nucleotides (a process called base calling).

NGS is an important technique in biomedical sciences.

Oncology The chief cause of cancer is mutagenesis and is caused by mutations occurring in somatic cells. The cancer genome is being studied by capillary-based cancer sequencing for more than a decade. The constraint was that it could be applied to only limited samples only. As per the new advents, the NGS has proved to be a promising technique in studying large-scale cancer genome projects worldwide, which comprises pediatric cancer genome projects. This technique provides precise diagnosis and classification of the disease; a more accurate prognosis may be used for potential identification of mutations induced by drugs. This type of sequencing provides a path for the personalized management of cancer. Many initiatives are taken to put NGS in the analysis of cancer genomes in clinical practice, mainly to identify mutations in tumors that can be treated by targeted by specific drugs.

Microbiology In microbiology, the NGS is used to characterize pathogens by their specific genomic sequence rather than morphology, staining molecules, and metabolic criteria with a genomic definition of pathogens. The study of genome defines essential information required by microbiologists like drug resistance and presents the relationship of different pathogens required to track the origin of infection outbreaks. Recently NGS is used to trace the outbreak of methicillin-resistant *Staphylococcus aureus* (MRSA) in a neonatal intensive care unit in the United Kingdom. Analysis by NGS of the pathogens shows the precise origin of MRSA and revealed a protracted outbreak due to a single hospital staff member. Thus, in microbiology also, this technique is proved to be highly beneficial.

Medical Treatment Mosaic mutations are acquired as a post-fertilization event, and accordingly, they demonstrate at a varying frequency within the cells and tissues of a single. Capillary sequencing may bypass these variants as they frequently occur with a refinement that falls below the technology's sensitivity. NGS sequencing provides a more sensitive read-out and can identify variants in just a few percent of

the cells, including mosaic variation. The sensitivity of NGS sequencing can be enhanced by increasing the depth of sequencing. Thus NGS can be utilized for interrogating DNA from maternal blood or chasing the levels of tumor cells from the circulation of cancer patients.

1.6 Spectroscopy of Single-Molecule and Mixtures

Spectroscopy is mainly about the interaction of electromagnetic waves with matter. It is used to identify the molecules, determine the structure, and study the mechanism of action. They are used to study a single molecule to determine the component in the mixture. UV-visible spectroscopy remains a routine life science technique to determine the concentration and purity of DNA and proteins. NMR spectroscopy is used to determine the structure of a protein in a solution. X-ray crystallography is more convenient than NMR to determine the protein structure, but the NMR structure is biologically more relevant because it is an aqueous solution in which the protein performs a function in the cell. NMR studies have given valuable insight into the mechanisms of enzymes and other proteins (Billeter et al. 2008). However, due to technical limitations, some proteins cannot be studied by both of these techniques; cryo-electron microscopy is also coming up as an alternative.

The invention of ZMW (zero-mode waveguide) has made it possible to take a single molecule spectrum even when the solution has a high concentration of target molecule-like protein. The detailed chemical mechanisms are studied using the technique called femtochemistry. ZMWs are extensively used in various NGS (next-generation DNA Sequencing) techniques. In NGS, a single DNA polymerase immobilized in a ZMW aperture is observed in action. The incoming nucleotides in a template or new strand are determined generally using fluorescence properties. In this way, the sequence of DNA being amplified is determined.

IR spectroscopy and its recent version, FT-IR, are used to identify and quantify the molecules in mixtures. Raman spectroscopy and FTIR are emerging beneficial techniques for diagnosis.

1.7 Informatics and Simulations

Every field of science and business has been transformed by increasing computing power and information technology. In biological sciences, it helps design intelligent experiments, narrowing down possibilities, and sometimes even deciphering information about processes that are not accessible through any other technique yet. For example, simulation of protein folding has given valuable insights into the mechanism of protein folding, and it can predict the structure of proteins. The experimental techniques are not adequate to probe the detailed protein folding mechanism, which is very fast (few microseconds to milliseconds). The folded structure of some proteins is also not feasible to determine experimentally, specifically for membrane proteins. Protein structure prediction has been one of the most critical scientific

challenges because the structure of a protein can open the path toward deciphering its function, mechanism, and designing drugs on its basis. In 2013, Martin Karplus, Michael Levitt, and Arieh Warshel were awarded Nobel prizes to recognize their contribution, which will solve the problem of protein folding and structure prediction someday (Levitt 2014). The best protein structure prediction methods are announced every 2 years through a competition called CASP (critical assessment of structure prediction). The artificial intelligence system called alpha fold, housed at Google Inc. subsidiary DeepMind, has performed the best in CASP14 held in 2020 (Alam and Shehu 2021).

Nevertheless, these knowledge-based methods performing the best in CASP competitions so far are only up to 90% accurate. These methods are based on the experimentally known structure of a similar sequence of proteins. The other approach is de novo structure prediction, which is based on interatomic forces that fold the protein. But given many atoms of the protein molecule itself and presence of the surrounding solvent, such calculations are too much for the presently available commuting power in most labs. The approximation of forces accounted for interatomic interactions is also not perfect. But in the near future, more computing power and better actuation of forces involved are expected to determine the structures of new protein sequences by de novo methods. Although the structure prediction of new natural protein sequences remains uncertain, state of the art has reasonably advanced to design proteins like therapeutic antibodies, enzymes, etc. Michael Levitt had developed an algorithm for humanizing antibodies that can transplant the binding site from animal antibodies to the scaffold of human antibodies (Queen et al. 1989).

Regarding the simulation studies in general, Albert Einstein said, "everything that counts is not counted and everything that is counted, does not count." Therefore, the ultimate proof of the structure or any truth is experimentation, but simulations help narrow down possibilities. Such narrowing down of possibilities in other experiments can also be of immense help. For example, in silico drug design can narrow down the possibility to a few molecules, whereas testing molecules randomly will involve wastage of many resources and killing of a large number of animals in experimentation (Gray et al. 2016). Nowadays, simulation in biological sciences is used at various levels of detail, depending on the system's scale in simulation. Quantum calculations can be performed for a small drug molecule, but for the larger systems, the forces have to be accounted for, in a coarse-grained manner because the computing power available is limited. The systems of every level in biology are studied using simulations like the structure of drug molecules, cellular processes, cell-cell interaction, organs' functioning, and up to the whole organism. Computational science is crucial for the study of biological networks at the cellular and system level.

The massive data accumulated about biological systems and increased information storage capacity of computers has brought big data analytics in biological sciences. The number of databases about various biological sciences is increasing. The journal *Nucleic Acids Research* maintains a comprehensive catalog of biological informatics resources at its website: https://www.oxfordjournals.org/nar/database/c/.

This includes databases about research literature, protein structure, genomes of various organisms, secondary databases, and up to the ecological and biodiversity databases (Rigden and Fernández 2021). A comprehensive analysis of such massive information is possible through computational methods/algorithms only. The development of such algorithms and data structures is now a separate discipline within biological sciences called as bioinformatics, which is an integral part of biomedical research.

1.8 Automation and Miniaturization of Experiments (Robotic Liquid Handling Systems and Microfluidics)

The cost of the long run experiments has come down due to the miniaturization of experimental set ups through microfluidics. It has enabled the study of new forces and phenomena affecting systems at the micro- and nanoscales (Kohl et al. 2021). Microfluidics requires less volume of chemicals, and the portability of set up makes it possible to design point of care devices (Dabbagh et al. 2021). Automation of repetitive experimental work using robotics has made experimentation easy, more accurate, precise, and free of human errors (Tegally et al. 2020). Automated liquid handling systems are commercially available for proteomics, transcriptomics, genomics research, and diagnostic at a commercial scale. This had become feasible due to reduced cost of electronic circuit chips (Gach et al. 2017).

1.9 Microelectromechanical Systems in Biology (Bio-MEMS)

Microelectromechanical systems (Bio-MEMS) are being developed with more functionalities by integrating the optics, electronics, and mechanical systems in microfluidic channels (Ino et al. 2020). These Bio-MEMS can do precise manipulation of biosystems and also closely observe the behavior/response. Some new phenomena and forces emerge at these nanoscale dimensions, such as surface plasmon response, which is the interaction of electromagnetic waves with electrons. Similarly, such forces at work in biological systems can also be studied through such miniaturized experimental setups and detection schemes integrated into it. These miniaturized systems are also ultimately less costly for extensive scale experiments like high throughput assays in research and diagnosis. Like LFA (immunochromatographic lateral flow assays) and glucometer, some of these devices have come up as point-of-care kits for diagnosis (Huang et al. 2020).

Microfluidics has made it possible to create a microenvironment around cells similar to the environment, which they experience in tissue or organs. Various functionalities are integrated into microfluidic channels for 3D cell culture and an organ on chip (Thompson et al. 2020). The use of these organs on chips not only does away with ethical clearance required in animal experiments but also is less time-consuming and more controlled experiments, enabling a clear understanding of cause and effect relationship. The origin of these technologies can be attributed to

flow cytometry, wherein the fluid flow is controlled to line up the cells and count them using advanced optics. Quake et al. had proposed the idea of microfluidic cell sorters (Fu et al. 2002). If a good purity of shorted cells is achieved, they can be treasured for label-free sorting of sperms for gender selection in cattle, separating cancerous cells from blood, harvesting stem cells, etc. Microfluidic systems are designed to automate DNA profiling from minute forensic samples such as a touch of a finger (Woolf et al. 2020). These devices extract the DNA, amplify the STRs using PCR, and detect the size of amplified STRs to create the DNA profile (Hong et al. 2020). These all-in-one DNA profiling devices are used on-site, such as police stations and mobile forensic labs. Latest developments in next-generation DNA sequencing like oxford nanopore technologies are a cutting edge example of integrating molecular machines, microfluidics, and sophisticated sensors (Kumar et al. 2019). They use protein as nanopores through which enzymes ratchet the DNA. The processive enzymes inspire this process in cells that work on DNA (Patel et al. 2018). The integrated optics and electronics are used to sense the chemical identity of nucleotides passing through the nanopore to determine the whole genome's sequence ultimately. This Oxford nanopore NGS is very error-prone, but future developments are expected to make these devices accurate and so cost-effective that almost all can get their genome sequenced for use in personalized medicine (Cohen 2020).

1.10 Personalized Medicine

Personalized medicine (PM) shows the potential to precise the therapy with the best response, and it also promises safety to ensure better patient care. This approach also promises better health care by receiving an earlier diagnosis, risk assessments, and better treatments. It also provides opportunities to develop agents specifically targeted to a group of patients for whom the traditional methods have failed or not proven to be very useful. Besides, this could lead to effective patient-physician and provider-patient communication that can improve the patient health outcome and quality of care. To successfully utilize personalized medicine, a person needs to change the practice pattern and its management strategy. All stakeholders will also need to address barriers to implementation if we proceed down the path of harnessing the ability to alter individualized diagnoses and prognosis. It was found that certain drugs work well in one person while not in another (Vogenberg et al. 2010). For example, a drug prescribed to one patient may or may not function in another as everyone's metabolism and physiology are different.

It is well known to physicians for centuries that few medicines work better in certain patients but were not aware of the reason behind it (Adams 2008). For instance, ten people who take the same medication for seizures, heart disease, or cancer might respond very differently. One person might have severe, even life-threatening side effects, whereas another might experience few if any and may seem to sail through treatment, or an anticancer drug may shrink a tumor in one person but not in another.

One major cause of this difference is that people inherit variations in their genes in the form of short nucleotide polymorphisms (SNPs), and these even can affect how the body responds to certain medications. Pharmacogenetics studies how genetic variations in individuals affect response to medications (Lynch and Price 2007).

In fact, the pharmacogenetic and pharmacogenomic techniques have strongly impacted the genetic basis underlying specific drug responses in individual patients. The initial pharmacogenetic approach is based on identifying sequence variations in several genes that affect drug response, whereas pharmacogenomic studies encompass the sum of all genes (i.e., the genome). Most of the currently available drugs are metabolized by the cytochrome P450 (CYP 450) system (Mancinelli et al. 2000). The variations in enzymes caused by SNPs are responsible for variations in absorption, distribution, metabolism, and drug excretion in individuals. Hence, just a minor variation, such as one nucleotide base "mis-spelling," can have clinically profound consequences.

Pharmacogenomics is therefore leading to understanding of drug discovery, personalized drug therapy, and new perceptivities into disease prevention (Mancinelli et al. 2000).

In drug therapy today, the approach is to treat large patient populations as groups, irrespective of the possibility of genetically based divergences in drug response. On the other hand, pharmacogenomics may help concentrate effective therapy on smaller patient subpopulations that establish the same disease phenotype but are characterized by distinct genetic profiles.

Genes are DNA segments found in all human cells, and they can influence a person's response to medications. DNA is a crucial part of an interactive chemical operating system in the body, apprising how to behave and interact on a cellular level. A primary gene can exist in variants and can secrete different chemical messengers. It is those interactions that also affect drug activity in the body.

To exemplify, consider genetic variation in codeine metabolism. Roughly 5% of the population does not catalyze codeine to morphine; for that reason, codeine is ineffective because it provides no pain relief. Codeine converts to morphine using the CYP 2D6 enzyme. However, genetic variation in this enzyme can result in too little or too much enzymatic action, resulting in non-conversion or too little absorption (hence no therapeutic effect). For example, a patient may have a genetic variation that makes the drug stay in the body longer than expected, causing severe adverse effects, or another person might have a variation that makes the medication less potent. It is being tried to identify and then record as many genetic variations as possible. When a variation is identified, that might be matched with a particular medication response leading to personalized medicine approach (Fisusi and Akala 2019). Such studies in pharmacogenomics, have demonstrated benefit for a variety of conditions. Certain medications used for breast cancer have numerous toxic effects. However now prior to start medication, the genetic variations that are likely to affect the response of the patient to drug can be identified and based on this information the appropriate effective but with less toxic one can be selected for him (Colombo et al. 2013).

1.11 Exosomes in Cancer Immunotherapy

Exosomes are single membrane vesicular structures with the approximate diameter of 30–100 nm, secreted by different cell types like cancerous cells and cells of immunity (Bobrie et al. 2011). The major molecular components of exosomes are proteins, lipids, glycoconjugates, and nucleic acids (Trajkovic et al. 2008; Buschow et al. 2009). These play a role in remodeling the extracellular matrix (ECM) and the intracellular transmission of signals and molecules. In the case of cancer progression, it plays a dual role in promoting and suppressing cancers (Fig. 1.3).

Due to high mortality from cancer and its progression, newer approaches are being made in this area. These include cancer immunotherapy because it involves strengthening of the immune system and provide endurance to cells. It has been quite effective against melanoma, non-small cell lung cancer (NSCLC), and kidney cancer (Savina et al. 2005; Hsu et al. 2010; Savina et al. 2002). Exosomes released by oncogenic cells alter different cells by promoting stromal cells that cause invasion and metastasis to activate autocrine VEGF signaling in endothelial cells, leading to angiogenesis (Ostrowski et al. 2010; Lind et al. 2020). They can also express molecules that mediate immunosuppression, such as PD-L1 and transforming growth factor-β (TGF-β) (Robbins and Morelli 2014).

Though there are new aspects of immunotherapy, there has been a widespread interest in cancer therapy as there are new low-toxicity inhibitors in immunotherapy, as potential cancer markers, and are more specific methods of delivering the anti-cancer drugs. These play an essential role in the supply of molecules like proteins, nucleic acids, and lipid contents, accordingly bestowing intercellular communication and immune regulation (Xu et al. 2020). Different studies have shown that the exosome-mediated immune response is dependent on the functional link between several immune cells and tumor cells. Thus, a transparent approach toward understanding the cell-specific molecular events on exosomes will pave the way for developing novel potential exosome-based biomarkers and therapeutics. Current advances in molecular and functional profiling of exosomes have led to

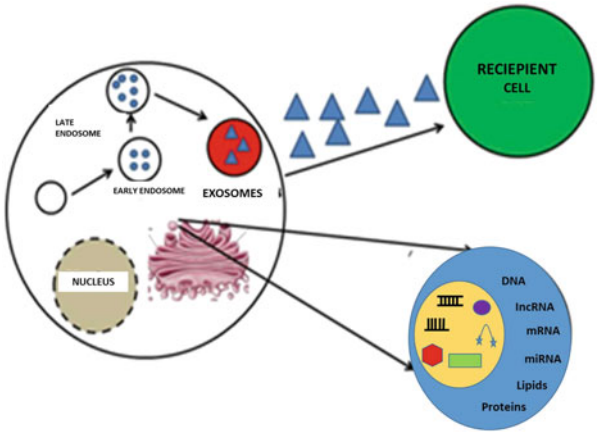

Fig. 1.3 Represents the biogenesis of exosomes. The exosomes are synthesized by endosomes and are single membranous organelles

identification of increasingly effective agents that show a potential behavior in cancer immunotherapies.

Although exosomes are potent anticancer agents, they have been shown to enhance anticancer immunotherapy only at modest levels. The main difficulty arises in production, separation, biocompatibility, and manufacturing practices (Ludwig et al. 2019). They are mostly manufactured from cell culture supernatants and plasma; hence, there are some limitations in their production and purity (Li et al. 2019). Large-scale production and other approaches that may achieve biocompatibility are required for immunotherapy (Sharghi-Namini et al. 2014), even if there is a need of more preclinical and clinical studies for validation. Exosome-based immunotherapy also requires specific international guidelines for their production and application (Normile 2018).

1.12 Neuronal Tracing Techniques

The first step in the field of biology is to establish the connection between structure and function. All the action and activity of the body parts and coordination are governed by the central nervous system (CNS) through the neuronal connections. The prime question arises how these neuronal cells of the brain are associated with themselves and other parts of the body. The development of the viral neuronal tracing technique can answer this question. Here, the neurotropic viruses are used as a tracer. A recombinant viral vector used in neuroscience research is AAV (adeno-associated virus). AAV can label a specific infected neuron but cannot do so in nearby neurons as these recombinant viruses cannot replicate (transsynaptic transmission). There is a need for such a viral vector that should replicate inside the infected neuron and infect the nearby neuronal population via the synaptic connection (monosynaptic transmission). The rabies virus can be such a viral vector that can be modified from transsynaptic neuronal tracer to monosynaptic neuronal tracer. RABV (rabies virus) can map the whole brain. It first infects one cell (starter cell) and replicates inside it. Thus, copies of RABV can be transmitted from the starter cell to the naive cell via a monosynaptic transmission (transmission through only the synapse). RABV transmission occurs in a retrograde manner, i.e., from the starter cell to the cell from where the starter cell receives synapse, thereby the whole brain map can be developed.

To prevent the transsynaptic transmission, RABV was first modified to infect only a specific neuronal cell type, i.e., monosynaptic transmission, and it can be done simply by deleting the viral envelope glycoprotein (G). To visualize the rabies virus infection, a fluorescent dye (**g**reen **f**luorescent **p**rotein—GFP) can be inserted at the place of deleted glycoprotein. Rabies virus with the deleted glycoprotein can also be pseudotyped with the engineered surface protein EnvA and represented as EnvA-pseudotyped-RABV. This pseudotyping results in a specific cell type infection. The EnvA-pseudotyped-RABV will infect only those with TVA (target cell surface receptor). Rabies virus is an enveloped virus, and its infection to the target cell is represented by the interaction between the viral envelope and target cell surface

receptor (TVA). Thus, a specific cell type can be infected via manipulating the rabies virus, and thus monosynaptic transmission can be achieved (Maksimović and Omanović-Mikličanin 2017).

The primary concerns of today's science are technological applications without destroying, over-exploiting, or depleting natural resources along with the production of materials that can be completely reused or reclaimed, reduced waste and pollution during production and consumption, and alternatives technologies without damaging health and the environment, which is the main aim of green technology. Green nanotechnology is one of the branches of green technology, green energy, green information technology, and green building, using green chemistry and green engineering principles. As a new and emerging area, nanoscience offers opportunities to apply green chemistry principles that create sustainability and protect and promote organisms' health and safety (Betts and Baganz 2006).

1.13 Miniaturized Bioreactors

Miniaturized stirred bioreactor (MSBR) development in the biomedical sciences is becoming a powerful tool for rapid and cost-effective bioprocess. Due to the low volume of MSBRs, there is an extent of scaling factor ($>10^5$) between these systems and the industrial scale process. This makes it challenging to establish industrially relevant conditions in the MSBR (Kumar et al. 2004). However, realizing conditions that reflect the large-scale process accurately can be challenging. Applications of MBRs include media development and strain improvement to process optimization. It can also be used for medium growth development, improvement of strain through metabolic engineering, or evolution in a directional path, called bioprospecting of natural products, all of which are methods to carry an enormous bioreactor burden enhanced using HT miniature devices. Specifically, MBRs may also reduce the labor intensity and cost of the vast number of cell cultivations necessary in the development of bioprocess, increasing the level of parallelism and throughput achievable, and as such are of growing interest (Lye et al. 2003; Weuster-Botz 2005; Al-Harbi 2012). When utilized for process development, such devices must be relied upon to mimic laboratory and pilot-scale bioreactors precisely. Growth kinetics and product expression can be optimized at a miniature scale and expected to scale-up quantitatively (Fig. 1.4).

1.14 Transcranial Magnetic Stimulation (TMS)

Several brain diseases occur via the hypoactivation of a particular brain region's neuronal population, causing disability. Although several antidepressants and psychotherapy have been developed to overcome the disability, they are less effective. There is thus an immense requirement to develop some newer therapies that can result in better treatment. One such therapy is transcranial magnetic stimulation (TMS). It is a noninvasive brain stimulation therapy based on Faraday's

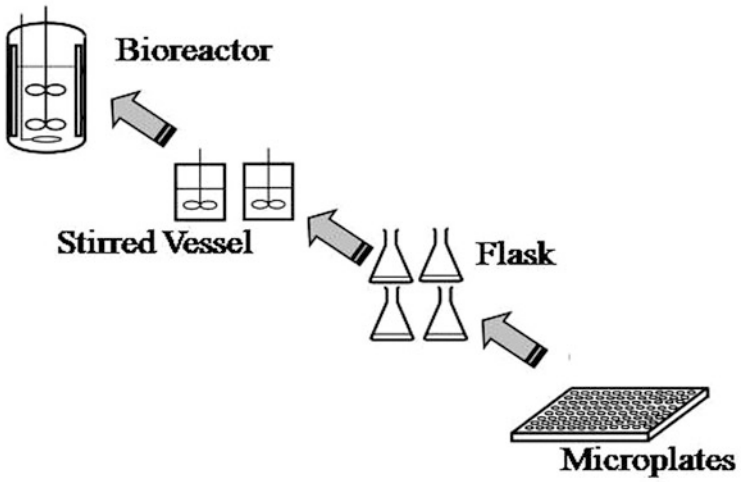

Fig. 1.4 Shows the fundamental assembly of miniaturized bioreactors

electromagnetic induction law. A magnetic field is induced on the scalp by an insulated copper wire coil connected to a magnetic stimulator. The magnetic field intensity is determined by using the "landmark," i.e., the brain's motor cortex. By targeting the motor cortex, we can know the best suitable place to locate the stimulation coil and the magnetic field's appropriate intensity to achieve sufficient stimulation. Using the above data, a specific brain region involved in the disease can be targeted. The induced magnetic field on the scalp via the coil generates an electric current that causes the neuronal population's depolarization, and thus specific response can be evoked (Fakhrullin et al. 2012). As compared to other medications, this technique does not show serious side effects. There may be headache and seizures in some cases during or following the TMS session. Patients who have epilepsy or head injury might be at high risk for the TMS session.

The TMS therapy session requires several sessions for several weeks, i.e., 5 days a week, and each session lasts for 20–50 min. However, the timing of the session depends upon the protocol used in the treatment.

1.15 Bionic Organism or Cyborg Cells

Bionic is the science of developing an artificial system that has properties of some living organisms. Bionics is an interdisciplinary science and can be compared with other interdisciplinary science, i.e., cybernetics. Both bionic and cybernetics are considered as two opposite sides of a coin. In this technique, living cells complement coatings of polyelectrolyte, magnetic and noble metal nanoparticles, shells of hard minerals, and nanomaterials of various complex types (Li et al. 2012). They are found to perform functions that are entirely different from their original

specialization. Such "cyborg cells" are used to find out a range of refreshing applications in areas of whole-cell biosensors, bioelectronics, toxicity microscreening, tissue engineering, cell implant protection, and bioanalytical chemistry. Recently, it has been found that it is used in determining the cell viability and function upon direct deposition of nanoparticles, coating with polyelectrolytes, polymer-assisted assembly of nanomaterials, and hard shells on the cell surface. For many practical applications, possible adverse effects of the deposited polymers, polyelectrolytes, and nanoparticles on the cell surface are also considered (Reuter et al. 2015). The idea of a bionic organism, or a cyborg, is a futuristic way to define a living system with some inorganic constituents designed to improve or alter its functionality. Cochlear implants and robotic prostheses are examples of cyborg devices, and under a broader definition, microchipping the cat or dog might make it a bionic pet. Two intriguing examples of familiar organisms with nanomaterial modifications are bionic mushroom and biohybrid yeast.

1.16 Conclusion

There have been tremendous technological developments in biomedical sciences, and innovation is the driving force. In the past few decades, technological progression has reached new heights with innovations, developments, and breakthroughs for precision in diagnosis and treatment of various diseases. Biomed-driven technologies are constantly evolving and growing exponentially; the example is the Covid-19 pandemic wherein a large number of vaccines have been produced in a record time through innovative technologies.

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Bioprospecting: Boon or Curse

2

Yusuf Akhter

Abstract

Bioprospecting is the investigation of organic material for industrially significant roles due to its exclusive biochemical properties. This chapter will be focussed on the activitiess that could frame the premise of new pharmaceuticals obtained from the bioprospecting. Verifiably, the vast majority of the dynamic fixings in medications have been common items, and characteristic items keep on forming a profitable wellspring of new medications. Given that most novel medication from the bioprospecting discovered in the parts of the world, which are under developed but carries most of the biodiversity. There should be some regulations, whereby access to biodiversity is conceivable under terms and conditions that are commonly adequate to protect this biodiversity. Following this idea, the United Nations delivered a system for saving the world's biodiversity while empowering the reasonable utilization of biodiversity by bioprospecting.

Keywords

Bioprospecting · Microorganisms · Pharmaceuticals · UNCBD · Metabolites · Compounds

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2.1 United Nations Convention on Biological Diversity.

The United Nations Convention on Biological Diversity (CBD) (www.biodiv.org) was one of the significant results of the Earth Summit in Rio de Janeiro in June 1992. The CBD has three fundamental objectives:

- The conservation of biodiversity.
- The sustainable use of the components of biodiversity.
- The sharing of benefits arising from the commercial and other utilization of genetic resources in a fair and equitable way.

Signatories to the CBD perceive that nations have sovereign rights over their hereditary and organic assets (i.e., biodiversity) inside their limits and consent to the conditions in the CBD for the conservation and economical utilization of biodiversity. Biodiverse-rich nations that have confirmed the CBD need to encourage access to their organic assets (Article 15.2). Such access must be as per fitting enactment (Article 15.1) and be on commonly concurred terms (Article 15.4) including earlier educated assent (Article 15.5). The source nation is relied upon to be engaged with synergistic innovative work ventures identifying with its biodiversity (Article 15.6), and the source nation should profit by innovation exchange (Article 16.2), from the consequences of research (Article 15.7) and from sharing of business benefits coming about because of utilization of its biodiversity (Article 15.7). Article 8 (j) likewise confers signatories to saving the conventional information of indigenous and nearby groups and to advancing their contribution in creating more extensive utilizations of their insight, be that as it may, there is little direction on how this may be accomplished. Since 1992, 192 nations and the European Union have marked or sanctioned the CBD, the striking special case being the United States. Nonetheless, issues identifying with access to organic assets have not been completely settled. Just around 25 nations have acquainted new directions with encourage get to, and most by far of nations still need to figure the suitable laws. To help the execution of bioprospecting under the CBD, the Conference of the Parties (the authority CBD body) embraced the Bonn Guidelines on 'Access to Genetic Resources and Fair and Equitable Sharing of the Benefits Arising out of their Utilization' (Secretariat 2002). These are not legitimately authoritative, but rather they are expected to enable all gatherings to take after accepted procedures in setting up bioprospecting understandings. Different expert bodies have reacted to the Bonn Guidelines with their own particular suggestions. For instance, the International Federation of Pharmaceutical Manufacturers and Associations has distributed its perspectives on "industry best practices" and the empowering steps that administrations need to take with respect to managing bioprospecting (IFPMA 2007). The Biotechnology Industry Organization of the United States has created itemized rules for its individuals about taking part in bioprospecting. These cover the general direct of bioprospecting, sharing of monetary advantages and of consequences of research, licensed innovation rights, and preservation and reasonable utilization of biodiversity. The association has additionally distributed a model Material Transfer

Agreement for use in bioprospecting tasks. There is an exceptionally valuable asset distributed by the International Institute for Sustainable Development as an "entrance and advantage sharing administration apparatus" and a going with handbook (IISD 2007). This gives a well ordered manual for acquiring earlier educated assent, achieving commonly concurred terms, concurring advantage sharing courses of action, and managing issues identifying with customary learning and protection. The Bonn Guidelines are probably going to be superseded by the Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization to the Convention on Biological Diversity (Secretariat 2011). This is another settlement under the CBD that was received in Nagoya in October 2010. It will be operational once 50 nations approve it (40 have done as such before the finish of July 2011). The point of the Protocol is to give more noteworthy legitimate sureness about all parts of bioprospecting. Specifically, it is expected to build up more unsurprising conditions for access to biodiversity and to guarantee fitting advantage sharing. The Protocol bargains more unequivocally than past archives with the utilization of customary information related with hereditary assets: contracting parties need to guarantee that nearby groups have given earlier educated assent and that there is reasonable and impartial advantage offering to the pertinent groups. The Nagoya Protocol likewise perceives that hereditary assets are seldom limited to a solitary nation and that customary information identified with utilization of hereditary assets is frequently shared by various groups. The protocol requests inclusion and participation of the applicable gatherings. A noteworthy shortcoming in the execution of the CBD concerning bioprospecting has been the moderate improvement of national frameworks for administering access to biodiversity. The Nagoya Protocol is express about the duties of signatories to make "national central focuses" and "equipped national experts" to make accessible data on the best way to get to hereditary assets and customary information and to be in charge of giving access to biodiversity. In Africa, a different scope of approaches and laws pertinent to access and advantage sharing is set up in a few nations, yet these are most created in South Africa through the National Environmental Management: Biodiversity Act (10 of 2004) ('the Biodiversity Act') and the directions go under this Act in 2008. The Biodiversity Act requires bioprospectors to acquire permission from the Government for bioprospecting including indigenous organic assets and for the fare of these assets. Earlier educated assent is required with landowners and indigenous groups previously; permission was issued. Profit sharing understandings must go to indigenous groups who utilize the asset customarily or who knew about its properties first (Wynberg et al. 2009).

2.2 Virtues of Bioprospecting

Bioprospecting has been a vital marvel of finding new medications since the beginning of human progress. A few numbers of individuals across the world have been utilizing in excess of 8000 types of therapeutic plants for the medicinal service needs. More than 800 therapeutic plant species are as of now being used by Indian

Table 2.1 List of few pharmacological products derived from herbs (Taylor 2000)

S. No.	Pharmacological products	Uses	Herb name
1.	Atropine	Anticholinergic	*Atropa belladonna*
2.	Codeine	Analgesic, antitussive	*Papaver somniferum*
3.	Cynarin	Cholerectic	*Cynara scolymus*
4.	Digitoxin/Digoxin	Cardiotonic	*Digitalis purpurea*
5.	Ephedrine	Sympathomimetic	*Ephedra vulgaris*
6.	Hyoscyamine	Anticholinergic	*Hyoscyamus niger*
7.	Methyl salicylate	Rubefacient	*Gaultheria procumbens*
8.	Morphine	Analgesic	*Papaver somniferum*
9.	Nicotine	Insecticide	*Nicotiana tabacuum*
10.	Physostigmine	Cholinesterase inhibitor	*Physostigma venenosum*
11.	Podophyllotoxin	Anti-neoplastic	*Podophyllum peltatum*
12.	Quinidine	Antiarrhythmic	*Cinchona ledgeriana*
13.	Theophylline	Diuretic, vasodilator	*Theobroma cacao*
14.	Tubocurarine	Skeletal muscle relaxant	*Chondodendron tomentosum*
15.	Vincristine	Anti-neoplastic	*Catharanthus roseus*
16.	Yohimbine	Alpha-2 adrenoceptor blocker	*Pausinystalia yohimbe*

home grown industry alone. In pharmaceutical industry, numerous outstanding and helpful medications have been gotten from leads given by the restorative plants (Table 2.1). Despite the fact that pharmaceutical firms and researchers keep on finding valuable use of segments from nature, their hunting techniques and applications have changed (Kumar 2004). The monetary estimation of plants or living creatures for pharmaceutical reasons for existing is gigantic and profiting not exclusively to the pharmaceutical ventures occupied with R and D yet to have nation and indigenous group additionally, who pick up from responsibility for natural assets and expect satisfactory remuneration for asset utilize, particularly after the Convention on Biological Diversity (CBD) in 1992. The Convention unmistakably sets up the control and sway of neighborhood office over the organic assets and its assorted variety (Kumar and Tarui 2004). With headway in subatomic science and accessibility of complex symptomatic instruments for screening, it has turned out to be really compelling for pharmaceutical firms to lead in exploring through bioprospecting (RAFI Communique 2006). In high-innovation labs, removals from natural examples experience quick and exact screening systems that consider the disconnection of chemicals showing a particularly focused on movement. In 1980, none of the US pharmaceutical industry spending plan was spent on inquiring about higher plants; at the same time today, it is evaluated that more than 200 organizations and research associations worldwide are screening plant and creature segments for therapeutic purposes. Disclosure of a few life-sparing medications including hostile to neoplastic medications (e.g., vinblastine, taxol, topotecan, and etoposide) in later past has recharged the enthusiasm of

pharmaceutical enterprises in bioprospecting. Endeavors are being made to confine hostile to HIV drugs from characteristic assets. No less than three hostile to HIV drugs, (+) calanolide A, (−) calanolide B (costatolide), and conocurovone, disconnected from plants are presently experiencing preclinical or early clinical trials (Taylor 2000). Prostratin and homoharringtonine, the other two against AIDS drugs separated from plants, are likewise under scrutiny with variable achievement. Bioprospecting joint efforts between pharmaceutical organizations and nations providing the restorative crude material and learning offer not just the income hotspot for immature nations, yet in addition open doors for society for better training and business roads. Numerous investigations have recommended that if the bioprospecting seek depends on the data and learning from neighborhood individuals, at that point, the estimation of bioprospecting advantages will be higher (Martin 2001).

2.3 Demons of Bioprospecting

There is a developing worry that various pharmaceutical firms and biotechnology organizations are investigating the woodlands, fields, and waters of creating world looking for natural wealth and indigenous learning with sole point of creating protected and beneficial items. Under most by far of cases, no cash has changed hands, and no acknowledgment has been given to indigenous groups who chose, kept up, and enhanced customary plant assortments for medication. Pharmaceutical firms are frequently blamed for deceiving nearby individuals by denying them access to learning and budgetary advantages. Numerous pharmaceutical firms guarantee that the procedure of bioprospecting includes components of high hazard and cost, and subsequently benefits are not critical. Accordingly, the greater part of the underdeveloped nations occupied with bioprospecting with multinational pharmaceutical firms proceed with their noteworthy part of just providers/exporters of crude materials for gathering of riches in the industrialized countries. The multinational organizations occupied with bioprospecting are allowed to patent biomaterials; however, there are no viable rules and conditions characterized for perceiving and compensating the commitments of indigenous individuals and other casual pioneers who are in charge of supporting, utilizing, and creating biodiversity. One of the persevering inquiries in the bioprospecting has been whether the examination and recognizable proof of dynamic therapeutic constituent in natural examples give the pharmaceutical firms the sole ideal on environmental territory in asset-rich areas or not (Zakrzewski 2002). So the inquiry frequently asked is: What gives pharmaceutical firms the privilege to patent any possibly dynamic mixes as their own disclosures, along these lines keeping the lawful cases of nearby occupants to sovereignties from the offer of such medications paying little mind to their imparting information to organizations? In spite the fact that bioprospecting assentions are authorized by the multilateral Convention on Biological Diversity, much of the time business bioprospecting understandings can't be adequately observed or implemented by source groups, nations, or by the convention itself (Zakrzewski 2002). In a few

cases, there is no control set up to guarantee that the source nations of these plants will be adjusted enough. The fiscal offer by multinational pharmaceutical firms to asset nations as a rule is not adequate. Numerous countries in the third world experience the ill effects of pulverizing weight of outside debt; hence, the money related offer by multinational firms frequently appeal them to auction their natural assets for allowance (RAFI Communique 2006). A few pharmaceutical firms don't offer specifically for access to biodiversity, rather work through middle people (RAFI Communique 2006). The go-betweens might be privately owned businesses, administrative, and non-benefit associations or even people utilized on contract premise. Consequently, it is usually troublesome for indigenous individuals and associations to know accurately with whom they are arranging or to whom they are giving their data and hereditary material. Awkwardness in environment because of over the top misuse of material assets is dependably a probability. The tropical rainforest districts of the world, which constitute over half of therapeutic plants, are vanishing (Moran 1992). This is basically because of huge number of business interests including bioprospecting.

2.4 Resolution to the Concerns

Attributable to the absence of appropriate direction and sufficient pay for nations providing the therapeutic plant species, the inquiries and questions of what should be possible to realize changes in the present framework should be tended to. Endeavors ought to be made to deal with the distinctions, assuming any, between the pharmaceuticals firms and the nations providing the plant materials. The disclosures through bioprospecting ought to be fairly shared between the pharmaceutical firms and nearby groups and indigenous individuals engaged with the revelation of normal items. Advantages of bioprospecting can be shared by the two gatherings in various structures like propel installment and sharing the income through sovereignty assertions (Smith and Kumar 2002). It is essential to plan a plan where the data and also access to the assets can be viably shared among firms and the nearby individuals with bioprospecting site. The terms and states of bioprospecting understandings under which indigenous individuals may profit fiscally ought to be clear and straightforward and free from vagueness. Preparing ability ought to be offered by multinational pharmaceutical organizations to the locals giving crude material to drugs. Giving employments, preparing, and aptitude to the source nations would profit neighborhood individuals with chances to advance. Different advantages can be conceded as far as types of gear and training and innovation are transferred. The scholarly respectability of indigenous individuals and other rustic individuals must be affirmed inside the Biodiversity Convention. This incorporates the privilege of indigenous individuals to profit by their conventions and virtuoso and a say in all basic leadership discussions. Without a persuading worldwide ethic or clear intension with respect to the universal group, indigenous groups and local governments ought to have each privilege and motivation to pronounce a ban on additionally gathering and new understandings. The privilege of indigenous groups

to state no to bio-privateers and to genuine bio-miners should likewise be guaranteed. No licensing of living items and procedures ought to be permitted in the future. Plants and living beings ought to be viewed as the sole property of indigenous individuals and governments. The present scholarly property frameworks don't and won't secure the interests of nearby occupants and casual group pioneers. It must be guaranteed in all bioprospecting understandings that a piece of advantage subsidizing goes to help ecological insurance in the locales providing the plants so as to guarantee long haul soundness of the characteristic biological community.

2.5 Conclusions and Future Prospects

Bioprospecting has been proposed as a potential intend to energize the protection and feasible utilization of biodiversity. The legitimate structure under the support of the United Nations is gradually being executed by biodiversity-rich nations, yet much still should be done if there is to be a bona fide assistance of bioprospecting. Maybe the execution of the Nagoya Protocol will give the important step. In any case, the hunger for bioprospecting by pharmaceutical improvement organizations has obviously reduced since the Rio Earth Summit in 1992, incompletely as a result of the complexities identifying with access and advantage sharing regularly without sufficient national administrative lucidity and institutional limit. Notwithstanding the proceeding with appearance of effective medication improvement ventures in light of characteristic items, there is an assumption that this approach might be excessively out-dated, making it impossible to be thought about genuinely screening of normal items for new leads. Different specialized issues without a doubt exist with the screening and disconnection of characteristic items; however, the prizes for defeating them would appear to legitimize the exertion required, and specialized arrangements are being depicted in the writing. For instance, cleansing and recognizable proof of regular items are accepted to be troublesome and moderate: high throughput division strategies combined with touchy diagnostic procedures can resolve this (Bugni et al. 2008; Hu et al. 2008). Normal items are artificially intricate: correlations of the compound properties of accumulations of regular items demonstrate that they all the more intently coordinate the "concoction space" of effective medications than accumulations of engineered chemicals (Grabowski and Schneider 2007; Ganesan 2008). Characteristic items are rumored to give excessively numerous false positives on present day screening examines; however, phenotypic tests are ending up increasingly prevalent, and it has been proposed that common items, with their medication like properties, are all around coordinated to such cell-based methodologies, and concentrates of regular items can be prepared to evacuate responsive mixes or even change over them into novel medication like structures (Rishton 2008). Regular items may just be accessible in little sums: procedures for coordinate union (Sunazuka et al. 2008) or generation by subatomic science (Kennedy 2008) have been quickly creating. While there is absolutely no single best approach to lead tranquilize revelation, similarly as there isn't a solitary panacea

for all sicknesses, it is without a doubt time for a new take a gander at the generally unexplored open doors given by current ways to deal with applying regular items in sedate disclosure. Maybe the lead should be given by the various scholastic gatherings dynamic in bioprospecting. Notwithstanding, these gatherings would stand more shot of accomplishment in the event that they could pool assets and work toward finding approved lead exacerbates that are probably going to be appropriate for improvement into meds for neglected remedial needs. The development of translational research and the foundation of focuses of translational research will empower scholastic gatherings to end up basic accomplices in pharmaceutical advancement. In spite of the confinements and affirmations of bio-theft, the bioprospecting with its potential as a rich and imperative wellspring of new remedial operators is a critical device for tranquilize revelation and research. In any case, the coordinated efforts between the pharmaceutical organizations and the nations providing the indigenous learning and restorative assets ought to be managed for commonly valuable relationship.

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Growing Need for Interdisciplinary Biomedical Research

3

Eshu Singhal Sinha, Prakash Kumar Sinha, and R. C. Sobti

Abstract

Biomedical research is the area of science devoted to the study of the life processes and diseases with the ultimate goal of improving health by generating preventive interventions and effective treatments for diseases. The fulfillment of this aim requires framing right questions, well-planned scientific experimentation, data analysis, and evaluation, which calls for experts from different areas of both life and physical sciences. **Biomedical research** has high expectations in supporting and accelerating medical research. However, limited success is achieved due to barriers in scientific translation. This raises demand to create new paths to solve problems that can be tackled only through interdisciplinary approaches. The potential of interdisciplinary research lies in the fact that this approach brings together knowledge and expertise from different fields and works in newer synergistic ways, thus enabling novel integrated perspectives on complex problems of medical science.

Keywords

Collaborative research · Integrated science · Translation medicine

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3.1 Introduction

Biomedical research is a broad area of science that involves the study of biological processes and diseases. It is aimed at benefiting the health sector by providing solutions to problems faced by biomedical profession, thus accelerating medical research. This field of research looks for ways to prevent and treat diseases through well-planned experimentation, observation, and analysis. However, biomedical research has achieved limited success due to a number of oppressing challenges such as inadequate statistical data, insufficient funds, and limited integrated research. These factors and, most importantly of these, limited interdisciplinary research contribute to a wide gap between biomedical research and clinic application (Musunuru et al. 2018) for which translation medicine poses a potential solution.

Translational medicine is an aspect of science that bridges the gap between findings in biomedical science and their effective clinical application (Jane-Budge et al. 2015). Despite the advancements made in the process of modern research, the field of translational medicine seems to be in its beginning. Translation of biomedical science into clinical application requires the facilitation of interdisciplinary collaborative research (Ravid et al. 2013) within academic and clinical environments of different areas ranging from molecular biology to physical and other associated sciences. Biomedical research itself is an interdisciplinary process that requires careful experimentation by several scientists including biologists, physicists, and chemists. To increase the productivity of biomedical research, there is a greater need for convergent interactions among researchers working in the field of genetic variations, cellular dynamics, behavioral sciences, nutrigenomics, environmental impact, and pharmaceutical variables (Fig. 3.1). This will be helpful in better evaluation for determining effective preventive interventions and treatments for diseases.

3.2 Interdisciplinary Research

Interdisciplinary research is described as "the integration of the analytical strengths of two or more often disparate scientific disciplines to solve a given biological problem" (Aboelela et al. 2007). Considering the importance of human health, attention toward issues associated with biomedical research is of prime importance. To find an optimum solution to any problem, researchers need to integrate knowledge gained from high-throughput molecular research with biophysical imaging studies, epidemiological studies, and data from clinical trials. This is also helpful in understanding what works/does not work and what is safe/unsafe. An association between socioeconomic status and health (Committee on the Organizational Structure of the National Institutes of Health 2003) also raises demand for interdisciplinary research.

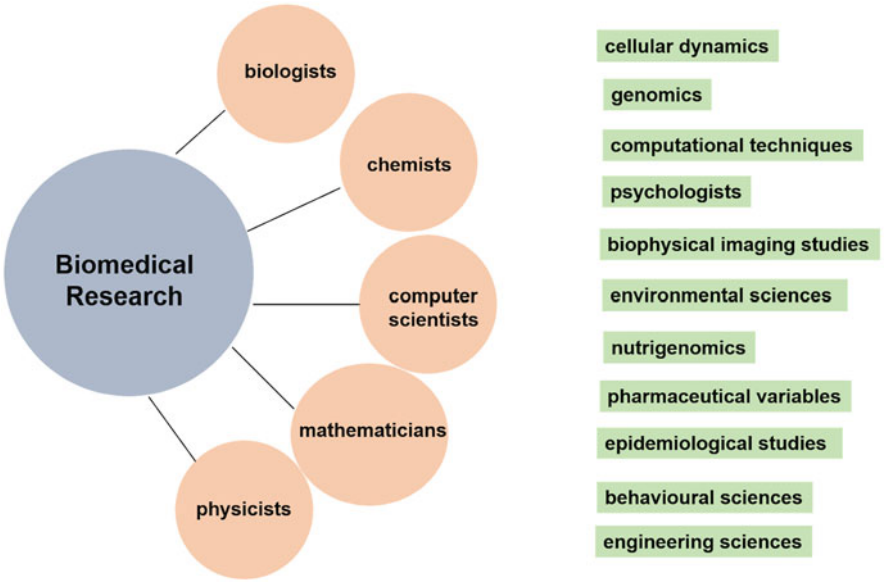

Fig. 3.1 Biomedical research is an interdisciplinary field of science and requires efforts by experts from different sciences including biologists, physicists, mathematicians, computer experts, and chemists. However, limited success is achieved in biomedical research due to barriers in scientific translation. To increase the productivity of biomedical research, there is a growing need for integrated convergent efforts from researchers working in the field of genetic variations, cell biology, behavioral sciences, nutrigenomics, environmental biologists, anatomists, engineering, biophysics, and pharmaceutical variables

The recognition of the need and value of interdisciplinary research is not new. Indeed, the history of medicine has demonstrated innumerable times that important advancements require interdisciplinary efforts. For example, the Human Genome Project involved expertise of biologists, chemists, computer scientists, mathematicians, and engineers, and laser surgery involved combined efforts from ophthalmologists, anatomists, and physicists (Committee on the Organizational Structure of the National Institutes of Health 2003).

Notably, many scientific problems require single disciplinary efforts and techniques. Hence, it is very important to understand that interdisciplinary research approach should arise out of necessity in response to a problem, which cannot be gripped by a single discipline. In past few years, fields that conventionally did not fondle interdisciplinary research have begun to recognize its necessity. Some examples where interdisciplinary efforts find an implication are discussed below.

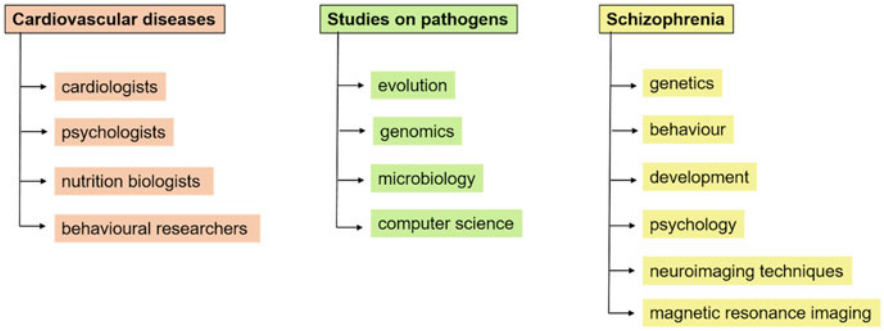

Fig. 3.2 Implication of interdisciplinary efforts in different fields

3.2.1 Cardiovascular Health and Diseases

Several studies have demonstrated that behavior, lifestyle, and traits such as smoking, lack of exercise, exposure to stress, anger, socioeconomic status, and diet influence the risk of coronary heart disease (Buttar et al. 2005; Phillips and Klein 2010). Hence, research collaborations among experts in areas including psychologists, cardiologists, nutrition biologists, and behavioral researchers (Fig. 3.2) would provide new advances in the prevention and management of cardiovascular diseases (Pellmar and Eisenberg 2000).

3.2.2 Studies on Pathogens

Increasing number of researchers involved in study of pathogens have shifted to interdisciplinary study of "pathogenomics," which involves using microbiology, computer science, and genomics along with information from evolution in a synchronized way (Fig. 3.2) to determine how pathogens interact with their host (Pompe et al. 2005). By using computational techniques, scientists are able to analyze a large amount of data obtained from bacterial genome sequencing for expeditious identification of features unique to a particular pathogen. This also helps in saving a lot of time via avoiding huge bench work/experimentation by providing a well analyzed hint about which epitopes can be potential immunogens for vaccine generation and which motifs of pathogens are responsible for host-pathogen interaction that might work best if targeted for therapy (Pompe et al. 2005).

3.2.3 Schizophrenia

Schizophrenia is a chronic mental disorder with abnormalities in perception, speech, emotional expression, and behavior. Hallucinations and social withdrawal commonly occur in the schizophrenic patients. Experts in the area believe that abnormal

brain development may be a cause of this disorder (Pellmar and Eisenberg 2000). This is supported by neuroimaging studies, demonstrating that some people with schizophrenia have abnormally large ventricles in the brain. A physiological link also exists between development of schizophrenia and impaired migration of neurons in the brain during fetal development (Jaaro-Peled et al. 2009). Some studies indicate a genetic predisposition to this disease (Gejman et al. 2010; Kukshal et al. 2012). Together, all these facts about schizophrenia suggest that synergistic efforts from multiple disciplines (Fig. 3.2) such as neuroimaging techniques, magnetic resonance imaging, genetics, development, and behavior would advance the understanding and treatment of this disease (Pellmar and Eisenberg 2000).

3.3 Increasing Urgency in Some Fields of Research

A greater sense of urgency pervades some fields of research globally including emerging infectious diseases such as the novel coronavirus disease-2019 (COVID-19), Zika virus, severe acute respiratory syndrome (SARS), etc. These warrant special attention on infectious diseases, their epidemiology, and regular monitoring of exposed people/masses. These diseases that have a huge impact on health and economy of the affected nations highlight the need for identification of the causative agents, rapid detection systems, and development of therapeutics. Success in these areas requires joint efforts from immunologists, experts in bioinformatics, economists, and scientists from various other fields in ways that might be difficult with current structural configuration of research.

The persistent threat of bioterrorism arising from spread of highly infectious causative agents further raises an alarm to set a roadmap for meeting futuristic challenges (Pompe et al. 2005). Beyond research, greater collaboration with the public health workers, emergency workers, and pharmaceutical industries is required. Huge data assessment and technology development also necessitate the involvement of nonbiologists, such as engineers, physicists, and computer experts along with biologists in such projects. Working in collaboration can play a pivotal role in framing appropriate questions and using the right ways to find an immediate solution by improving scientific knowledge about the subject that is important for limiting the spread of the causative agents and in developing vaccines and treatments.

3.4 Why Do We Need Interdisciplinary Research?

The life processes and medical problems do not fall sharply into subject categories. Thus, the issues and phenomena cannot always be studied independently by using tools, expertise, and knowledge from one particular discipline (Boon and Baalen 2019). Therefore, for deeper understanding of phenomenon, scientific collaboration across disciplinary borders must increase (Mumuni et al. 2016). In fact, many new fields in science such as tissue engineering, bioinformatics, and nano-biotechnology

have emerged which are hybrid fields and involve interdisciplinary efforts. Despite these new avenues, still some barriers to scientific translation exist, and further advancement is required. Interdisciplinary approaches set the stage for innovation by uniting together and ultimately opening new avenues/opportunities in research (Resnick 2011). The opportunities and needs thus generated, further raise questions about whether current research structure supports, or limits the adoption of collaborative research.

3.5 Future Directions for Interdisciplinary Research

Biomedical research is a long known area of science with well-established principles and goals. What is changing with time in biomedical research is the recognition of increasing need for interdisciplinary research. Some of the life phenomenon and causes of disease and death are found to be exasperatingly complex. Thus, studying physiological links among genes, environment, behavior, and disease are required for discovering treatment interventions for several diseases including AIDS, cardiovascular diseases, cancers, and schizophrenia. These conditions are difficult to be understood extensively by limiting oneself to the research scenario of individual investigators working in isolation in their own discipline. The collective framing of research questions by research personnel from different disciplines can result in better questions that can be addressed with comparatively higher chances of positive outcomes. However, the current scenario of educational practices and research funding mechanisms has created a system that sometimes presents challenges for interdisciplinary work (Pellmar and Eisenberg 2000).

The creation of favorable environment for interdisciplinary research and training may require some changes through redesigning of conventional research training programs. This will also require support from funding schemes calling for interdisciplinary training and research (Resnick 2011). All these inputs may play a strong role in synchronizing biomedical science with clinical needs and patient outcomes so as to deliver evidence-based outputs (Committee on the Organizational Structure of the National Institutes of Health 2003). Besides biological fields, the active involvement of disciplines such as physics, chemistry, mathematics, and computer science should also be sorted. This is where the interdisciplinary synergistic research may show its full potential. Bringing such different ways of thinking to the same bench is a great way to expedite research that neither individual research group, nor complete but isolated field, has thought before. In such collaborations, one side can cover the weaknesses of other discipline by suggesting alternate tools and techniques.

3.6 Concluding Remarks

Many biomedical problems can effectively be solved by researchers of the respective field. However, some problems are complex and cannot be tackled by a single discipline. Besides biologists, these require mutual knowledge and efforts from

experts working in different fields such as physics, engineering, chemistry, mathematics, and computer science that were traditionally considered peripheral to mainstream biomedical science. Thus, in past few years, there is an increasing demand for generating integrated working platforms, which work through sharing of knowledge and providing their tools, techniques, and expertise in order to supplement the success of biomedical research and apply the outcome in clinical practice. This will ultimately be beneficial to improve human health in long-run. One of the critical steps toward implementing collective intelligence in translational medicine can be the inclusion of redesigned research training programs and funding schemes that can support the interdisciplinary biomedical research.

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Interplay Between Theory and Experiment: A Future Approach for Biomedical Research

4

Rolly Yadav, Anamika Shukla, and Devesh Kumar

Abstract

Theoretical and computational chemistry have become indispensable in various fields of chemical research as they provide structures, properties, and reactivities of the molecules. Computational enzymology is a rapidly developing area and is testing theories of catalysis, challenging "textbook" mechanisms, and identifying novel catalytic mechanisms. Modeling of enzymes is contributing to the experimental study of enzyme-catalyzed reactions in the field of drug discovery, catalyst design, and interpretation of experimental data. In the present text, we would discuss some controversies and reaction mechanisms which were later resolved with the help of theoretical calculations.

Keywords

Metalloenzyme · Drugs · Substrates · Controversies

4.1 History

In late 1960s with advent of microprocessor technology (Moore 1965; Whitworth 1979; Brinkman et al. 1997), foundation of interdisciplinary scientific field, which is today known as computational chemistry, was laid. This field is an amalgamation of mathematics and computational science with scientific discipline such as physics, chemistry, and biology. A few decades ago, theoretical modeling played a minor role in understanding redox-active reaction of metalloenzymes. The theoretical methods were underdeveloped; due to this, either result were not accurate enough, or processing time was too long.

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But in today's scenario, the situation is changed with the development of methods and insights from application. Development of density functional theory (DFT) was reached at a stage where their accuracy was not far from most accurate. The breakthrough was incorporation of density gradient terms for the exchange part and of fractions of exact exchange (Becke 1993). During first year of its applications in transition-metal complexes, it was clear that results were quite accurate (Bauschlicher 1997; Siegbahn 1996). Decades of experience gained from study of small models of transition metal containing complexes gave idea for further improvements in method to be used and how to address mechanism studies of large organometallic complexes; as surprisingly, small model methods were quite helpful in gaining insights in action of mechanism of biomolecules. Transition-state structures and individual reaction steps turned were not entirely dependent of the size of the model for understanding of reaction mechanism. There are two originally different approaches in study of enzymatic systems. First is the cluster model approach, which uses small or truncated model system; this small model approach had a potential to elucidate main features of mechanism. First study using this model was done in 1997 on methane monooxygenase enzyme (MMO) (Siegbahn and Crabtree 1997). Second approach is treating small core active site of the enzyme with extensive QM methods using DFT, while rest of the system is described by molecular mechanics; hence, this approach is called as QM/MM model (Warshel and Levitt 1976).

The first application of QM/MM on the mechanism of galactose oxidase was made in 2000 (Mulholland and Richards1993). Both approaches have been developed over the years from their original form. Nowadays, with improvement in computer technology, QM cluster models can handle quite big model, i.e., with more than 200 atoms, and even larger QM core can be used in the QM/MM approach.

Nowadays, theoretical model calculation can be regarded of equal importance in determining mechanism of metalloenzymes. Experimental methods have advantage that they are studied on the actual system, but spectroscopically guarding of short lived species, electron transfer, and interpretation of results is quite troublesome. In both approaches, accuracy of the results should be there. In theoretical modeling, accuracy of the results depends on the accurate choice of the method and real system under consideration. More than two decades of experience in this area has made the understanding of limitations and applicability on different models to reach a mature stage.

4.2 Introduction

Enzymes are green "catalysts," ever since their potential is recognized; researchers have wondered and argued about how they work (Mulholland 2007). Better understanding of the mechanism by which they work and performs biological transformation reactions will lead to advances in the area of designing new drugs (as many drugs are inhibitors, which will eventually bind to enzymes and prevent them from further functioning), designing of new and better biomimetic or engineered catalysts,

and analysis of effect of mutations will help in predicting metabolism of pharmaceuticals. Thus, there is a great demand of development of protein catalyst for the practical applications in the field of pharmaceutical, chemical, and biotechnology industries. Computational modeling has a major role in understanding these enzymatic processes due to unstable species such as reaction intermediates and transition state, which cannot be studied directly via experiments in these complex systems (Mulholland 2005; Garcia-Viloca et al. 2004; Warshel 2003; Martí et al. 2004).

Enzymology raises rigorous debates and controversial proposals about mechanism of reaction performed by enzymes. Identifying and sticking to one proposal seems too difficult, and it is often hard to differentiate between alternative proposals. Some of the controversies in the field of enzyme catalysis are low "barrier-hydrogen" bonds (Cleland et al. 1998; Mulholland et al. 2000; Schutz and Warshel 2004; Molina and Jensen 2003), the role of enzyme dynamics in catalysis (Garcia-Viloca et al. 2004; Warshel 2003), and quantum tunneling (Masgrau et al. 2006). Controversies are hard to resolve by experimental analysis because of the complexity of the system. Atomistic simulations have offered a potentially vital role in these debates by providing a molecular level picture of action of mechanism in enzymes and in the interpretation of experimental data. It is now possible by theory to reach unprecedented level of chemical accuracy of (1 kcal/mol) in accord with experimental data (Claeyssens et al. 2006).

In this article, some of these debates and complementary behavior of theory and experiment have been tried to discuss.

4.2.1 Cytochrome P450 (P450)

From a catalytic point of view, P450s are fascinating because they oxidize a wide range of relatively inert substrates both stereoselectively and regiospecifically. Thus, Cytochrome P450 is a nanomachine (Shaik et al. 2005) that operates by means of the catalytic cycle (Hata 2004; Shaik and de Visser 2004; Ogliaro et al. 2000a, b) depicted in Fig. 4.1, that it gets triggered by entry of substrate, followed by series of electron and proton transfer reaction transforming the inactive resting state to the catalytically active species, and thereby, insertion of oxygen into substrate is believed to be universal among all P450 isoforms (Ortiz de Montellano 2004). Although there is difference in protein environment due to which redox partners and electrons sources are different in isoforms of P450 (Munro et al. 1996, 2007; Matsunaga et al. 2002). The catalytic cycle mainly consists of eight steps, the catalytic cycle begins with the resting state (**1**) in which water molecule is bound to the distal position to the ferric-ion. The iron is hexacoordinated with Fe (III) oxidation state, d-block orbitals contains five electrons in total and results in low spin doublet electronic configuration. Arrival of substrate (any drug like compound, alkane, alkene, etc.) displaces the distal water, which results in pentacoordinated ferric-porphyrin (**2**) high spin sextet species. This ferric complex so formed is relatively better electron acceptor than the resting state and hence accepts electron from a reductase protein leading to a high spin ferrous complex (**3**). Molecular

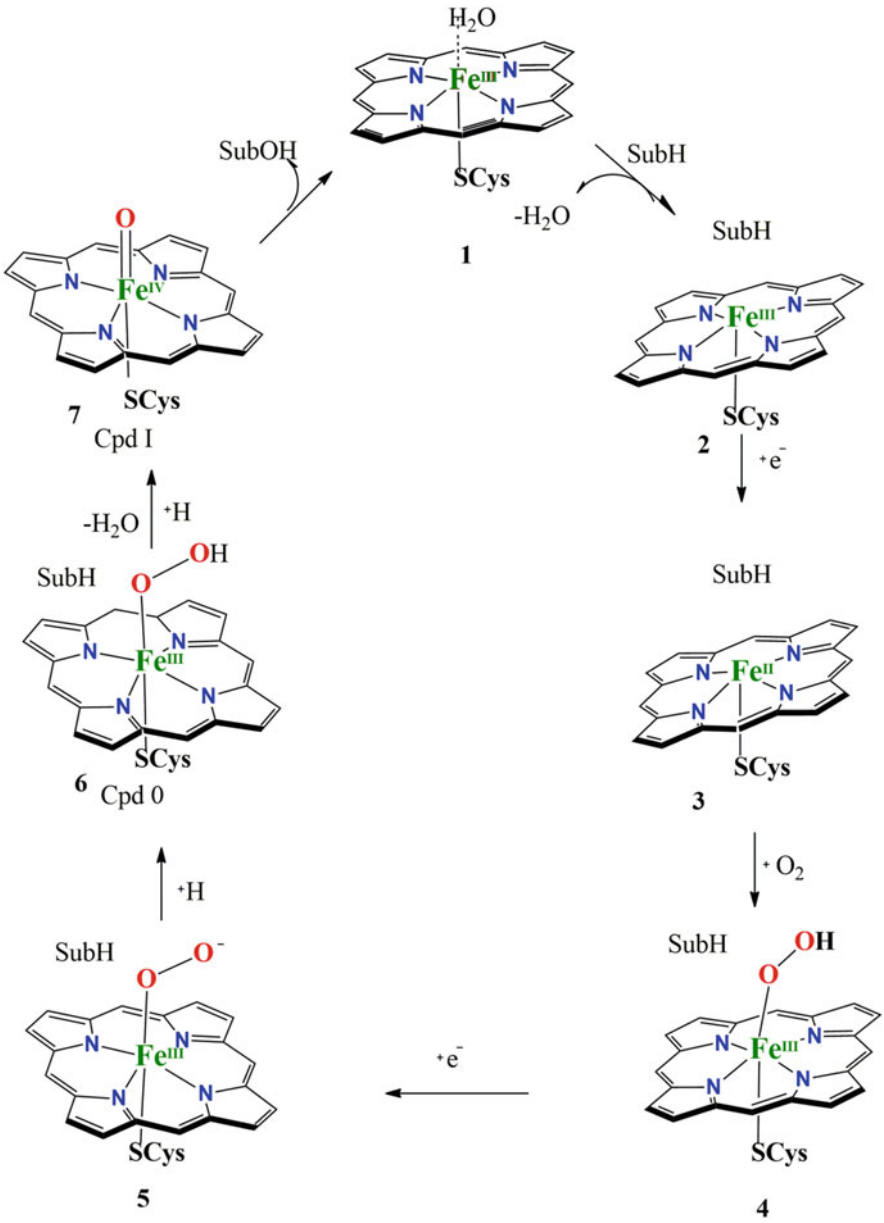


Fig. 4.1 Catalytic cycle of P450 after entry of substrate SubH

oxygen subsequently binds on the distal side, to produce ferrous dioxygen complex (**4**), which has a singlet spin state and is a good acceptor of electron. The second reduction occurs, which is also the rate determining step in the catalytic cycle, to generate a ferric–peroxo ion species (**5**), this is a good Lewis base hence it readily

gets protonated to form ferric-hydroperoxide species (**6**), which is called Cpd 0. This is a precursor for the formation of the primary oxidant Cpd I. As it is also a good Lewis base so it accepts proton to released water and results in the formation of ferryl-oxo species (**7**) compound I, i.e., Cpd I. This proton transfer step of conversion from Cpd 0 to Cpd I is quite fast. This oxidant performs oxygen insertion reaction in substrates, and then products leave the protein pocket, and water molecule enters inside and binds to the distal side of the ferric ion restoring the resting state (**1**). In the P450 catalytic cycle, electron transfer processes mostly originate from either nicotinamide adenine dinucleotide (NADH) or nicotinamide adenine dinucleotide phosphate (NADPH) coenzymes, with sequential delivery of two electrons through auxiliary electron-transfer proteins. Early studies on synthetic models and chloroperoxidase (CPO) suggested an iron(IV)-oxo heme cation radical ([heme (+•)-FeIV=O] or Compound I) as the active species able to abstract a hydrogen atom from a substrate and rebind the hydroxyl group to form an alcohol product (Sono et al. 1996; Groves 2005; Rutter et al. 1984; Zaks and Dodds 1995). But the role of active oxidant had always been the topic of debate between theoreticians and experimentalists. This problem originated due less sophisticated tools available to detect the electron transfer processes in later stages of catalytic cycle of P450. The Cpd I active oxidant as suggested by DFT studies was short lived and spectroscopically never been trapped at that point of time. The possibility for a second oxidant species in the cycle of P450 enzyme was sensed indirectly due to various results from reactivity studies of P450 for different substrates, lifetime measurements of short-lived intermediates, and product distributions of P450s and site directed mutagenesis (Shaik et al. 2007). As can be seen, there was a gray area between theory and experiment. Recently reported work of Rittle and Green (2010) gave the firm evidence of existence of Cpd I and its catalytic properties using various spectroscopic studies such as UV-Vis, EPR, and Mössbauer effect. They were successfully able to generate substrate-free ferric sample of the thermophilic CYP119, which was then reacted with *meta*-chloroperbenzoic acid (*m*-CPBA) at 4 °C, leading to the formation of pristine Cpd I efficiently. Cpd I was characterized, and its existence was detected for first. In addition, they further studied its reactivity (oxidant power) with a selection of substrates, which identified Cpd I as a potent oxidant ruling out all experimental findings. Thus, the first firm evidence of the capacity of P450 enzymes to form Cpd I was much later provided through experiments, which showed theory as an efficient tool in this field.

4.3 Experiment That Created Doubt

4.3.1 Site-Directed Mutagenesis

The protonation machinery in $P450_{cam}$ basically consists of two main residues: the alcohol side chain of Thr252 and carboxylic acid group of Asp251. As the name suggests "site-directed" mutagenesis, some key residues, which are involved in proton transfer in conversion of Cpd 0 to Cpd I, were altered (or mutated) (Newcomb

and Toy 2000; Vaz et al. 1998). The reactivity patterns for the wild type enzyme and mutated ones were different. The reaction profile for mutated enzyme with cis and trans 2-butene yielded more epoxidation products compared with hydroxylated (Vaz et al. 1998) ones, thus showing likeliness for epoxidation in mutated enzyme. DFT studies showed that the proton affinity of Cpd 0 is as high as 334 (Harris and Loew 1998) or 330.1 kcal/mol (Ogliaro et al. 2002a, b), which allows barrier less second proton transfer and thus collapses to Cpd I. EPR/ENDOR studies of Davydov et al. (2001) showed that in the T252A mutant of P450cam formation of Cpd I was blocked and catalytic cycle was thought to be terminated at Cpd 0; hence, it was not able to hydroxylase camphor, but surprisingly its was able to form epoxide in double bonds of camphene (Jin et al. 2003), although sluggishly than WT enzyme. The study mutated both the proton transferring residues T252A/D25N1 and hence completely disrupted the proton transfer machinery; the results were quite surprising as it showed little activity toward camphor hydroxylation. These studies made researchers think that either Cpd 0 showed different reactivity in different mutants (that include the T-to-A mutation) or may be different amount of Cpd I is present in different T → A mutants. Also, in cases where epoxidation and hydroxylation mechanisms were competitive, a regioselectivity switch was observed (Vaz et al. 1996, 1998). Based on these studies, a two-oxidant scenario was hypothesized by both theory and experiment. We shall review here some of the DFT calculations associated with the reactivity of Cpd 0 toward substrates (Shaik et al. 2005).

4.3.1.1 Ethylene Epoxidation by CPD 0 and CPD I

To test the validity of two oxidant present in catalytic cycle of P450, comparative studies for epoxidation via both oxidant Cpd 0 and Cpd I were performed by two groups, Shaik and Yoshizawa. They both tested epoxidation of ethylene (Ogliaro et al. 2002a, b; Kamachi et al. 2003) with truncated model of enzyme active site that involves protoporphyrin IX macrocycle without side chains. Shaik et al. studied the reaction profile with cysteniate ligand has been replaced by thiolate; the epoxidation barriers for ferric(III) hydroperoxy species and iron(IV) oxo species were compared. It was found and can be seen in Fig. 4.2 that barrier for Cpd 0 was very high (37–44 kcal/mol) as compared with Cpd I with barrier height of 13.0 kcal/mol. The Cpd I performs the epoxidation reaction on two spin states due to degeneracy. It follows nonsynchronous epoxidation mechanism; high spin (HS) state involves cationic intermediate and can lose stereochemistry, whereas the low spin state (LS) proceeds via concerted mechanism. On the other hand, Cpd 0 catalytic activity involves single spin state. The results of Yoshizawa et al. were in agreement to the present study with slight variation in energy values due to difference in the choice of axial ligand (methyl mercaptan in place of thiolate). Later experimentally conclusion was drawn that the ferric-hydroperoxo complex is a sluggish oxidant as compared with Cpd I for epoxidation reactions (Park et al. 2006).

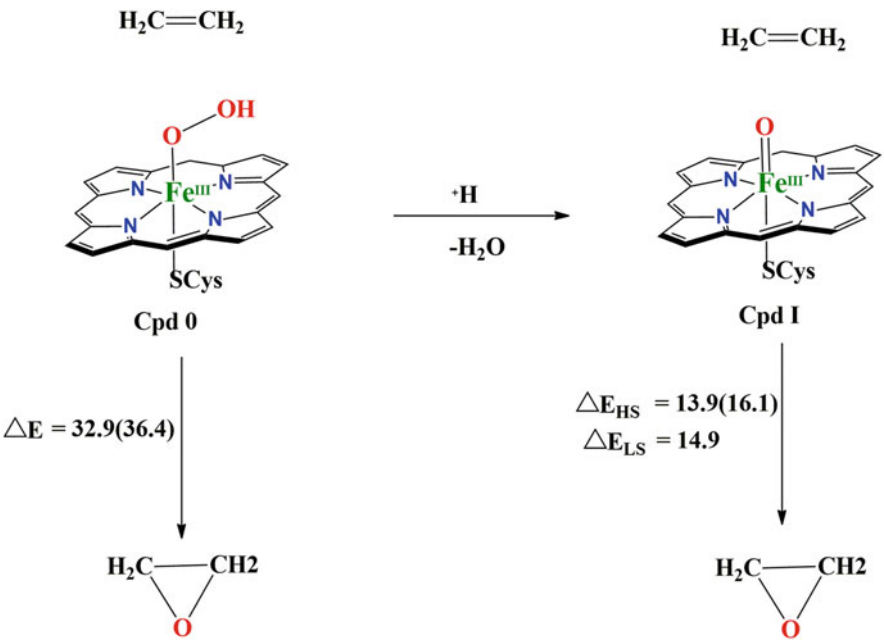


Fig. 4.2 Epoxidation of ethylene by Cpd 0 and Cpd I with energies in kcal/mol

4.3.2 DFT and QM/MM Studies of Alkyl Sulfide

4.3.2.1 Sulfoxidation by Cpd 0 and Cpd I

Sulfoxidation reactions were also studied by several groups in the search of potent oxidant. Using truncated P450 model complexes, quantum mechanical calculations were employed in both Cpd 0 and Cpd I using dimethyl sulfide (DMS) as their active substrate (Sharma et al. 2003; Kumar et al. 2005; Li et al. 2007; Porro et al. 2009; Rydberg et al. 2008; Kumar et al. 2011a). Figure 4.3 depicts the potential energy curves of DMS with Cpd 0 and Cpd I. Sulfoxidation reaction with Cpd I involved single transition states unlike C=C epoxidation and C–H hydroxylation reactions. Here, two electrons are transferred from DMS to heme for S–O bond formation. The reaction is stepwise and involves two spin states. However, the difference between ^{4}**TS**SO and ^{2}**TS**SO is considerably, so one can say sulfoxidation proceeds via SSR (single state reactivity). The barrier heights are different for quartet and doublet due to the electron transfer in different orbitals. In doublet spin state, the electron transfer process occurs by filling of a_{2u} and π^*_{xz}, and quartet spin state involves electron transfer in a_{2u} and $\sigma^*_{z^2}$ orbital. The orientation of substrate to oxidant in rate determining transition state depends on the orbital in which electron is being transferred like in case of electron transfer in π^*_{xz}, the substrate attack from sideways, whereas in quartet spin state $\sigma^*_{z^2}$ involved the substrate attacks from top. Now when Cpd 0 is used as a oxidant, the barrier height for S–O bond formation were quite high; this gain suggested that Cpd 0 is a sluggish oxidant in comparison with Cpd I.

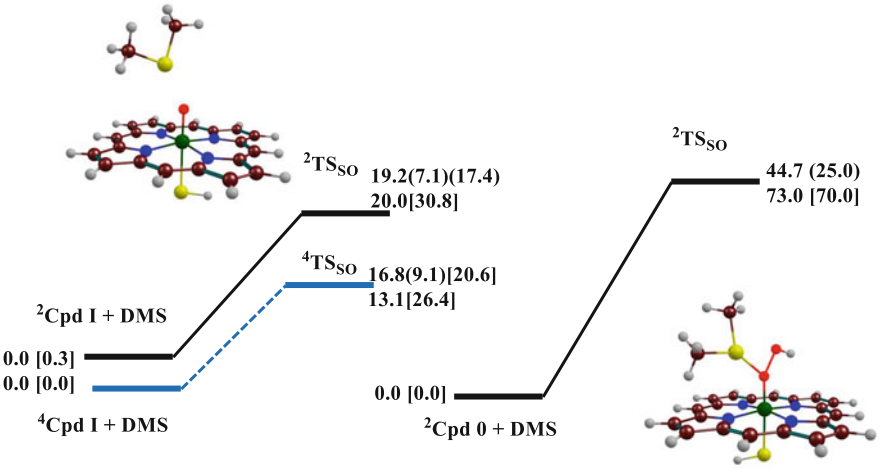


Fig. 4.3 Potential energy profile of sulfoxidation of DMS by Cpd I and Cpd 0

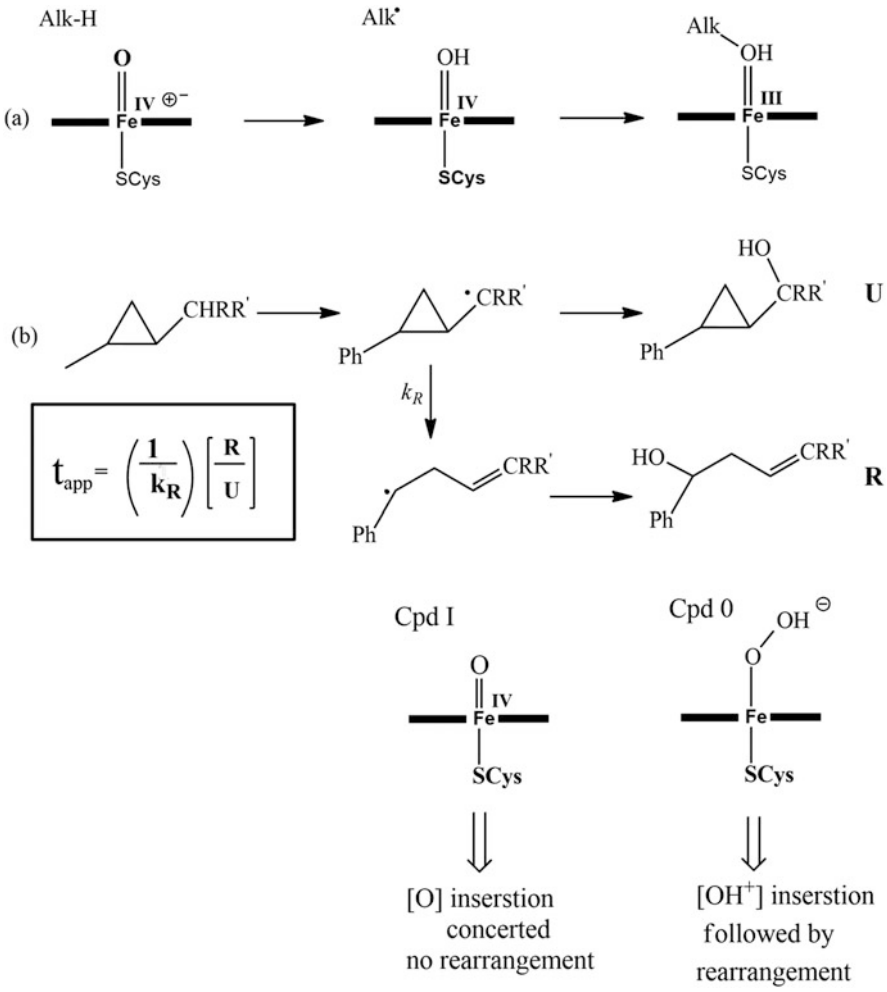
4.3.2.2 Radical Clock Experiment

Rebound Controversy and Its Resolution by TSR Scenarios

The mechanism for C–H hydroxylation by Cpd I as active oxidant was given by Groves et al. (1978) shown in Scheme 4.1a. It was first coined "rebound mechanism," then only its initial step involves hydrogen abstraction from the alkane (Alk-H) by Cpd I. Subsequently, the alkyl radical (Alk) can either instantaneously rebound to ferric hydroxy intermediate to give unarranged (**U**) alcohol product complex keeping original stereochemistry of the alkane preserved or can undergo skeletal arrangement first and thereafter rebound to form a rearranged (**R**) alcohol product. The rebound mechanism justifies key experimental data of partial loss of stereochemistry and geometrical rearrangement (a) Ortiz de Montellano and De Voss (2002) (b) Auclaire et al. (2002), large intrinsic kinetic isotope effects (KIEs) when hydrogen in transition is replaced by deuterium (Groves and McClusky 1976; Sorokin et al. 1993; Gelb et al. 1982; Sono et al. 1996; Woggon 1996; Meunier and Bernadou 2002; Fretz et al. 1989; Ortiz de Montellano and Stearns 1987; Audergon et al. 1999).

The apparent radical lifetime (τ_{aap}) calculation study was first done by Ortiz de Montellano Scheme 4.1b using bicycle [2.1.0] pentene. They basically calculated the ratio of unarranged (**U**) to rearranged (**R**) product yield [**U/R**], divided by rate constant for rearranged free radical clock (K_R). The lifetime of radical intermediate was found to be short lived but finite ($t = 50$ ps).

The rebound mechanism was well accepted and looked solid until Newcomb and colleagues (Newcomb et al. 1995, 2000; Newcomb and Toy 2000; Toy et al. 1998) started investigation through ultrafast radical and carbocationic clocks. The determination of quantity [U/R], with one of the probe substrates depicted in Scheme 4.1b, lead to the conclusion that if radical intermediates are involved in the catalytic cycle



Scheme 4.1 (**a**) Rebound mechanism suggested by Grooves and McClusky. (**b**) Apparent lifetime (τ_{aap}) of a putative radical intermediate from the ratio of rearranged (**R**) to unrearranged (**U**) alcohol products produced from P450 hydroxylation of a substrate probe, here bicycle [2.1.0] pentene. (**c**) Probe that can distinguish between radical and carbocationic rearrangements, and the mechanistic proposal of C–H hydroxylation via two oxidants, Cpd I and Cpd 0

of P450s, then these will have a lifetime of 100 fs or less (Newcomb et al. 1995, 2000), which means that real intermediate species is not possible. In P450 mutant species where Cpd I is assumed to be absent, the ratio [**R/U**] was larger than wild type enzyme (Ortiz de Montellano and De Voss 2002; Auclaire et al. 2002).

This ought to conclusion that rearranged product originate from the non-radical intermediates, which get generated due to presence of another oxidant species which becomes active in mutants. Substrate that can distinguish between radical and carbocationic rearrangement pattern was subjected to validate this hypothesis, and

results from this suggested that intermediate is a carbocation not a radical in the hydroxylation reaction of alkane. Newcomb hence proposed that alkane hydroxylation proceeds via two oxidants present in enzyme Cpd I and its precursor Cpd 0 without involvement of radical intermediate Scheme 4.1c. Cpd I allow concerted mechanism with insertion of [O] in C–H; on the other hand, Cpd 0 involves insertion of hydronium ion (OH+) in C–H bond and generates a protonated alcohol, which undergoes rearrangement for carbocationic species. From the discussion in above section one can say that Cpd 0 is a sluggish oxidant. It can only play role where Cpd I is suppressed or not present. Thus, the two-oxidant mechanism is not reconcilable from experiment or theory from reactivity study of Cpd 0 and Cpd I. But indeed Newcomb studies lead door open to intrude further into action of mechanism as *experimental results pointed in the direction of products that behaved as they are originated from two different sources*.

The role of theory comes into the picture, and it has already offered a reasonable resolution (Ogliaro et al. 2002a, b; de Visser et al. 2001; Shaik et al. 2002; Yoshizawa et al. 2001; Kamachi and Yoshizawa 2003) of the main mechanistic problems depicted by experiment with the help of two-state reaction (TSR) mechanism (Ogliaro et al. 2002a, b; de Visser et al. 2001; Shaik et al. 2002).

Let us first give a brief revision to our knowledge of Cpd I from theory and experiment and throw some basic light on the origin of idea of two state reaction (TSR) mechanisms (Shaik et al. 2002, 2005). The very idea of "two-state reactivity" is based on the fact that Cpd I has two closely lying (nearly degenerate) spin surfaces due to its molecular orbital arrangement (de Visser et al. 2003). The five 3-d orbitals of metal split into the characteristic $3t_{2g} - 2_{eg}$ pattern. The $3t_{2g}$ further disassemble itself into one nonbonding $(\delta_{x^2-y^2})$ and two antibonding metal and distal oxygen orbital (*xz, *yz). σ^*_{xy} and $\sigma^*_{z^2}$ are two high lying antibonding virtual orbitals. σ^*_{xy} originates from the mixing of σ orbitals on nitrogen and orbital of metal center, and $\sigma^*_{z^2}$ is the outcome of mixing of orbital along O–Fe–S axis, i.e., mixing of $3dz^2$ of Fe, 2pz orbital on oxygen, and lone pair of thiolate axial ligand. The orbital occupation of metal looks like $\delta^2_{x^2-y^2}$,$*xz^1$, $*yz^1$, hence the oxidation state of iron is IV Fe (IV). Apart from these metal orbitals, there are two high lying orbitals of porphyrin, a_{1u} and a_{2u}; these two orbitals are degenerate (Ghosh 1998) in pure heme macrocycle, but in case of P450 enzymes, there is mixing of a_{2u} and σ orbital of axial sulfur ligand, which results in increase in energy of a_{2u} with respect to a_{1u}. Thus, due to lower energy, a_{1u} is doubly occupied in ground state, and a_{2u} is singly occupied, and overall ground state electronic configuration of Cpd I becomes $\delta^2_{x^2-y^2}$, $a_{1u}{}^2$, $a_{2u}{}^1$, $*xz^1$, $*yz^1$. These three unpaired ferromagnetically and antiferromagnetically couples to give what we called quartet and doublet spin states, respectively, with same orbital occupation. All the orbitals are shown in Fig. 4.4. The extensive density-functional calculations (DFT) reveals that energy difference between quartet and doublet spin states are within 1 kcal/mol. The ordering and energy of these states depends on the axial ligand effects and perturbations in protein environment (Green 1999, 2001; Schöneboom et al. 2002; Kumar et al. 2011b; Latifi et al. 2013) (Fig. 4.4).

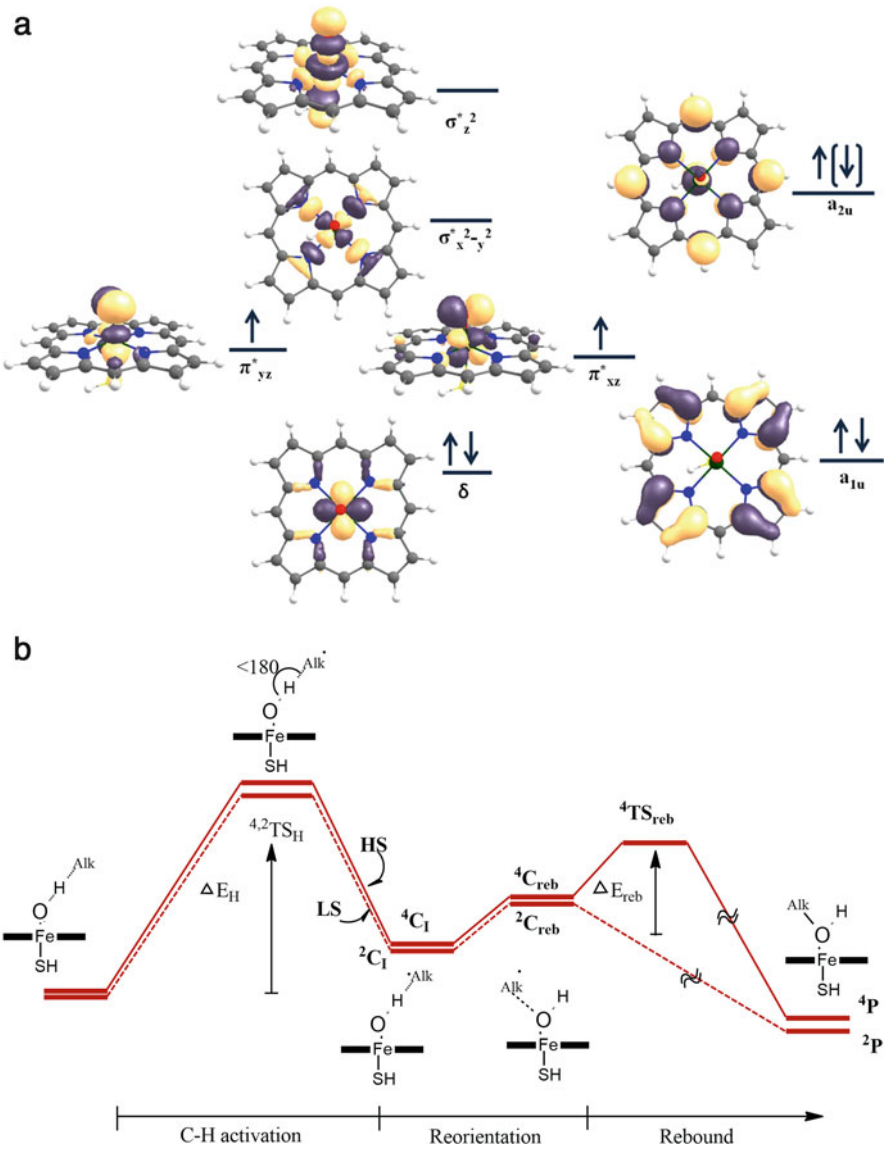


Fig. 4.4 Typical reaction profile for C–H hydroxylation (Alk-H is the substrate) showing two-state reactivity (TSR) due to the closeness of the two spin-state profiles

Cpd I has two closely lying spin states quartet and doublet, which are referred as high spin (HS) and low spin (LS) states, respectively. These states follow rebound mechanism (Kumar et al. 2003).

It can be seen from Fig. 4.4 that reaction pathway involves C–H activation phase that involves barrier and nature of this transition state is hydrogen atom abstraction type. After this step, alkyl moiety attains a radical character, and this moiety is

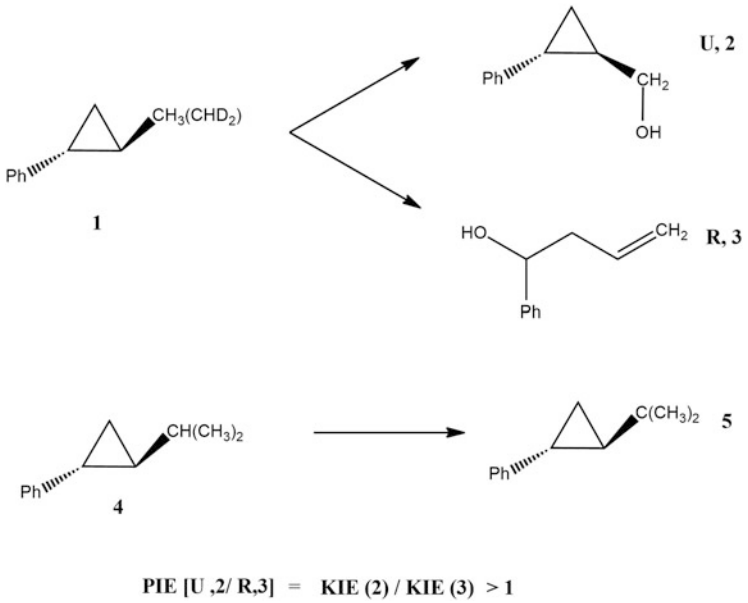
loosely coordinated to ferric iron-hydroxo intermediate by C···HO hydrogen bond, followed by reorientation process in which radical "reorients" itself away from OH bond in a manner so that it can have a direct C···O bond formation. The last step of the process is a rebound phase, which allows C–O bond formation to give away the alcohol product.

The HS and LS profile stays close in energy till PorFeOH/Alk,· and then both states bifurcate. The LS manifold follows effectively concerted ones, the radical snaps out of the C···HO interaction from iron-hydroxo species (Ogliaro et al. 2000a, b; de Visser et al. 2001; Shaik et al. 2002) pathways leading to a product formation, while HS surface offers considerable barrier what we call rebound transition state. The reason behind the rebound barrier in HS surface is due to promotion of electron from substrate to high lying virtual orbital $\sigma^*_{z^2}$, and on LS state, the promotion of this substrate electron takes place two a much low lying orbital *xz. Hence, it can be inferred that radicals on the HS profile will have a significantly long lifetime. Contrast to HS, LS will have intermediate with shorter lifetime, and its upper limit will depend on the frequency of rotational modes, which establish a rebound position. Thus, TSR provides two-state information on the lifetime of the radical containing both "ultra-short" radical and "normal" radical (Shaik et al. 2004).

Now, this picture was extended to explain the radical clock experiment result from Scheme 4.1 that says that amount of rearranged product **R** mainly arrives from HS manifold and the unarranged product **U** is the outcome of both HS and LS manifold, or most of the time LS state. Apparent lifetime, which is derived from the ratio [**U**/**R**], cannot be regarded as a true lifetime since it is also taking relative yields of HS and LS in account. Taking example of C–H hydroxylation, the yield of product due to HS surface, i.e., rearranged product, will be small, and majority of product will be unarranged product type, which is outcome of LS manifold, and this will lead to unrealistically short apparent lifetime calculation. But the matter of the fact is that radicals exist on HS surface and have normal lifetime; apparent lifetime manifestation is a result of assumption that both the products arise from single radical intermediate. TSR mechanism can accommodate both the KIE results (de Visser et al. 2013), which shows that the transition state is hydrogen, abstraction type in bind activation step, and also it was able to explain that two-state rebound step too can lead to controversial lifetime if [**U**/**R**] is justified by single radical intermediate, which can partition between immediate rebound and first rearrangement followed by rebound (Kumar et al. 2004).

4.3.2.3 Products Isotope Effect and TSR

In kinetic isotope studies for two probe substrates **1** and **4**, it was indicated by Newcomb et al. (2003) that products of rearranged **R** (**3**) and unarranged **U** (**2**) for this clock substrate respond differently on their isotropic substitution of scissile C–H bonds. It can be seen from Scheme 4.2 that the kinetic isotope effect for unarranged is large than the rearranged due to which there exist a product isotope effect (PIE), which is greater than unity PIE [*U*,**2**/*R*,**3**] > 1. These results rule out the possibility of product arising from common intermediate and are indicating its support in the direction of products of reaction mechanism by P450 with two processes. PIE $\neq 1$



Scheme 4.2 Rearranged (R,2)) and unarranged (U,3)) products of substrate 1 and its observed product isotopes effect (PIE), and substrate 4 with its sole product 5

indicates that either mechanism is TSR or it is two-oxidant mechanism. Ogliaro et al. (2000a, b) anticipated that PIE will not be equal to unity with TSR scenarios, since **U** and **R** originate from two different H abstraction transition state. But, as objected by Newcomb et al. (2003) based on the studies of methane hydroxylation and allylic hydroxylation using TSR results, the PIE would be less than one, entirely opposite to the experimentally measured quantities, i.e., *PIE* [*U*,**2**/*R*,**3**] > 1. Hence, his findings ruled out TSR scenario and again supported two-oxidant hypothesis. The resolution of this puzzle was addressed by theory using DFT studies on two of the substrates used by Newcomb et al. (2003) and their theoretical determination of PIE[*U*,**2**/*R*,**3**] quantity. Kumar et al. (2004) studied the reaction profile of trans-2-phenyl-methyl cyclopropane with DFT (B3LYP) calculations and found product isotope effect for the same, which revealed PIE > 1, the results from TSR scenario for the probe were in direction of experimental results.

Since the structure and position (along H-abstraction coordinate) of transition state of LS and HS ($^{4,2}TS_H$) are different, the corresponding KIE will also be different, i.e., $KIE_{LS} \neq KIE_{HS}$. This will lead to intrinsic PIE_{int} on the rearranged **R** and unarranged **U** product, defined by equation below:

$$PIE_{int} \equiv PIE_{TSR} = [U_H/U_D/R_H/R_D]_{int} = (KIE_{LS}/KIE_{HS})$$

This equation is based on the fact that **U** arises from effectively concerted LS pathway, while rearranged product arises from the stepwise HS pathway. The

deviation of $PIE_{int} = 1$ and its direction (<, >1) will depend upon the structures of $^{2}TS_{H}$ versus $^{4}TS_{H}$ (Shaik et al. 2007). Intrinsic PIE can be determined by the calculation of intramolecular KIE on the rearranged and unarranged products since the masking of intramolecular isotope effect is negligible or small. TSR scenario reveals that PIE > 1 because doublet transition state is "central" and quartet TS is "late" (Shaik et al. 2007). It was also inferred from these studies that PIE_{int} also depends on the nature of the substrate and the structure of $^{2,4}TS_{H}$ as well.

4.3.2.4 Quantum Mechanical and Molecular Mechanical Application in Biomedical

The description of chemical reactions involves bond making and bond-breaking or other processes that involves charge transfer; change in electronic structure requires use of quantum-mechanical (QM) methods. But their extensive computational cost restricts the study to only several tens to hundreds of atom (nearly 100–200). This problem was resolved by considering treatment of chemically active region by QM (i.e., substrate, cofactors, and important residues if needed) and rest of the system containing residual protein and solvent by less exhaustive MM method (Senn and Theil 2007b). The resulting schemes are commonly referred to as combined or hybrid QM/MM methods. They enable the modeling biomolecular systems at reasonable computational cost and provide the necessary accuracy. The QM/MM concept was introduced, as early as 1976, by Warshel and Levitt, who presented a semi-empirical QM/MM treatment for a chemical reaction in lysozyme, exactly 30 years ago, a method that addressed all the aspects of QM/MM calculations in the field of enzymology. Singh and Kollman (1986) after 10 years from Warsehl and Levitt combined ab initio approach to QM (Hartree-Fock) with molecular mechanics. They were first to report QM/MM geometry optimization with coupled ab initio. Field et al. (1990) developed the method by coupling semi-empirical method (AM1 or MNDO) with CHARMM force field.

The generalized model of QM/MM can be understood by Fig. 4.5, where the system is partitioned into two parts. The inner region (I) is treated with QM methods, and the rest of the system (protein) is treated by classical mechanics, region (II). The part where these two interact is called boundary region. This region requires careful attention; it can also contain additional atoms, which are not a part of the system and are used to cap the QM region or there could be some atoms at this boundary, which are part of both QM and MM region (Senn and Thiel 2007a, b).

There are two approaches to do QM/MM calculations. (1) Subtractive scheme: these methods involve two set of calculations; first MM energy of whole system is calculated using MM methods, followed by calculation of energy of QM region (active site) at QM level of theory. After this, QM energy is added to MM energy and MM energy of QM part is subtracted from this sum. In this method, entire QM/MM interaction of the system is handled at MM level. This type of calculation is problematic where there is electrostatic interaction. (2) Additive scheme: this comprises of sum of QM energy of QM region, MM energy of MM region, and coupling term of QM/MM (i.e., boundary). This scheme is preferred in most of the calculations as it includes both bonded and non-bonded terms present at the QM/MM

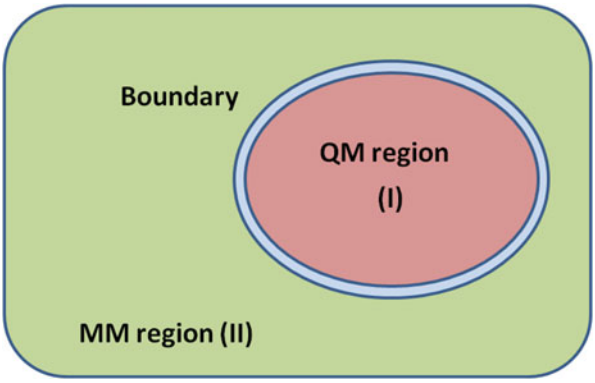


Fig. 4.5 Pictorial representation of QM and MM parts in QM/MM method

boundary, both handled at the MM level of the theory, and it also counts electrostatic interaction terms, which are modeled explicitly by QM method (Shaik et al. 2011).

It is beyond the scope of this article to throw light on all studies performed by QM/MM methods, but few of them are discussed, which covers different areas of biomedical field.

4.4 Establishing and Testing Reaction Mechanisms

4.4.1 Lysozymes

The hypothesis for reactions mechanism is developed from the information from experimental data such as structural kinetics and mutational data (Vocadlo et al. 2001; Kirby 2001).

But such data cannot give complete the idea of the possible reaction mechanism and could also indicate multiple mechanisms for the same. To elucidate the likely mechanism from these experimental data alone is far from trivial task. One can say this because even in textbook, some of these data presented is wrong (Vocadlo et al. 2001; Bowman et al. 2008). Hen egg white lysozyme (HEWL) is such an example of the first enzyme whose x-ray crystallography structure has been solved (Phillips 1967). Lysozymes are glycosidase, which are present in peptidoglycans that occur in cell walls of bacteria, and here, it catalyzes the hydrolysis of bond between *N*-acetylmuramic acid (NAM) and *N*-acetyl-D-glucosamine (NAG) (Van der Kamp and Mulholland 2013). Based on the structure of HEWL, the mechanism proposed by Phillips (1967) was present in the textbooks of biochemistry. It involved oxocarbenium ion intermediate when proton is transferred from Glu35 to glycosidic oxygen and subsequently results in the cleavage of glycosidic bond. Later experiment by Koshland (1953) suggested formation of covalent intermediate due to bond formation between Asp52 and NAM, and crystallography and mass spectrometry experiments also indicated the same (Vocadlo et al. 2001; Kirby 2001). It was still unclear whether covalent mechanism is used or not because these studies used wild type HEWL and unnatural substrates.

Free energy profiles for protonation of glycosidic oxygen and cleavage of glycosidic bond by Glu35 was calculated using QM/MM at the PM3-CHARMM27 level of the theory. They observe spontaneous formation of covalent bond with Asp52. At higher level of QM theory when same was studied, results were in accord with experimental predictions suggesting Koshland-type mechanism. The transition state was oxocarbenium ion like for glycosidic bond breakage. Thus, the very first QM/MM studies were helpful in elucidating the possible mechanism and also concluded that lysozymes provide good electrostatic stabilization for such species (Warshel and Levitt 1976).

4.4.2 Citrate Synthase

Citrate synthase catalyzes the first reaction mechanism in citrate acid cycle, i.e., conversion of oxaloacetate into citrate acid. This conversion reaction is an important model in the study of carbon-carbon bond formation in biological systems (Van der Kamp and Mulholland 2013). The reaction involves initial hydrogen abstraction from acetyl-coenzyme A (acetyl-CoA), followed by condensation of acetyl-CoA with oxaloacetate to form citryl-CoA. The last step is the hydrolysis of stable intermediate citryl-CoA to form citrate and CoA. The nature of this deprotonated acetyl-CoA intermediate was uncertain. Proposals suggested it to be either enolate or enol of acetyl-CoA or enolic form that has so called "low-barrier" hydrogen bond between His274 residue and acetyl oxygen (Van der Kamp and Mulholland 2013).

QM/MM tried to address this problem and elucidate the mechanism. QM/MM studies were done for hydrogen abstraction reaction firstly at AM1-CHARMM2765 level and after that at higher level of the theory using MP2/6-31+G(d)-CHARMM2766. Results from both set of calculation indicated in favor of enolate formation with bond between His274 and conserved water molecule, which gives stabilization to the species and discarded the "low-barrier" hydrogen bond formation (Mulholland et al. 2000; Van der Kamp et al. 2007).

QM/MM modeling using SCS-MP2 was applied in analysis of enantioselective conversion of fluoroacetyl-CoA to fluorocitrate (Van der Kamp et al. 2011). AM1-CHARMM27 MD simulations were performed with enzyme model with oxaloacetate, and fluoroacetyl-CoA bound (PDB code 4CSC) revealed that fluoroacetyl group can give both the pro-S and pro-R proton to the proton abstracting Asp375. Proton transfer along (r = d(OAsp375H) − d(CFaCoAH)) reaction coordinate generated five TS structures (Fig. 4.6) and hence five potential energy surfaces for each reaction by performing geometry optimization at B3LYP/6-31+G(d)-CHARMM27 level and SCS-MP2/aug-cc-pVDZCHARMM27 level to calculate energies. The energy difference for the pro-R abstraction and Pro-S abstraction between reactant state and enolate state are in favor of pro-R abstraction by ~2 kcal/mol leading major product 2R,3R-fluorocitrate. This result is in accordance with experimental major and minor product distribution, and different fluorocitrate enantiomers are result of inherent energy distribution between two enolates.

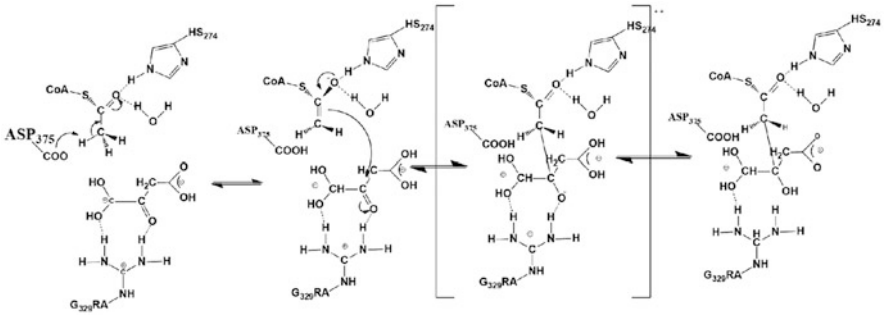


Fig. 4.6 Reaction mechanism for carbon−carbon bond formation in citrate synthase proposed by high-level QM/MM calculations. Proton transfer to Asp375 leads to an acetyl-CoA enolate intermediate, which later attacks the carbonyl carbon of oxaloacetate to create citryl-CoA intermediate, an unusual proton transfer from Arg329 appears to be required. All structures are optimized at B3LYP/631-G(d)-CHARMM27 level

Formation of acetyl-CoA enolate, studied with high-level of QM/MM in CS also indicated, is not a rate limiting step (Van der Kamp et al. 2010). This would imply that condensation of acetyl-CoA with oxaloacetate could be the possible rate limiting step for the mesophilic forms of the enzyme (Eggerer 1965), although condensation and hydrolysis will have similar barriers (Kurz et al. 2009). Mechanism for condensation process is still unclear, and proton donation to the formal carbonyl oxygen of oxaloacetate is suggested from several residues, which include several histidines in the active pocket (Karpusas et al. 1990; Remington 1992) controversial proton donation from Arg329, which is also supposed to donate proton to carbonyl oxygen (Remington 1992).

Van der Kamp et al. tried to elucidate this unclear step by performing calculation considering different proton donors in this reaction step (Van der Kamp et al. 2008). AM1-CHARMM27 calculation results depicted Arg329 as best possible donor; later optimization at 31+G(d) CHARMM27 level of theory also repeated the same result, i.e., Arg329 as proton donor residue (including enolate formation, Fig. 4.6). Ab initio QM/MM energy calculation by MP2/aug-cc-pVDZCHARMM27 energies is in accordance with so far results from theory and experiment (Alter et al. 1990) (barriers of 14.2 kcal mol^{-1} and 14.7 kcal mol^{-1}, respectively), confirmed the likelihood of proton donation by Arg329.

4.5 QM/MM Modeling of Drug Target Enzyme

4.5.1 Fatty-Acid Amide Hydrolase (FAAH)

This enzyme is mainly involved in the degradation of fatty acids amides that are biologically relevant, and hence, it is a prime target for the treatment of anxiety, hypertension, and pain (Minkkila et al. 2010). Its protein contains unusual catalytic triad of Ser-Ser-Lys function of which are modeled by QM/MM reaction modeling

(Lodola et al. 2005). B3LYP/6-31+G(d) corrected PM3 CHARMM27 generated potential energy profiles are consistent to the barrier heights calculated from experimental observations reaction of oleamide, which indicated neutral Lys142 deprotonates the Ser241 with Ser217 and thus acts as a proton shuttle. Another group Tubert-Brohman et al. (2006) performed QM/MM-FE Monte Carlo simulations, using PDDG/PM3 as QM method and supported the role of Lys142Ala mutant on the hydrolysis rate of different substrates. The acylation of oleamide in FAAH occurs with high energy conformational substrates of the enzyme substrate complex; this was highlighted in QM/MM and MD simulations studies (Lodola et al. 2007, 2010). QM/MM calculation also provides direct insights into the binding modes of carbamic acid lead compound (Ladola et al. 2008), which cannot be predicted by docking and quantitative structure activity relationship (QSAR) studies (Lodola et al. 2011a). The binding modes were later confirmed by x-ray crystallography (Mileni et al. 2010). The knowledge gained from these results was used in investigation of better inhibitors (Mor et al. 2008). QM/MM studies on reaction mechanism of three carbamate compounds gave further insights (Lodola et al. 2011b).

The first reaction step was modeled using two reaction coordinates creating a 2-D potential energy surface; one is proton abstraction from Ser241 by Ser217 coupled with nucleophilic attack by Ser241, and another is proton transfer between Ser217 and Lys142 (Lodola et al. 2011b). Energy calculation for the approximate TS and intermediate was carried out at B3LYP/6-31+G(d)-CHARMM27 level (Lodola et al. 2005) in which predicted rate of inhibition in carbamylation of Ser241 depends upon the formation of first tetrahedral intermediate in all three cases (Lodola et al. 2011b). These results also pointed out that electron-donating substituents on the inhibitors does not produce significant effects on the key TS in reaction due the fact that Ser241 and Ser217 mainly involves proton transfer and no increased inhibition potency can be seen, but these substituents do effect the inherent reactivity (Lodola et al. 2011a). Hence, QM/MM calculation results of FAAH inhibitors can be used in better drug design.

4.5.2 Zn Enzymatic Drug Metabolism: Antibiotic Hydrolysis by Metallo-β-Lactamases Enzymes

Zn is an essential metal ion in living organism (Andreini et al. 2009). Zn ions provide major drug resistance in bacteria (Fretz et al. 1989). Zinc is silent to most spectroscopic methods, and calculations can therefore provide an excellent complement to the experimental work. Over the years, a large number of different zinc-dependent enzymes have been studied with quantum chemical methods, using both the cluster approach and various QM/MM techniques (Smith et al. 2011; Amata et al. 2011; Chen et al. 2012; Zhang et al. 2012; Wong and Gao 2011; Wu et al. 2010). Metallo-β-lactase (MβLs) is a zinc-dependent metelloenzyme, which is responsible for the degradation of β-lactom moiety present in antibiotics like penicillins, cephalosporins, and carbapenems (Frere 1995). Any of the inhibitors are not able to disrupt catalytic activity of MβLs representing serious clinical threat (Meini et al. 2013). Talking about classification of MβLs, they are divided into three subclasses, B1, B2, and B3,

and require one or two Zn (II) ions present inside protein that should be catalytically active. B1 and B3 active site has tetrahedral Zn binding sites (ZnA) and a second tetrahedral/trigonal bipyramidal binding site (ZnB), which is also common in B2 subclass. Despite exact no. of metal ions required to cleave the antibiotics being unclear (Sgrignani et al. 2012), the B1 and B3 classes is catalytically active with use of one or two Zn ions (Crowder et al. 2006; Sgrignani et al. 2012). Besides, B2 subclass require single ZnB metal ion, and it is usually found in degradation of carbapenem antibiotics in disfunctioning resistant gram-negative bacteria (Vidossich and Magistrato 2014). QM/MM method-based calculation has been employed in better understanding of the mechanism in some family members of this enzyme (Ackerman and Gatti 2013; Dal Peraro et al. 2004, 2007; Diaz et al. 2000, 2001; Simona et al. 2007; Suarez et al. 2002; Wu et al. 2010; Zhu et al. 2013). Enzymatic mechanism study was performed by Simona et al. (2009) in degradation of CphA by *Aeromonas hydrophila*, which belong to B2 subclass.

QM (Car–Parrinello)/MM MD simulation were performed to study hydrolysis reaction for different water content in initial active site taken from the crystallographic structure that contains CphA and partially hydrolysed biazapine complex. The MM part was treated using AMBER force field, while the QM region was treated at DFT/B3LYP level (Becke 1988; Zhao et al. 2012). Model ES1 considers coordination of Zn ion by Cys221, Asp120, and His263 with carboxylate group of Bia completing its coordination sphere. In model ES2, carboxylate group is replaced by Wat-A, but both models have Wat-B molecule placed right in between the His118, which are supposed to be possible H-bond acceptor during first reaction step.

Modeling of reaction mechanism with thermodynamic integration revealed in ES1 is the Asp120 residue, which activates the attacking water molecule by deprotonating it from hydroxide nucleophile. After this step, nitrogen of the β-lactum moiety gets coordinated with Zn ion by displacing carboxylate group of Bia. The barrier for this step is found to be 15 ± 3 kacl/mol. Subsequent intermediate formed after TS has a distorted tetrahedral geometry with Cys221, His263, nitrogen of β-lactum, and carboxylate in its coordination sphere. Second protonation step is mediated by Asp120, and it transfers hydrogen that it has abstracted in first step to the N of β-lactum with energy barrier 15 ± 2 kcal/mol; hence, overall reaction barrier is nearly 24.3 kcal/mol. The data produced here is inconsistent with experimental barrier that is reported to be 14 kcal/mol, whereas in ES2 complex, there is a synchronous exchange of proton, which leads to product formation in single step with energy barrier 15 ± 3 kcal/mol. The process in the chronological order involves deprotonation by His118 of Wat-B, protonation of N1 by Wat-A, readily becoming Zn-bound hydroxide and last transfer of proton from His118 to the Zn bound hydroxide to restore Wat-A. This mechanism has striking similarity with the work performed by Dal Peraro et al. (2007) for B1 catalyzed MβL CCrA in complex with cefotaxime. The modeling results indicate that ZnB is of prime importance in hydrolysis of β-lactam antibiotic, and this structural motif could be the target for drug design studies of inhibitors for different MβLs (Vidossich and Magistrato 2014) (Fig. 4.7).

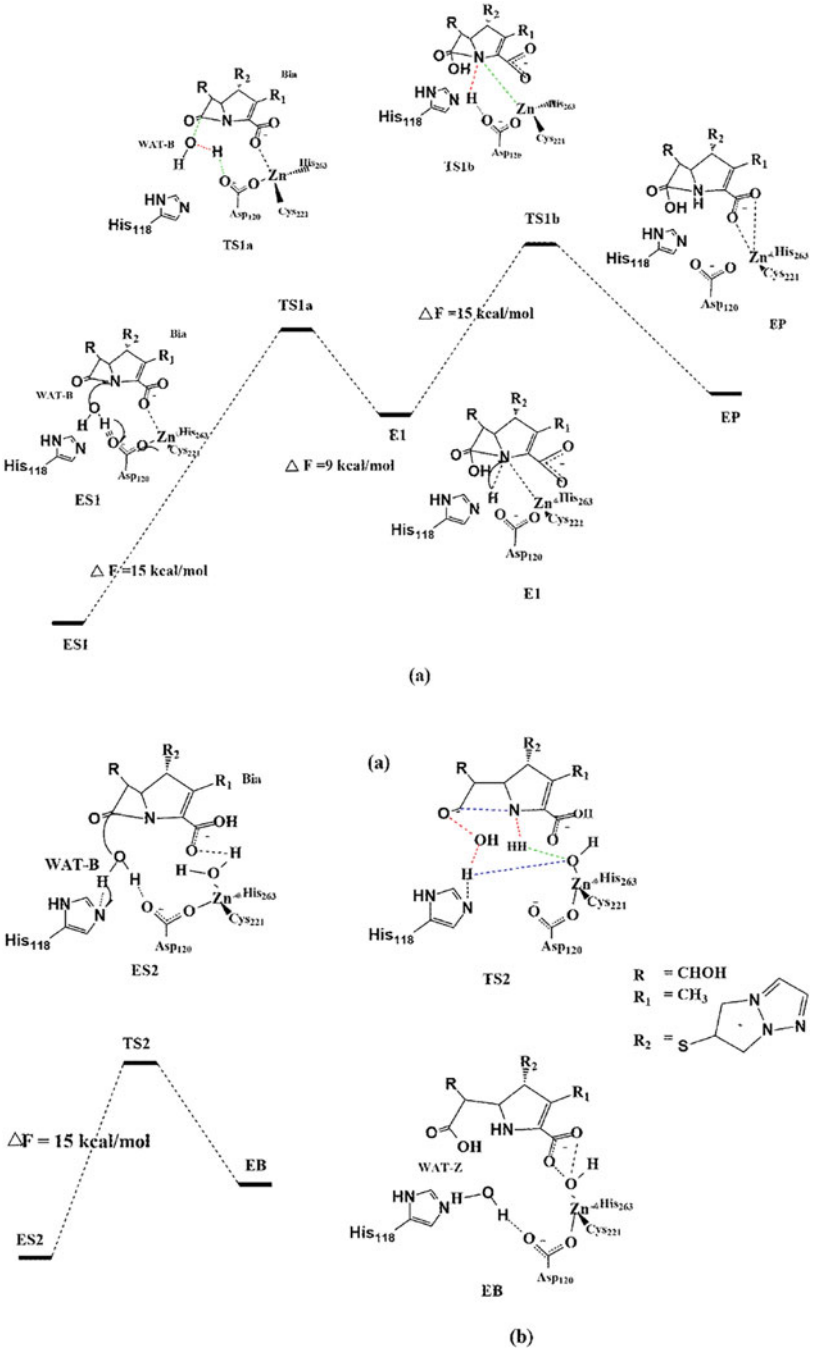


Fig. 4.7 Reaction mechanism of biapenem hydrolysis proposed in. In the transition states, bonds that are formed or broken are indicated as red and green dashed lines, respectively. The two-step and one-step reaction mechanisms are shown in (**a**) and (**b**), respectively

4.6 Summary

Understanding reaction mechanisms of metalloenzyme is important challenges in the drug industries. With known reaction mechanisms, the drug/substrate may be modified for efficient action. Since these reactions are much fast, its mechanism cannot be identified and sometimes two or more experimental setups come up with controversies. With the help of modern day computational tools, these controversies can be resolved, and also the short lived intermediates can be easily characterized. Hence, the interplay between theory and experiment will play important role for the studies of enzyme reactions.

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Past, Present, and Future Perspective of Biomedical Innovation in India

5

Joseph L. Mathew and Thalakkotur Lazar Mathew

Abstract

Innovation to design, develop, and deploy biomedical technologies, for use in health care, has been actively pursued in our country for several decades. Excellent work done by individuals, institutions, and organizations laid the seeds for a biomedical innovation culture in the country. Some of these seeds resulted in revolutionary technologies such as the Jaipur foot, Kalam-Raju coronary stent, Chitra heart valve, and SBMT ventilator. In addition, organized efforts by various governmental agencies and research projects in engineering and medical institutions have given further impetus for innovation to develop biomedical devices. At the same time, these initiatives have taught several hard lessons for current and future innovators. The exigencies created by the COVID-19 pandemic demanded an accelerated pace of innovation to address the urgent needs and uncertainties created by the pandemic. This led to extremely rapid revival, refinement, and recycling of existing technologies. However, most of the so-called COVID-inspired innovations in the country are merely imitations, improvisations, and improvements in existing technologies. Very few can be classified as novel innovations, and almost none are inventions. Failure of these technologies to address the real needs of end-users, limited laboratory testing, absence of clinical validation, and infringement of intellectual property rights makes them unsuitable for use in the healthcare sector. This represents a tremendous wastage of human, material, and financial resources. The way forward is for

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innovators (and institutions/organizations facilitating innovation) to consider all aspects of the innovation cycle, starting from the generation of ideas to address the real needs of end-users, designing of prototypes, laboratory testing, clinical validation, regulatory approval, commercialization, and post-marketing surveillance. A simplified framework—the KNOW ESSENTIALS algorithm—is presented here, which can help to consolidate the gains of past innovation efforts and encourage genuine innovation in the country.

Keywords

Biomedical innovation · Healthcare technologies · Innovation pathway · Innovation ecosystem · COVID-19

5.1 Introduction

Biomedical innovation to design, develop, and deploy healthcare technologies has been active in our country during the past few decades. Excellent work done by individuals, institutions, and organizations laid the seeds for a biomedical innovation culture in the country. Some of these seeds resulted in the world-famous Jaipur foot, Kalam-Raju coronary stent, floor-reaction orthosis, CytoScan, Chitra heart valve, and SBMT ventilator. In addition, organized efforts by the Society for Biomedical Technology (SBMT), National Program on Smart Materials, Structures and Systems (NPSM, NPMASS), Stanford India Bio-design program, National Hub for Healthcare Instrumentation Development (NHHID) Chennai, and Biomedical Instruments and Devices Hub (BIDH) Chandigarh have paved the way for developing innovative technologies. Besides, research projects funded by the Department of Science and Technology (DST) through Indian Institutes of Technology (IITs), Indian Institute of Science (IISc), and some Engineering and Medical institutions have given further impetus for innovation to develop biomedical devices. However, many of these efforts have resulted in reports and publications, rather than products and processes. At the same time, these initiatives have taught several hard lessons for current and future innovators.

The exigencies created by Coronavirus Diseases 2019 (COVID-19) (World Health Organization 2020a) put a spotlight on the need for rapid innovations to urgently meet the needs created by the pandemic (World Health Organization 2020b). At the time of writing this, there are almost 80 million cases worldwide, with 1.75 million deaths (COVID-19 Coronavirus pandemic 2020). India alone has over 10 million cases, and our country occupies the second place in terms of the number of people affected by the virus (COVID-19 Coronavirus pandemic 2020). The full impact of the virus goes beyond the number of cases and includes adverse impacts on the economy, healthcare system, and political discourse. In fact, the pandemic has dramatically altered every aspect of life. However, a proverbial silver lining in the dismal cloud caused by the pandemic is the innovation in various fields. In fact, the wave of innovation has even been declared as a "benefit" of COVID (Luo and Galasso 2020; Sahasranamam 2020).

This chapter highlights some of the important innovations that have taken place in India over the past few decades and the lessons to be learnt from them. It also provides a glimpse into COVID inspired "innovations" and their limitations. Finally, it presents a way forward to encourage true innovation in the biomedical field.

5.2 Background to Biomedical Innovation in India

The paradigm of healthcare technology acquisition in India is neatly summarized in Fig. 5.1 adapted from lectures by Prof. T Lazar Mathew (n.d.). Traditionally, biomedical technologies used in health care were (and to a large extent, still are) "imported" from other countries. For many decades, these imports occurred from developed countries, although recent years have witnessed lower cost importations from some developing countries also. The costs related to dependence on imports resulted in efforts at "imitation" of foreign technologies. The euphemism "reverse engineering" is applied to the process of imitating an imported technology, disregarding the intellectual property issues, and attempting to recreate the technology in the local setting. Success of imitation efforts was naturally restricted to low-end health technologies. However, the entrepreneurial spirit of Indian industrialists and scientists led to the next stage, viz., "improvisation." These improvisations occurred on imported and imitated technologies initially and gradually led to efforts at developing low(er) cost solutions to local needs. However, the problem with improvised technologies (colloquially referred in Hindi as *jugaad*) is the lack of consistency, unclear safety, and limited responsibility of developers. The focus of improvisation is restricted to quick-fix solutions, without concomitant emphasis on long-term sustainability.

The next step in the pathway, viz., "indigenization" of biomedical technologies resulted in the creation of several medium-tech, medium-cost biomedical products, with technologies from other spheres extrapolated to health care. This not only made

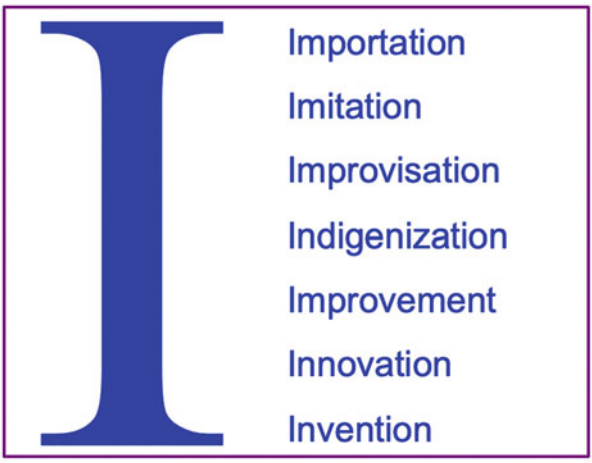

Fig. 5.1 Healthcare technology acquisition in India. (Source: Lectures on Health Care Management by Prof. (Dr.) T. Lazar Mathew)

India less reliant on imported technologies but also enabled the country to export some technologies to other resource-limited countries. However, high(er)-end healthcare technologies remained outside the ambit of such efforts. Indigenization led to "improvements" with adaptations, to make them applicable for use in the local context. The penultimate step toward self-reliance in healthcare technology is "innovation." This is the process wherein novel technology components are developed, or existing technologies are adapted for novel uses. Biomedical innovation in India started a few decades back and has picked up pace very rapidly in the last 20 odd years. The final step of self-sufficiency, viz., "invention" of new technologies remains largely out of reach at present. Capacity and capability of a country to invent technologies usually coincides with the stage of becoming fully developed, hence India is expected to follow this trajectory in the ensuing years.

5.3 Biomedical Innovation in India: Lessons Learned from the Past

A careful study of the past efforts in biomedical innovation is crucial to understand the current efforts for innovation, especially in the context of COVID-19. Some of the landmark developments are summarized here. These are highlighted to emphasize lessons for current and future innovators, rather than being a compendium of salient innovations.

The Jaipur Foot Dr. P K Sethi and Sh Ram Chandra Sharma designed a revolutionary prosthesis for people with foot amputations, as far back as 1968 (Jaipur leg 2020). At that time, such people had limited options that were mostly unwieldy, had limited functionality, and were uncomfortable. The Jaipur foot prosthesis was made of polyurethane resulting in lighter weight and water resistance. It also had an aesthetic appeal as it was designed to look and function like a normal foot. From the medical perspective, the prosthesis was far superior to then available prostheses, because it offered greater range of movements in the amputated foot (including inversion-eversion and dorsiflexion). Below-knee amputees were able to sit, run, squat, climb, walk, and swim, thereby dramatically improving the quality of life. Many of these functions could be performed by above-knee amputees as well. The total contact socket provided better sensory feedback to the wearer and prevented edema (Jaipur leg 2020).

In addition to the revolutionary prosthesis, the innovative duo also built a novel delivery platform for this great technology. Through this, the prosthesis was provided free of charge, at the center in Jaipur. Patients could walk in without prior appointment, and the complete process from assessment, fitment, and discharge home was completed within 1–3 days. The center even provided board and lodging to poor patients (Jaipur foot 2020). Not surprisingly, the Jaipur foot was widely acclaimed as one of the greatest innovations of the twentieth century (Bhargava 2019). To this day, it is a sterling example of an innovation ecosystem comprising

(1) assessment of need, (2) product design and development to address the actual need, and (3) a platform to deliver the health technology to the end-users.

Society for Biomedical Technology (SBMT) This was an interministerial initiative encompassing the Defence Research and Development Organization (DRDO), Department of Science and Technology (DST), Department of Rural Development, and Ministry of Health. The Society was formed in 1993 under the leadership of Dr. APJ Abdul Kalam (Founder Chairman) and Dr. Lazar Mathew (Founder CEO), with the goal of bringing the benefits of high-end technologies developed for defense, space, atomic energy, etc. to the common man (Society for Biomedical Technology 2020; Defence technology spinoff for healthcare 2020). The Society was successful in creating many landmark technologies, including Kalam-Raju coronary stent, floor reaction orthosis, CytoScan, laparoscopic surgery training simulator, slit lamp microscope, eye laser, cardiac stress test system, titanium bone and dental implants, cochlear implant, and speech enhancer (Affordable healthcare technology 2020). One of the most important developments was the indigenous ventilator, which became very important during the COVID pandemic.

Kalam-Raju Coronary Stent This product was envisioned to make coronary stents affordable in India. The DRDO provided material and manufacturing inputs, and the Nizam's Institute of Medical Sciences (NIMS) provided design and testing inputs (Kalam-Raju coronary stent 2020). The stent was initially manufactured by ancillary units of the defence sector, hence the cost of development, manufacturing, marketing, etc. were absorbed. Thus, the product could be sold in the market for as little as Rs 15,000/- compared with more than Rs 80,000/- for imported stents. Clinical efficacy was established through real-world use in patients at NIMS (Kalam-Raju stent–Missile Man's invention saved many lives 2020). The initial high demand from hospitals resulted in plummeting of the prices of imported stents also. However, being a technology development, there was no marketing strategy or business model for the stent. Hence, aggressive marketing by commercial manufacturers and the practice of offering commissions to physicians rapidly built-up resistance even in government hospitals (Krishna 2020). The availability of imported drug-eluting stents finally elbowed the indigenous stent out. Thus, an indigenous novel technology completely faded out of existence in the country, although some other countries readily accepted it.

SBMT Floor Reaction Orthosis (FRO) This was developed as a remedy for polio patients (having quadriceps paralysis) (Kalam 2020). It was based on the cantilever principle, wherein application of body weight creates reaction forces from the floor, causing turning movement locking the knee joint (Floor reaction orthosis 2020). The extremely light weight of the calipers (350 g compared with 3.5 kg of existing calipers) was achievable by using glass filled polypropylene (Menon 2020). Working in a mission-mode manner under the inspiration of Dr. Abdul Kalam, over 50,000 units were manufactured and distributed free-of-charge to polio patients (Tanwar and Kumar 2015). The Artificial Limb Manufacturing Corporation of

India (ALIMCO) was the main agency involved in production and distribution (Lower limb orthotics calipers 2020). However, this excellent biomedical innovation was also edged out by commercial manufacturers with stronger marketing tactics.

CytoScan This was a computer-based system capable of acquiring and analyzing the image of single cells, to detect and classify cell abnormalities based on image processing and pattern recognition (Cytoscan 2020). Statistical programs to analyze large volumes of cell data were also developed, with the ultimate goal of automated classification of cells as normal or abnormal. In a sense, this was one of the first steps in India, to use artificial intelligence in diagnosis of cervical cancer (Defense bio-engineering and electro-medical lab 2020). The technology was validated at two leading oncology institutions in the country and field tested by screening rural women for cervical cancer, in two districts of Andhra Pradesh under Project Tulasi (Mudur 2020). Besides the technological revolution, this was a unique model of doorstep healthcare delivery to a highly disadvantaged population. However, this program also faded away due to lack of marketing strategies.

Ventilator As far back as 1995, the SBMT conceived a project to develop an indigenous ventilator. The development team comprised Dr. T. Lazar Mathew (representing SBMT), Sh Arun Joshi (DMRL/DRDO Hyderabad), Dr. Gourie Devi and GS Umamaehswara Rao (NIMHANS Bangalore), and engineers from PSG College of Technology (Dr. P. Radhakrishnan, Dr. PV Mohanram, Dr. PC Angelo, Sh. S Benedict Biju, and Sh. P Gopishankar). The team designed a proof-of-concept model (Fig. 5.2a) and a bench model (Fig. 5.2b) that were together used to create a working prototype. The prototype was evaluated for clinical use at NIMHAS Bangalore (Fig. 5.2c, d). Following successful clinical trials, the ventilator was launched on 31 August 2002 (Angelo PC, personal communication). Thus, the entire process from conception of the novel idea to a clinically useful product was completed within 7 years. The technology was transferred to a Coimbatore-based company, Pricol Medical Systems, which successfully developed a commercial model. Later, Pricol Medical Systems transferred the technology to M/s Skanray of Mysore (Ventilator 2020; Biju B 2020), which was able to market a sizeable number of units in competition with imported ventilators. Almost two decades after its rollout, the COVID pandemic revived interest in this indigenous ventilator. The company received a contract for delivering 30,000 ventilators to meet the exigencies created by the pandemic, bringing to fruition the seeds of innovation sown around three decades previously.

The next step was to develop a critical-care ventilator (Critical care ventilators 2020). This was achieved successfully by designing and developing Inventa—a volume and pressure controlled model with mandatory and assisted breaths (Kannan 2020). It was priced at approximately 30% of the comparable imported product. Subsequently, the DST sanctioned a project to develop an infant ventilator, which was also successfully developed (Fig. 5.3). The foundation for the current indigenous critical-care ventilators were thus laid, several years before the pandemic created a necessity.

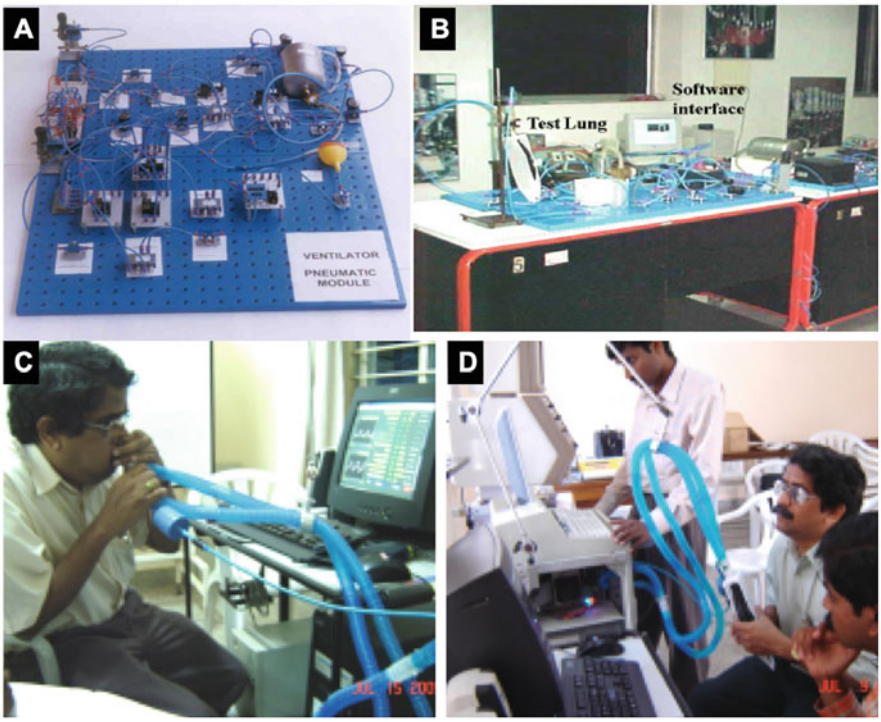

Fig. 5.2 Design and development of India's first indigenous ventilator

Chitra Heart Valve Prof. M.S. Valiathan successfully developed a state-of-the-art cardiac valve at the Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST) (Chitra heart valve 2020). The indigenous heart valve had several unique features including in-vitro noise reduction, blood flow resistance reduction, greater durability on account of high quality materials, and good manufacturing (Raghu 2020). The cost was almost a quarter of the imported counterpart, making it a great leap in indigenization of high-end, critical-care technologies (Sankarkumar et al. 2001). This revolutionary indigenous valve has stood the test of time for nearly three decades. Subsequently, an oxygenator and blood bag were also developed by the same team and marketed widely.

Real Time Quantitative Automated Micro-PCR Systems BigTec Bangalore under the leadership of Dr. Chandrashekaran Nair successfully developed battery-operated diagnostic platforms using miniature Bio-MEMS-based "PCR-on-Chip" technology, which were marketed by Molbio Diagnostics Pvt. Ltd. (Mobio diagnostics for the real world 2020). This has the potential to revolutionize the field of diagnostics with accurate diagnosis, rapid turn-around time (sample to result in less than 1 h), and detection of multiple diseases at one go. The initial experience with diagnosis of tuberculosis (TrueNAAT) suggests that simplified sample

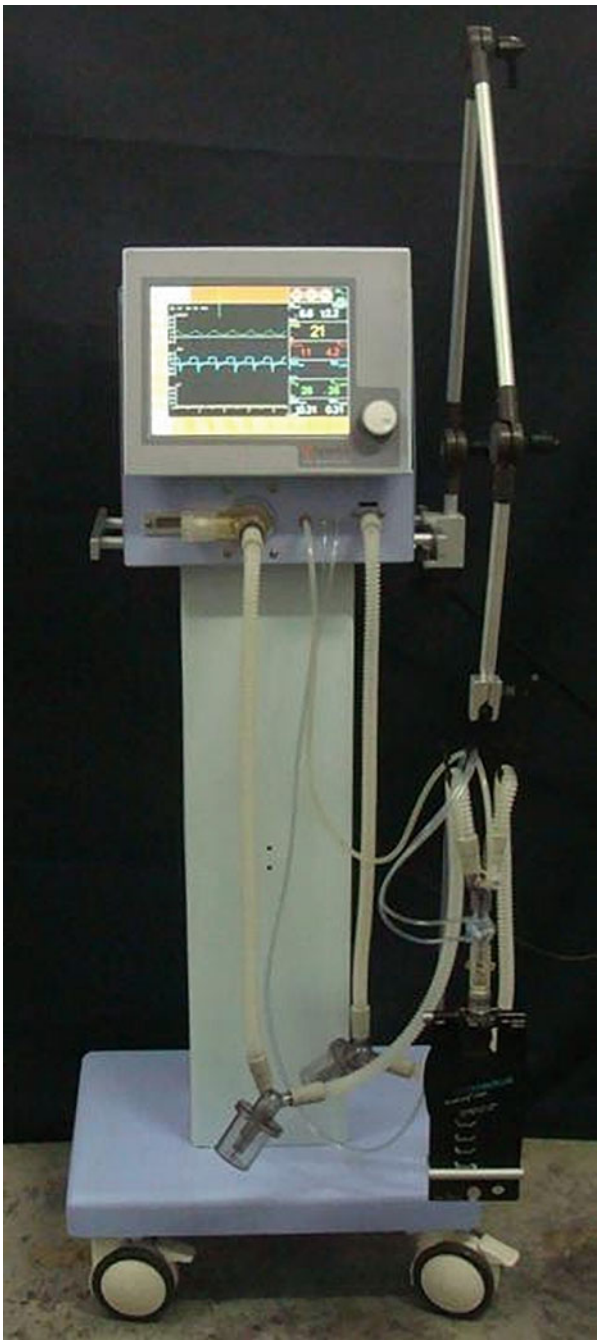

Fig. 5.3 Indigenous infant ventilator

processing, absence of the need to refrigerate test reagents, and non-dependence on electricity could make it ideal for point-of-care diagnosis in nonhospital settings as well (Truenat™ MTB-RIF Dx 2020; Molbio publications 2020). The innovations in this field paved the way for development of similar diagnostic platforms for other infectious diseases, including COVID-19.

5.4 Contemporary Advancements in Biomedical Innovation

Stanford India Biodesign Program This was a unique program led by Prof. Balram Bhargava at AIIMS New Delhi, in collaboration with IIT Delhi, through international leadership and support provided by Stanford University (Stanford-India biodesign 2020). The program was designed to bring together medical professionals and engineering/technology experts to design novel solutions for local needs and then take it to the next level by building a business model to manufacture and market the products. Thus, the program aimed to take novel technologies to the end-user. A few start-up companies were established to make this a reality. Some of the better-known technologies from this program include fecal incontinence device, limb-immobilization device, neonatal resuscitation device, patient transfer device, fetomaternal parameter monitoring system, portable hand sanitization device, auditory impairment screening device, soft tissue biopsy device, abdominal paracentesis device, assistive device for enteral tubes, and aspiration device. However, only a few of these are in actual use in clinical environments (Million+ patients helped 2020).

National Program on Smart Materials, Structures, and Systems (NPSM and NPMASS) This unique program spear-headed by the Ministry of Defense was initiated to address the burning need for indigenous technologies for the country across multiple streams (Aatre 2020). One stream was dedicated to health care (including medical technologies). Coining the slogan "Project for a Product," Dr. Lazar Mathew was able to translate several innovative ideas to commercially viable healthcare products within a short span of time. Some of the salient innovations include uropathogen detection and antibiotic sensitivity sensor with a ready-to-use kit for rapid culture of uropathogens (developed by Prof. Suman Kapur at BITS Pilani), lab-on-chip for cardiac markers using the micro-cantilever principle to detect antigen-antibody interaction by measurement of voltage fluctuation (developed by Prof. Ramgopal Rao at IIT Bombay,), miniature drug delivery systems, noninvasive detection of analytes, and microfluidic-based micro-PCR (Sukshma 2020).

National Hub for Healthcare Instrumentation Development (NHHID) Chennai The Department of Science and Technology established the NHHID at Anna University, Chennai, to harness the capabilities and skills of engineers and industrialists for healthcare needs in the country (NHHID 2020). The Hub has succeeded in developing commercial prototypes of healthcare devices, undertaking R&D services and product validation from healthcare industries, providing

calibration and testing of medical devices with certification, and manpower training. Some of the salient innovations with potential for clinical use include data acquisition system for neuromuscular and cardiac parameters, antibiogram device, RFID-based infant theft prevention system, hand-held milk adulteration detector, and ophthalmic lesions detector (software to analyze fundus images) (NHHID healthcare device innovations: the gateway for healthcare product realization 2020).

Biomedical Instruments and Devices Hub, Chandigarh Recognizing the potential for clinician-driven innovative development of healthcare devices, the DST recently established this Hub at PGIMER Chandigarh (Biomedical instruments and devices hub: a centre for innovation, design and clinical validation 2020). The focus of this Hub is to identify unmet clinical needs that can be solved through indigenous innovation, test prototypes beyond the laboratory in a real-world clinical environment, and bridge the gap between engineering/technology solutions and clinical needs of users (viz., healthcare professional and consumers). Salient developments through the Hub include the Artificial Breathing Capability Device, machine for double volume exchange transfusion in neonates, infusion alarm, cricoid pressure sensor, device to estimate integrity of intestinal mucosa by measuring impedance, a novel device for use with endoscopic ultrasonography to treat intra-abdominal cysts, virtual reality-based rehabilitation programs for children with neuromuscular problems, hand-held indigenous pupillometer, and a teaching aid to assist endotracheal intubation (Biomedical instruments and devices hub: a centre for innovation, design and clinical validation 2020).

Closed Loop Anesthesia Delivery System (CLADS) Prof. GD Puri at PGIMER Chandigarh, developed the first-of-its-kind computer-based anesthesia management system (Puri 2020), wherein depth of anesthesia measured by EEG electrodes is communicated to infusion pumps delivering anesthetic agents. This enables an automated control to maintain the desired level of anesthesia in patients. It has been clinically validated in a variety of patients undergoing various surgical procedures under general anesthesia (Puri et al. 2007; Mahajan et al. 2017). CLADS has also opened up the possibility of automating the delivery and control of other medications using electronic monitors to guide dosage, flow-rate, etc.

AgVa Ventilation Device This ventilation device was developed by an engineer, Diwakar Vaish in collaboration with Prof. Deepak Agrawal of AIIMS New Delhi (AgVa ventilator 2020; AgVa Healthcare 2020). The device is capable of ventilation without using compressed air and can be trained to deliver ventilation based on patients' respiratory patterns and volumes. A display unit linked to an app permits easy visualization of ventilation graphics, notably breathing curves and lung volumes. It is claimed that the ventilator consumes only 100 watts of energy and hence can run on a small battery. One of the main applications of this device is to provide ventilation for normal lungs (as in patients with neurological brain injury). The innovators also claim that it can be used safely at home as it does not require an ICU setup for its operation. However, the technical specifications of these ventilators

are not available in the public domain. The company has also developed different version of their basic ventilator, and there was high hope that it would be useful for COVID patients (Srivastava 2020).

Artificial Breathing Capability Device (ABCD) This device developed in 2018 by Prof. Joseph L Mathew at PGIMER Chandigarh, in collaboration with Prof. Manu Sharma (Panjab University), Dr. Navin Kumar (IIT Ropar) and Dr. Sukesha (Panjab University) is the country's first clinically validated automated device to replace manual ventilation (Mathew et al. 2018). The device enables physicians to automate the compression of self-inflating bags (such as Ambu bags) at preset peak inspiratory pressure, preset ventilation rate, and preset inspiration time. Thus, the device provides several features of a ventilator, at a much lower cost (WIPO IP Portal 2020). In addition, the smart device has inbuilt safety features, including cough alert, leak alarms, and disconnection alarms. ABCD underwent extensive laboratory testing for efficacy and safety including 396 combinations of user settings suitable for adult and pediatric patients in 2019 (Mathew et al. 2020). Laboratory testing also included nonstop functioning for 60 continuous days (Mathew et al. 2020). Thereafter, the device was also tested on a patient simulator in PGIMER Chandigarh, followed by clinical trials in real patients in the Emergency ward at PGIMER (Artificial breathing capability device 2020). The design of ABCD has been liberally imitated during the COVID-19 pandemic, but none of the imitated designs have been successful (Fig. 5.4).

Besides the above examples, considerable efforts were also made at IIT Delhi (by Dr. SK Guha and Dr. Sneh Anand), IIT Bombay, and I.I.Sc, to develop biomedical devices and equipment to meet the needs of the country. These prototypes need to be clinically validated and marketed.

5.5 Lessons Learnt from Pre-COVID Biomedical Innovation in India

It is important to draw lessons from the pre-COVID era of biomedical innovations in India, in order to understand the challenges, limitations, and potential for innovative healthcare solutions in the country. Despite a plethora of innovative ideas, imitations, and improvisations, fueled by ingenuity, imagination, and enthusiasm, there are very few products that have been successfully commercialized. The proportion of commercialized products that are in actual use by healthcare professionals is even smaller. One of the major reasons for this is that most of the innovations are merely technology solutions without considering end-user needs (Mathew 2019). Naturally, such products are not welcomed for use in healthcare settings. Very few innovative products have been adequately tested in the laboratory for robustness, precision, and reliability. In fact, most have undergone only cursory lab validation at the prototype stage. Naturally, almost none of the innovative products have been clinically tested in patients or hospitals or other clinical settings. This deficiency makes physicians wary of using these technologies (Mathew 2019).

Fig. 5.4 Artificial Breathing Capability Device (ABCD) being used in a child

The other important lessons are the need to have a strong scientific background, with consideration of all aspects of the innovation cycle (from an idea to a prototype, to commercialization and post-marketing surveillance) (Mathew 2018). A mission-mode approach and multidisciplinary teamwork is required to make a commercially viable product. The failure of several useful technologies to be commercially successful showed that a clear business plan (commercial model) is essential to ensure that innovative technologies are not crushed by market forces. These considerations are essential to ensure that the full benefit of indigenous technology reaches the common man.

5.6 "Innovations" Stimulated by the COVID-19 Pandemic

The onset of the COVID-19 pandemic witnessed a flurry of innovations geared toward the pandemic. The term "innovation" is loosely applied as most of the products are at best, merely imitations, improvisations, and indigenization efforts. The range of technologies claimed to be innovations or inventions have been recently compiled in a detailed review article by the authors (Mathew and Mathew

Table 5.1 Developments in response to the COVID-19 pandemic hailed as innovations

Potentially life-saving technologies • Critical-care ventilators • Devices to automate manual ventilation • Devices to provide humidified oxygen at high flow rates • Adaptors for splitting ventilator output • Oxygen delivery splitters
Critical, but not life-saving technologies **Personal protective equipment (PPE) products** • Face masks • Face shields and splash guards • Intubation boxes • Aerosol guard boxes
Sanitation products and solutions • Indigenous hand sanitizers • Ultra-violet light emitters • Ozone • Silver-based products • Others
Solutions to reduce contact • Foot-operated sanitizer dispensers • Contactless sanitizer dispensers • Personal guards at desks, counters, etc. • Automated door openers • Foot-operated door openers
Diagnostic testing kits • Indigenous antibody-based testing kits • Indigenous antigen detection kits • PCR based diagnostic kits • Micro-PCR kits • Loop-mediated isothermal amplification (LAMP)
Information Technology solutions • Tele-health solutions (conventional and adapted versions) • Tele-education solutions (for professionals, students, public, etc.) • Tele-meeting solutions (i.e., platforms for communication) • Remote patient monitoring systems repurposed for COVID • Apps for various applications
Repurposing/Retrofitting/Adapting existing technologies for healthcare applications • Wearable devices adapted for COVID monitoring and/or tracking • GPS enabled technologies adapted as COVID trackers • Artificial intelligence (AI)–driven platforms for automation of radiological diagnosis, patient monitoring, environmental monitoring, etc. • Robots for contact-less delivery of medications, etc. • Drones for sanitization, transportation of small objects, and public health messaging

2020), hence are not replicated here. A broad list is summarized in Table 5.1. Besides these, there are numerous apps designed for various functions related to management of COVID-19.

Ventilators Early in the pandemic, the government estimated that approximately 75,000 ventilator units would be required in the country within 3 months. The public sector company HLL Lifecare Limited was tasked with arranging 60,000 ventilators through various local companies (Sharma et al. 2020). The long standing need to have a license to produce ventilators (as essential medical equipment) was waived off.

At that time, Skanray Technologies had the technical know-how to manufacture the SBMT ventilators (described previously). However, the capacity to deliver the government order of 30,000 units within a few weeks was lacking. A collaboration with Bharat Electronics Limited (BEL) made this possible by diverting huge amounts of workforce, raw material, supplies, and floor space. Thus, the BEL-Skanray Pavan CV 200 ventilators were produced (Sharma et al. 2020). This ventilator was also equipped with a robust battery permitting electricity-free operation for several hours, and an inbuilt compressor made it possible to function without an external source of compressed air. It is theoretically able to deliver tidal volume as low as 50 ml, hence could be used in children also. In short, the manufacture and deployment of these ventilators for the COVID pandemic were not innovative, but upscaling of production.

Technical know-how of a somewhat different nature was also available with AgVa Healthcare, which received an order to deliver 10,000 units within a short span of time. This was facilitated by collaboration with Maruti Suzuki Limited. Similarly, the Andhra Pradesh MedTech Zone (AMTZ) was tasked with producing another 13,500 ventilators (Sharma et al. 2020).

In addition to mass-production of ventilators based on pre-existing technology, several manufacturing companies were able to provide parts/components to be incorporated into the ventilators. Many of these parts could be produced using 3-D printing technology (Sharma et al. 2020).

Respiratory Support Devices The COVID pandemic spawned the imitation and improvisation of a slew of devices to mechanize and/or automate the compression of self-inflating bags (SIB), such as Ambu bags. Although the concept of mechanizing the compression is not novel (Bergman 2020a, 2020b), the effort to provide a high degree of precise control for successfully ventilating patients was pioneered by the Artificial Breathing Capability Device (ABCD) discussed previously. In response to the anticipated shortage of ventilators, many individuals and institutions attempted to replicate the design of ABCD (Indian Institute of Technology Kanpur: Standard Chartered Bank first entity to fund ventilator prototype by IIT Kanpur 2020; BW Online Bureau 2020; Bora 2020; Watch: how the world's lowest-cost 'made in India' ventilator 2020; Indian Railways develops low-cost ventilator 'Jeevan', seeks ICMR approval 2020; Nanda 2020; Hyundai Motor India develops automatic AMBU bag actuator prototype 2020; Rana 2020; Karnataka based startup comes up with automatic AMBU bag 2020; SCTIMST ties up with Wipro 3D to make automated ventilators 2020; COVID-19: NIT-Durgapur builds artificial breathing device 2020; Khanna 2020). However, unlike ABCD, all these devices failed to consider and correct the main problems of manual ventilation, viz., lack of control over compression pressure, the need for plateau pressure during the inspiratory phase, positive end-expiratory pressure, ventilation volume, and dyssynchrony

with patient events such as cough, spontaneous breaths, etc. Despite these limitations and the complete lack of clinical validation, prototypes of several such devices were widely publicized in the lay media and social media platforms (Indian Institute of Technology Kanpur: Standard Chartered Bank first entity to fund ventilator prototype by IIT Kanpur 2020; BW Online Bureau 2020; Bora 2020; Watch: how the world's lowest-cost 'made in India' ventilator 2020; Indian Railways develops low-cost ventilator 'Jeevan', seeks ICMR approval 2020; Nanda 2020; Hyundai Motor India develops automatic AMBU bag actuator prototype 2020; Rana 2020; Karnataka based startup comes up with automatic AMBU bag 2020; SCTIMST ties up with Wipro 3D to make automated ventilators 2020; COVID-19: NIT-Durgapur builds artificial breathing device 2020). Naturally, these devices were unsuitable for clinical use and attempts to do so resulted in mishaps (Singh 2020; Outlook Web Bureau 2020; 'Approved by centrally accredited lab': Gujarat govt defends locally developed 'Dhaman-1' ventilators 2020).

Recognizing the dangers posed by the use of such devices in clinical settings (Singh 2020; Outlook Web Bureau 2020; 'Approved by centrally accredited lab': Gujarat govt defends locally developed 'Dhaman-1' ventilators 2020) and the absence of technical specifications for such devices (to guide innovators and users), recently a set of detailed technical specifications was published by the lead author of this chapter (Mathew 2021). These are summarized in Table 5.2 and encompass both efficacy and safety parameters.

Most of the other so-called innovations in response to COVID, listed in Table 5.1, are mere imitations and improvisations of existing (national and international) technologies, hence are not discussed further. A limited SWOT analysis of these innovation efforts is presented in Table 5.3.

5.7 Lessons from COVID-Related Innovations

Innovation should be regarded as a process, not only an outcome. In other words, the journey is as important as the destination, and the pathway to develop a product ultimately determines its usability. The process (journey) should ideally start with a detailed appraisal of the need for the new technology (Mathew and Mathew 2020). Assessment of need requires a thorough understanding of the problem and limitations of existing solutions. This requires active engagement of end-users of health technologies (viz., healthcare professionals and/or patients and/or public) from the inception of the innovation efforts. These stakeholders best understand the limitations of existing health technologies and hence the requirement for new/innovative technologies. They are also in the best position to assess the success (or failure) of innovative solutions.

Unfortunately, many innovators bypass this crucial step and develop prototypes based on their limited understanding of the problem and the needs of end-users. However, innovative prototypes require extensive laboratory testing, to be followed by clinical validation (for efficacy and safety) in real-world healthcare settings. Only then can such technologies be processed for regulatory approval and ultimately reach

Table 5.2 Efficacy and safety specifications for devices automating manual ventilation

Efficacy specifications	Safety specifications
• Device should provide pressure-controlled ventilation, or volume-controlled ventilation, or both modes • Devices merely compressing Ambu bags without measuring and/or controlling either pressure or volume should not be developed • Peak inspiratory pressure (PIP) in the range 10–40 cmH_2O • Tidal volume (V_T) in the range 200–800 ml for adults and 50–300 ml for infants/children • Ventilation rate (VR) in the range 10–30/min (for adults) and 15–50/min for infants and children • Inspiratory time (T_i) in the range 0.4–3.0 s (for adults) and 0.25–3.0 (for infants and children). The ratio of inspiration time to expiration time (I/E) should be in the range 0.25–1.0 • Positive end expiratory pressure (PEEP) in the range 5–15 cmH_2O • Set parameters should be delivered without lag (i.e., within the first two breaths) • Device should permit users to change desired ventilation parameters without switching off and restarting the system • Device should permit connection with an air-oxygen blender so that variable FiO_2 can be provided as per clinical need	• Compliance with established safety norms for electrical equipment, medical devices, ventilatory support equipment, and anesthetic equipment • Self-regulatory checks for mechanical integrity, electronic integrity, ventilation circuit integrity, and prevention of inputting nonphysiological ventilation parameters. • Auto cut-off if patient coughs during inspiration, followed by auto-resumption with the original settings • Real-time display of ventilation parameters delivered in each breath • Audio and visual alarms in case of deviation of delivered parameters from preset values • Audio and visual alarms in the event of ventilation circuit disconnection, endotracheal tube blockage, and SIB displacement • Battery charging status and available battery life display • Internal cooling system to prevent overheating of parts/fire hazard • For devices designed to be used in COVID-19 patients, exhaled air should be vented out without risk of environmental contamination • Laboratory testing for at least 60 days to ensure fail-safe functions

Table 5.3 SWOT analysis of biomedical innovations during the COVID-19 pandemic

Strengths	**Weaknesses**
• Multidisciplinary • Multi-sectoral • Flexible • Focus on products (rather than prototypes)	• Lack of originality • Duplication • Failure to analyze need • Limited lab testing • Absence of clinical validation
Opportunities	**Threats**
• Time available during the lockdown • Industry support • Funding (by govt agencies and NGOs) • Lay media encouragement • Social media platforms	• Lack of sustainability • Dependence on low-cost imported parts/raw materials • Disregard of intellectual property issues • Lack of regulation of innovation

the market. Thereafter, post-marketing surveillance is essential to ensure effectiveness (which is beyond efficacy) and on-going safety for the end-users (Mathew 2018; Mathew and Mathew 2020).

The COVID-inspired innovations in India were borne out of the desperate need for quick solutions, fuelled by lay media focusing on feel-good stories, and supported by financial and other support by governmental and private agencies. However, the emphasis on rapidly achieving outcomes resulted in short-circuiting the innovation cycle described above. The failure to consider the clinical (or public health) needs of end-users in the COVID-inspired innovation race has resulted in considerable wastage of resources (human resource, financial resources, material resources, and the most important resource-time) (Mathew and Mathew 2020). The desperation also resulted in a total disregard of intellectual property rights (IPR) issues, with two consequences. First, existing technologies were liberally plagiarized in the guise of responding to the pandemic. Second, plagiarized ideas were liberally disclosed in the lay press and social media platforms, creating difficulties for genuine innovators to patent their ideas, prototypes, or products.

The other serious consequence of short-circuiting the innovation cycle is the limited emphasis on safety. Quick-fix solutions for COVID generally focus on proving efficacy (i.e., the ability of the technology to work) without a concomitant emphasis on establishing patient safety. This is largely because confirmation of safety (in the context of healthcare environments) is cumbersome, expensive, and time-consuming. However, establishing safety of users of health technologies is critical, because many biomedical innovations can have (anticipated and unanticipated) adverse effects if they are not rigorously tested and designed to be fool proof. Table 5.4 summarizes an assessment of efficacy and safety of various "innovations" in response to the COVID pandemic.

The development of innovative respiratory support devices (by automation of Ambu bag compression) provides a classic case study of the two issues described above. On account of shortage of ICU ventilators, the high cost of these machines, and the need for an ICU set-up to use them, the actual "need" was (and is) for devices to ventilate patients safely at a lower cost. However, innovation efforts focused on developing devices that merely compress Ambu bags (at preset rate, pressure, etc.). Although this could be achieved very rapidly, it did not benefit the healthcare system as the clinical need (of ventilating patients safely) was not addressed. Consequently, most of these devices remained mere prototypes. Attempts to use these devices in clinical services without adequate testing for patient safety resulted in disastrous consequences (Singh 2020; Outlook Web Bureau 2020; 'Approved by centrally accredited lab': Gujarat govt defends locally developed 'Dhaman-1' ventilators 2020).

During the early phase of the pandemic, the inventors of the Artificial Breathing Capability Device (ABCD) were approached by government officials offering significant resources for rapidly scaling-up production of the device. At that time, ABCD was undergoing clinical validation in non-COVID patients; hence, the inventor team politely declined the offer until clinical efficacy and safety were confirmed. Further, ABCD was not designed for use in COVID patients, wherein the clinical requirements are somewhat different from many other conditions. However, these limitations did not deter the government agency from offering large

Table 5.4 Assessment of efficacy and safety of various innovations in response to the COVID pandemic

Box 1. COVID-19 inspired innovations in India	Availability of efficacy and safety norms for development	Potential risk for harm (due to limitations in efficacy and/or safety)	Laboratory testing done prior to release	Clinical validation conducted prior to release	In clinical and/or public health use
Critical (life-saving) equipment and devices					
Critical-care ventilators	Yes	Very high	Limited	No	Yes
Devices to automate manual ventilation	No	Very high	Limited	No	No
Devices to provide humidified oxygen at high flow rates	Yes	High	No	No	No
Adaptors for splitting ventilator output	No	High	No	No	No
Oxygen delivery splitters	No	Moderate	No	No	Yes
Oxygen generators	Yes	Moderate to high	Yes	Limited	Limited
Personal protective equipment (and components)					
Indigenous PPE kits	Yes	High	Yes	No	Yes
Indigenous face masks	No	Moderate to high	No	No	Yes
Indigenous N95 (and other specialized masks)	Yes	High	Yes	No	Yes
Face shields and splash guards	Yes	High	No	No	Yes
Intubation boxes	No	High	No	No	Yes
Aerosol guard boxes	No	High	No	No	Yes
Sanitization solutions					
Indigenous hand sanitizers	Yes	Moderate to high	No	No	Yes
Ultra-violet light emitters	Yes	Moderate to high	No	No	Yes
Ozone-based disinfection	Yes	Moderate to high	No	No	Yes
Silver-based products	Yes	Moderate to high	No	No	Yes
Other disinfectants	Yes	Moderate to high	No	No	Yes

(continued)

Table 5.4 (continued)

Box 1. COVID-19 inspired innovations in India	Availability of efficacy and safety norms for development	Potential risk for harm (due to limitations in efficacy and/or safety)	Laboratory testing done prior to release	Clinical validation conducted prior to release	In clinical and/or public health use
Sanitization tunnels	No	Moderate to high	No	No	Yes[a]
Disinfection chambers	No	Moderate to high	No	No	No
Hand hygiene testers	No	Moderate	No	No	No
Solutions to reduce contact					
Foot-operated sanitizer dispensers	No	Low	No	No	Yes
Contactless sanitizer dispensers	No	Low	No	No	Yes
Personal guards at desks, counters, etc.	No	Low	No	No	Yes
Automated door openers	No	Low	No	No	Yes
Foot-operated door openers	No	Low	No	No	Yes
Sampling kiosks	No	Moderate to high	No	No	Yes
Consultation kiosks	No	Moderate to high	No	No	Yes
Robots for contactless delivery of medications etc.	No	Moderate	No	No	Yes
Drones for sanitization, transportation of objects, and public health messaging	No	Moderate	No	No	Yes
Devices to reduce hand contact with face	No	Low	No	No	No
Social distancing tags	No	Low	No	No	No
Contactless dustbins	No	Low	No	No	Limited
Digital stethoscope	No	Low	No	No	Limited

(continued)

Table 5.4 (continued)

Box 1. COVID-19 inspired innovations in India	Availability of efficacy and safety norms for development	Potential risk for harm (due to limitations in efficacy and/or safety)	Laboratory testing done prior to release	Clinical validation conducted prior to release	In clinical and/or public health use
Wearable devices for remote monitoring of vital signs	No	Moderate	No	No	Limited
Technologies for diagnosis					
Indigenous antibody-based testing kits	Yes	High	Yes	Limited	Yes
Indigenous antigen detection kits	Yes	High	Yes	Limited	Yes
PCR-based diagnostic kits Micro-PCR kits	Yes	High	Yes	Limited	Yes
Loop-mediated isothermal amplification (LAMP) kits	Yes	High	Yes	No	No
Contactless thermometers	Yes	Moderate	Yes	No	Yes
Technologies claimed to be for treatment					
Indigenous remedies	No	High	No	No	Yes
Ayurvedic products	No	High	No	No	Yes
Home-based remedies	No	High	No	No	Yes
Vaccines					
Indigenous vaccines	Yes	High	Yes	Yes	Yes
Information Technology solutions					
Tele-health solutions	Yes	Low to moderate	Limited	No	Yes
Tele-education solutions	Yes	Low	No	Not applicable	Yes
Tele-meeting solutions	Yes	Low	No	Not applicable	Yes
Remote patient monitoring systems	Yes	Moderate	No	No	Yes
Apps for various applications	Variable	Low	Variable	Not applicable	Yes

[a] Now discontinued

grants to other innovators for mass producing similar devices. As expected, none of these imitation devices can be used in clinical settings.

The inventors of ABCD recently redesigned the device to make it suitable for use in COVID patients. They applied for a grant to another government agency inviting applications for COVID solutions. The comprehensive application included design and development of a new device along with rigorous laboratory testing followed by clinical validation. The proposal was readily approved; however, the timeline was slashed to 6 months (as the funding agency assumed that the pandemic would be over within that duration) and the project was sanctioned only for developing a prototype with limited testing (COVID-19: DBT-BIRAC supported products and technologies compendium 2020). This type of short-sighted focus on the innovation outcome (rather than the process) results in the situation wherein the innovation pathway remains incomplete and the benefit of the novel technology does not reach the people who need it.

5.8 Future Perspectives: The Way Forward

It is important to build up the foundation of biomedical and healthcare technology innovations of the past few decades and consolidate the limited gains from the recent COVID-related accelerated innovation. This will help current and future innovators to develop usable technologies, without repetition of past mistakes. The following framework (Table 5.5) adapted from the KNOW ESSENTIALS tool for health technology assessment (Mathew 2011) can help innovators, funding agencies, non-governmental organizations, and even the general public to design and develop innovative technologies that are appropriate for use. It can also help to build up the innovation ecosystem that was recently proposed for the country (Mathew and Mathew 2020). KNOW ESSENTIALS is an acronym wherein each letter represents a specific issue to be considered (Mathew 2011). The points mentioned should ideally be considered before innovation work begins but also can be used to assess an already-developed innovation, at any stage in the innovation cycle.

5.9 Conclusion

Innovation for producing useful and usable technologies for improving healthcare has been going on in our country for the past few decades. The excellent work by individuals, institutions, and organizations working singly or jointly has led to the development of several technologies with positive impacts on health care. These initiatives laid the seeds for a biomedical innovation culture in the country. At the same time, these efforts have taught several hard lessons for current and future innovators. The exigencies created by the COVID-19 pandemic demanded innovation due to multiple, urgent needs created by the pandemic. While this led to laudable efforts across disciplines, most of the COVID-inspired innovation in the

Table 5.5 Framework to assess and assist healthcare innovation in the country (adapted from the KNOW ESSENTIALS tool for health technology assessment)

	Criteria	Remarks for innovators intending to develop innovative technologies	Remarks for agencies (e.g., funding agencies) facilitating innovation
K	Knowledge	Is there sufficient knowledge of the problem?	Does the innovator team have an appropriate level of knowledge, experience, and expertise to understand the problem and work towards its solution?
N	Need	Is there a clear understanding of the needs of the end-user?	Have the innovators considered the need and does the innovative technology meet the real need? Has the problem been identified and defined appropriately?
O	Outcomes of interest	Are the outcomes clearly defined (as opposed to open-ended)?	Does the innovative technology meet the desired outcomes?
W	Who is the target	Are the target end-users identified?	Who are the potential end-users of the innovative technology? Can the target user base be broadened by different applications of the technology?
E	Evidence of effectiveness or efficacy	How will the effectiveness (or at least efficacy) of the innovation be objectively measured?	Is there evidence of effectiveness (or at least efficacy) in the target end-users w.r.t the outcomes of interest?
S	Evidence of safety	What are the safety considerations for end-users?	Have all safety considerations (for end-users) been considered, and does the innovative technology meet them? How has safety of end-users been established? Is there a risk of safety issues appearing later?
S	Social issues	Are there any ethical, social legal or other issues (ELSI) that can impact the innovation?	Have the innovators considered the potential ethical, social, legal, or other issues (ELSI) associated with the innovative technology development and/or deployment and/or its use in healthcare? Is there a risk of ELSI appearing late(r) in the innovation cycle?
E	Economic considerations	Is the innovation cheaper than existing solutions? If not, does it add value?	What are the economic implications of the innovative technology? Is the final price to be paid by the end-user

(continued)

Table 5.5 (continued)

	Criteria	Remarks for innovators intending to develop innovative technologies	Remarks for agencies (e.g., funding agencies) facilitating innovation
			commensurate with the value provided? Would payers of healthcare (public and private sector) show willingness-to-pay for the innovative technology?
N	Novelty	Does the innovation add any new knowledge?	Is there true novelty in the design, development, or deployment of the innovative technology, or does it merely represent replication of existing technologies?
T	Timeframe for innovation	How soon can the innovation reach the end-user (from the stage of idea to certified product)?	Does the innovative development require an unduly long time period to complete all the steps of the innovation cycle? Is there a risk of the innovation becoming redundant by the time the cycle is complete?
I	Integration with existing technologies	Can the innovation be readily integrated with existing systems in healthcare?	Does the innovative technology require a stand-alone delivery system, or can it be used with other technologies for the same (or other) healthcare problems?
A	Alternatives	How does the innovation score over other alternatives (in use) for the same problem?	What are the limitations of existing technologies addressing the same problem, and what value advantage does the innovation have over them?
L	Likely impact of not having the technology	What is the likely impact if the innovation was not developed?	Are there likely to be adverse impacts on end-users and/or the healthcare system if the innovative technology was not designed, developed, or deployed?
S	Sustainability	Does the innovation provide a temporary or sustainable solution to the need it seeks to meet?	Does the innovative technology provide sustained efficacy and safety for end-users in terms of the needs required to be addressed? Will there be need to restart the innovation cycle if the technology is not sustainable?

country appears to be limited to imitation, improvisation, and improvements in existing technologies, or merely broadening the application of such technologies. It has also raised new challenges and problems including the total disregard for intellectual property rights. Very few of these technologies were developed to address the real needs of end-users; hence, most of them are not suitable for use in the healthcare sector. The way forward is for innovators to consider all aspects of the innovation cycle, from the generation of ideas to meet real-world needs to the stage of product deployment. The modified KNOW ESSENTIALS algorithm can be used by innovators and innovation facilitators to consolidate the gains of past innovation efforts and encourage genuine innovation in the country.

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Systems Biology Approaches to Study Disease Comorbidities

6

Tammanna R. Sahrawat

Abstract

Unraveling the relationship between disease phenotypes and disruptions in the underlying cellular functions is an important challenge of contemporary biology and medicine. The traditional approaches to study disease focus primarily on individual genes or proteins related to certain phenotype. Gene-based approach to establish factors that predispose an individual to disease, involved identification of specific genetic defects and single nucleotide polymorphisms and copy number variations. These approaches, though successful, are insufficient since function of various cellular components is exerted through intricate networks of regulatory, metabolic, and protein interactions. On the other hand, the systems biology-based network approaches can facilitate the development of better diagnostic markers and the discovery of core alterations for human complex diseases by system wide analysis on disease diagnosis and the identified disease-responsive genes and modules. Complex diseases result from variations in a large number of correlated genes and their complex interactions rather than by alterations in individual genes. Systems network biology approaches enable investigation of complex interdependencies among a cell's molecular components, to identify significant underlying relationships between apparently distinct disease phenotypes. Such approaches can further facilitate the development of better diagnostic markers and the discovery of core alterations for human complex diseases by system wide analysis on disease diagnosis and the identification of disease-responsive genes and modules.

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Keywords

Protein interaction network · Gene interaction network · Biological networks · Computational biology · Systems network biology

6.1 Introduction

The discovery of genes and genetic modules that drive disease has been enabled by study of biological networks. Systems biology aims to investigate multiple biomolecular components and their dynamical behaviors to understand nonlinear interactions that characterize phenotypic alterations leading to complex etiologies of multifactorial diseases such as cancer, diabetes, Alzheimer's disease, etc. The study of complex diseases using traditional approaches such as genetic studies was difficult as multiple factors such as genetic, epigenetic (e.g. environmental factors), cells, and tissue types, and their interactions are involved in the occurrence and development of complex diseases. Furthermore, additional difficulty arises from disruption of normal behaviors of the complex molecular networks of genes and proteins resulting in molecular defects associated with complex diseases. Therefore, for understanding complex diseases, a new paradigm of systems network biology has emerged in view of the availability of large-scale genomic, transcriptomic, proteomic, and metabolomic data that has enabled the identification of risk factors of complex diseases, personalized medicine, and so forth.

Traditional classification of diseases was mainly based on the observed and correlation between pathological analysis and clinical syndromes, which has been widely recognized to have shortcomings on sensitivity and specificity (Loscalzo et al. 2007). In contrast, the network-based systems biology approach can facilitate the development of better diagnostic markers and the discovery of core alterations for human complex diseases by system wide analysis on disease diagnosis and the identified disease-responsive genes and modules (Gijsen et al. 2001).

Network systems biology has facilitated the investigation of complex diseases and their co-occurrence by using all available molecular interaction and ontology data. It is necessary to study disease comorbidities as it is a major problem at the clinical level as it results in the increase in the patient mortality and also complicate the choice of treatment strategies. Furthermore, comorbidity cases are typically associated with polypharmacy (the use of multiple drugs), which can decrease treatment efficacies and can cause unexpected adverse effects, further adding to the disease spectrum in a given patient (Von Lueder and Atar 2014).

6.2 Systems Network Biology Perspective to Study Disease Co-occurrence

Biomolecular networks are integral parts of cellular systems and play a fundamental part in giving rise to life and maintaining the homoeostasis in living organisms. Complex diseases are believed to arise from the dysfunction of these networks rather than single components like genes or proteins (Erler and Linding 2010).

The availability of high throughput genomic, transcriptomic, proteomic, and metabolomic data provides the opportunity to investigate the essential mechanisms by biomolecular networks, in which each node is a biological molecule, and each edge represents the dysfunction interaction or association of a pair of molecules related to disease complex diseases. Hub proteins in PPI networks correspond to essential proteins having a higher probability of involvement in the disease (He and Zhang 2006). Therefore, the omics datasets can be integrated into higher level understandings of a disease process. There exist various biomolecular networks, namely, protein–protein interaction network [PPIN], gene regulatory network [GBN], metabolic network [MBN], and signal transduction network [STN]; these networks interacts with each other to build complex network of interactions (Hu et al. 2016). The human genetic interactions are one of the most important predictors of a link between diseases. Any malfunctions observed in these networks can indicate a presence of disease phenotype.

6.3 Analysis of Disease Co-occurrence Using Biological Networks

The study of disease similarities can provide information of etiology, common pathophysiology, and/or suggest treatment that can be appropriated from one disease to another (Butte and Kohane 2006). Different diseases in the same disorder class may exhibit concordance in protein networks and biological processes, which can be viewed in terms of a "diseasome." To investigate comorbidity between diseases, various types of information such as genetic, symptomatic, and phenotypic in association with penetrance models and medicare data have been used (Rzhetsky et al. 2007; Suthram et al. 2010; Mathur and Dinakarpandian 2012).

Two important computer languages, viz., R (Team RC 2013) and python (Van Rossum and Drake Jr 1995), provide a programming environment for detailed, data-driven biological network analysis and visualization. Many packages are easily downloadable from bioconductor using R platform such as "neat" (efficient network enrichment analysis test) (Signorelli et al. 2016) Version 1.2.1 that integrates gene enrichment analysis (GEA) tests with information on known relations between genes, represented by means of a gene network. Clustering biological functions using gene ontology and semantic similarity can be performed using ViSEAGO (Brionne et al. 2019). Online open source software cytoscape (Shannon et al. 2003) can be used for integrating biomolecular interaction networks with high-throughput expression data to decipher the associations among protein-protein, protein-DNA,

and genetic interactions. RCytoscape (Shannon et al. 2013) merges the powers of R and Cytoscape, thereby resulting in a synergistic tool that combines statistical and data handling facilities with the powerful network visualization and analysis capabilities of cytoscape software.

Another common observation is that a complex disease may lead to the appearance of another phenotype through physiological changes referred to as pleiotropy. Example it has been reported that patients with type 1 diabetes mellitus having hyperglycemia can develop diabetic retinopathy. Thereby treatment of DM (Type I) can prevent the occurrence of the complications associated with it. However, this is not the case when two or more diseases are co-occurring owing to shared genetics or shared diseased pathways. Therefore, it is important to identify the underlying pathways using phenome-wide association studies (PheWAS) that detect genotype-to-phenotype associations by combining and linking genotype data with detailed clinical data from health records (Denny et al. 2013; Bush et al. 2016). Genome-wide association studies (GWAS) can be used to detect pleiotropic risk variants, while studying comorbidity of diseases, which may not otherwise be found in single-disease studies (Ellinghaus et al. 2016). BUHMBOX (Bellou et al. 2020; Gao 2020) is one such online tool that can distinguish between pleiotropy and heterogeneity where the latter refers to misclassification in which a subgroup of cases in one disease is genetically identical to another disease (Ellinghaus et al. 2016).

To understand complex relationships between multifactorial diseases in a population, a systems network biology approach involving computational biology helps to elucidate their underlying common factors. Some of the approaches are as follows:

6.3.1 Using Protein-Protein Interaction to Study Diseases

Proteins interact with one another to form physical or functional interactions, and any perturbations may alter the interactions causing disturbance in the biological functions and leading to one or more diseases. Therefore, studying protein-protein interactions (PPIs) can help us interpret the underlying mechanism of a disease and its relationship with other diseases (Baudot et al. 2009).

Studying the co-occurrence between two or more diseases involves construction and analysis of the PPIs. The PPIs can be retrieved from two types of databases:

1. Primary PPI databases such as MINT and InAct that contain data from the experimental evidence in the literature and use a manual curation process.
2. Metadatabase or a predictive database that include STRING and UniHI, which use the interactions defined in primary databases to computationally predict and refine the interactions.

The PPI data retrieved from the databases can be visualized and analyzed using various bioinformatics tools like Cytoscape, NAViGaTOR, POINeT, Gephi, and Pajek (Leong et al. 2013). These network analysis tools have the ability to take data

from varied sources. They perform various analysis using different algorithms to identify various critical hub nodes that may be responsible for various diseases along with gene ontology (GO) annotations by various plugins such as BiNGO and ClueGO, which use gene ontology (GO), Reactome, and KEGG annotations (Maere et al. 2005; Bindea et al. 2009; Kanehisa et al. 2017; Fabregat et al. 2018).

6.3.2 Using Gene-Gene Interaction Networks to Study Diseases

Gene products, i.e., the proteins, work together to perform a particular task. One of the important genetic interactions is that between the genetic variants, which can be lethal or have a suppressor effect. It is important to study the complex networks of these genes to understand the complexity of the diseases and their co-occurrence (Bebek 2012). Co-expression networks of the genes can be constructed to study the correlations between the expressions of the different genes (Cho et al. 2012). Like the PPI data, analysis of gene-gene interactions also involves the retrieval of genes from the databases such as gene database (NCBI), KEGG or WikiPathways, BioGRID, IRefIndex, and I2D (Zuberi et al. 2013). This is followed by building an analysis of a gene-gene interaction network using various bioinformatics tools like Cytoscape application GeneMANIA and CytoCluster (Zuberi et al. 2013; Li et al. 2017).

6.3.3 Pathway Enrichment Analysis or Functional Enrichment Analysis

Pathway enrichment or functional enrichment aids in achieving deep biological insights of the functional roles of the proteins, genes, and metabolites from multi-omics datasets by comparing the activity of the pathways or biological processes of interest in two or more states or cohorts to be compared. Three approaches for pathway enrichment are as follows (Paczkowska et al. 2020):

1. Over-representation analysis approach gives information about the number of genes in each pathway and whether the pathway is upregulated or downregulated. MetPA is a software based on this approach (Khatri et al. 2012).
2. Functional Class Scoring Approach uses all available molecular measurements for the analysis of the genes. An example is gene set enrichment analysis (GSEA) software that analyzes set of the genes that are differentially expressed, which can later be visualized using EnrichmentMap plugin in Cytoscape (Khatri et al. 2012; Reimand et al. 2019; Subramanian et al. 2005).

6.3.4 Disease-Gene Interaction

Disease-gene networks can be used to identify the dysregulated genes of a particular disease that are responsible for causing other diseases too. DisGeNET is a repository that contains information about genes, genetic variants, and their interaction with the complex human diseases including Mendelian, environmental, and rare diseases (Bauer-Mehren et al. 2010). DisGeNET integrates data from expert curated repositories, GWAS catalogues, animal models, and the scientific literature. The information provided in it is accessible through a web interface, a Cytoscape App, an RDF SPARQL endpoint, scripts in several programming languages, and R package (Piñero et al. 2016). The information retrieved can be used to decipher information of a gene and its cross-talk with the other diseases.

6.4 Identifying Critical Hub Proteins and Probable Therapeutic Targets

In the omics era, the study of interconnections amongst genome, transcriptome, proteome and metabolome using systems biology approaches has led to increasing awareness of complex diseases and their comorbidities. Studies based on the systems network biology approaches revealed genes that frequent between breast and bone cancer were found to be enriched in various signaling pathways such as cell cycle, transcription misregulation in cancer, p53 signaling, breast cancer, integrated breast cancer, FOXO signaling pathway, and DNA damage pathways, thereby indicating that they may be responsible for common molecular origin of these cancers (Sahrawat and Kaur 2017). Another study identified common molecular signatures and pathways that interact between inflammatory bowel disease and colorectal cancer, and the indispensable pathological mechanisms revealed 177 common differentially expressed genes (DEGs) between the two diseases (Al Mustanjid et al. 2020). Using a systems biology network approach involving PPI network analysis, critical protein was identified, viz., HSPA8/HSC70, which could be targeted to affect the regulation of CFTR (cystic fibrosis transmembrane conductance regulator), which is mutated in cystic fibrosis (Sahrawat and Bhalla 2013). The genes and their protein products may act as potential biomarkers for early detection of predisposition to co-occurrence of diseases and potential therapeutic targets based on the common molecular underpinnings of comorbid diseases such as diabetes, depression, and cardiovascular disease (Sahrawat and Talwar 2020) and age-related diseases such as Alzheimer's disease and diabetes for better understanding of pathophysiology (Sahrawat and Dwivedi 2020).Thus, systems biology approach may hold great promise to identify probable therapeutic targets for diseases that are comorbid.

6.5 Concluding Remarks

In systems biology, the individual molecules of the biological systems do not exist in isolation. On the contrary, they work in conjunction with one another to produce a specific phenotypic pattern specific to a given cell type. Study of bimolecular networks using systems biology approaches can be used to identify biomarkers and critical control proteins that can play an important role in the understanding disease etiologies and design treatment regimens for diseases that are comorbid due to the common genes/proteins in the pathways of complex human diseases. The elucidation of such common genes/proteins in comorbid diseases can go a long way for better quality treatment for the patient in a timely manner.

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Bioinformatics: The Interactome of Multidisciplinary Approaches

7

Veena Puri, Sanjeev Puri, and R. C. Sobti

Abstract

Bioinformatics is an amalgamation of multidisciplinary sciences that has played a significant role in extending tremendous benefits through comprehending the biological information in a quick and precise manner. The recent opening of multiple "omics" technologies have provided plethora of information; the bioinformatics has provided means to process this humongous information in more systematic and precise ways to provide potentially beneficial biological information. Continuing efforts and zeal that set the premise for mapping of *Haemophilus influenza* genome potentially led to successful decoding of the human genomic information in a very ambitious human genome project. The systematic breakdown and understanding of huge nucleic acid sequence data out of human genome has been an important milestone of this field. Taking on with the futuristic and additional developments in the field of bioinformatics has now brought this field at the stage where now functional outcome has proceeded from mere prediction to a reality. All the aspects of biological functioning, viz., genomics, proteomics, metabolomics, transcriptomics, RNAiomics, and kinomics to drug development, etc. are now being revolutionized, (courtesy of the bioinformatics).

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Data analysis · Databases · Genomics · Proteomics · Bioinformatics

7.1 Introduction

Bioinformatics is a fast-growing field that is bringing in revolutionary changes in almost all the fields of biological sciences. In order to comprehend the biological data, various tools of bioinformatics, initially utilized genomic as well as the proteomic data. To start with, the genomic data initially entailed the information out of the human genome mapping, and later on, data out of proteomics, transcriptomics, metabolomics, etc. continue to challenge the scientist. The enormous body of the data gets further accentuated when considering large number of organisms whose omics information is getting uploaded every day. Handling of such data is indeed a daunting task, which has inclined the focus on the systematic data gathering, processing analysis, and giving a right interpretation. Thus, start from gathering the data to interpretation, there is an amalgamation of multiple disciplines, viz., biochemistry, genetics, microbiology, biotechnology, data science, mathematics statistics, engineering, etc. (Fig. 7.1).

Together, the multiple disciplines led to the final outcome of the biological data interpretation, which has actually contributed toward birth of this new discipline, the bioinformatics. Today, the bioinformatics has even gone beyond the realm of the data management, analysis, and interpretation to a level where its applications are helpful in drug development, toxicokinetic, disease diagnosis, and marker

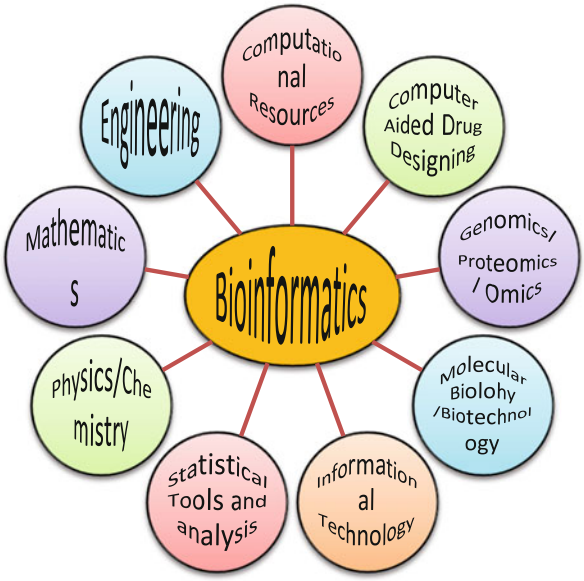

Fig. 7.1 Spoke and hub of interdisciplinary field of bioinformatics

identification, thus revolutionizing the field of clinical medicine including personalized medicine.

The information retrieved from vast array of the genomic data formed the very important basis for our processing and understanding of both the basic biological information on its application in the field of medicine (International Human Genome Sequencing Consortium 2001). Such efforts clearly dates back to a time point when genomic sequencing was being thought to provide much needed answers to the biological functionality. Even though genome sequencing of *H. influenza* (Fleischmann et al. 1995) set the ball rolling, it was the whole genome sequencing of different organisms including the human genome project that the field of bioinformatics saw unprecedented growth both in its utilization and applications. It was well realized that no doubt the genomic information is pertinent to its function, but the emphasis has be on the gene product for making it functionally applicable. Having sequenced the human genome (Luscombe et al. 2001; Subramanian et al. 2001), the bioinformatics has been utilizing comparative and functional genomics and proteomics to better understand the genes and their products for better understanding of the biological information. Subramanian et al. (2001) pointed out that comparative genomics and proteomics besides helping to locate human gene has also enabled to fully comprehend the gene's function. This is a significant contribution of the bioinformatics toward biological data, which in a time to come would bring applications at a personalized level.

7.2 Bioinformatics Databases

Today, the collection of biological information has reached to a level where its systematic storage has enabled the researcher to retrieve the information with ease, update the existent information, and manage it. The huge amount of biological information is filling up the space that the task of bioinformatician is dauntingly getting overwhelmed. The challenge is to get to the most comprehensible information from the hugely available biological data. Bioinformaticians are coming up with novel strategies to put this data in more systematic way that resulted in development of huge number of databases today. As per the 2020 report published in the month of January, a total of about 1637 different databases were cited in the journal of *Nucleic Acids Research* (Neerincx and Leunissen 2005). Off course, the number is increasing continuously by the time this review appears; it is envisaged that about 500–1000 new databases would have certainly been added to the existing one. This shows the amount of challenge and the necessity of process owing to newer and newer species that are being decoded or diseases being understood to count a few. These databases are now equipped with the collection of information that are providing and proving to be the most invaluable for lending huge support to the execution of biological research. A large number of databases have been generated encompassing nucleic acid, proteins, peptides, metabolite set of resources, viz., DNA and protein sequences, structural information of different macromolecules, gene sequencing data, 3-D structure, and metabolomic data, thus enabling researchers to retrieve the

Table 7.1 Important databases (Baxevanis 2001)

Primary databases	Contains • Experimental data repositories • Sequence databases • Structure databases			
DNA/ nucleotide sequence databases	Ensembl (EBI/Wellcome Trust Sanger Inst.)	GenBank (NCBI)	DNA Data Bank of Japan (DDBJ)	European Nucleotide Archive (EMBL-EBI)
Protein sequences	UniProtKB (UniProt Knowledge Base) UniProtKB/Swiss-ProtUniProt KB/TrEMBL	NCBI Protein		
Primary structure databases	Protein Data Bank (PDB)	Nucleic Acid Database	Cambridge Structural Database	
Secondary databases	Store curated datasets			
Sequence related	ProSite	Pfam	Enzyme	REBase (restriction enzymes)
Structure related	DSSP Database of Secondary Structure Assignments	HSSP Homology-derived Secondary Structure of proteins	Dali: Proteins comparing protein structures in 3-D	
Relational/ specialized databases	Curated Datasets of specialized types			
	Gene expression data Protein expression data—Human Protein Atlas	Pathway-related KEGG, Reactome, Metacyc	General human genetics databases, e.g., HGMD, OMIM	General polymorphism databases, e.g., dbSNP

huge amount of stored information and process to gain insight into biological processes, which otherwise were more intricate and complex. Plethora of information is now available and far more is now getting flooded with more user-friendly databases, which provide multi-omic approaches. Similar accessibility about different databases have also been elaborated in different observations (Subramanian et al. 2001; Manohar and Shailendra 2012). Some of the commonly available databases are presented in Table 7.1.

These databases are actually categorized as primary and secondary databases. The primary databases include EMBL (European Molecular Biology Laboratory), NCBI (GenBank at the National Center for Biotechnology Information) and DDBJ (DNA Database of Japan) while the secondary databases include secondary databases,

Table 7.2 The International Nucleotide Sequence Database Collaboration (INSDC) repositories

Data category	DDBJ	EMBL-EBI	NCBI
Next-generation read sets	Sequence Read Archive	European Nucleotide Archive (ENA)	Sequence Read Archive
Genome sequence archive	GEA		GenBank
Data annotation/ assembled sequences	DDBJ		GenBank
Genotype /Phenotype archive	JGA	EGA (European Genome-Phenome Archive)	
Samples	BioSample		BioSample
Projects	BioProject		BioProject

Protein Information Resource (PIR), UniProtKB/Swiss-Prot, Protein Data Bank (PDB), Structural Classification of Proteins 2 (SCOP), and Prosite. These databases are often presented after their proper curation based on their information of protein structure, function, domains, and classification (Pevsner 2015).

These represent the central databases of nucleotide sequences and proteins. The interesting attractive feature of these databases is sharing of deposited information on daily basis among each other. And together, these are the constituents of the International Nucleotide Sequence Database Collaboration (INSDC) consortium (Table 7.2), which represents consolidated information starting from raw data retrieved from different samples and the experimental designs to functional annotation through the process of data assembly and their alignments (Manohar and Shailendra 2012). An identification system of the sequences deposited has been taken into consideration by INSDC to provide much needed information about the origin and the nature of the data (Amaral et al. 2007). Based on the data types, this identification system uses the accession number (AN) as represented by the grouping of one to three letters and five or six digits. These identifiers have further been designated to represent sequence identifier (GI GenInfo Identifier) that corresponds an assignment to every nucleotide or protein sequence (Protein ID). This GI identifier is individual and neither transferable and nor modifiable. Different codes and/or prefixes that are used to represent these sequences include: GB (GenBank) and emb (EMBL), e.g., Xenopus laevis leptin gene that has the following Accession: NM_001095714.1 GI:148233266.

In order to make sense of the fast-developing pool of molecular biological data, bioinformaticians have developed large collections of tools. Since all biological systems tend to be complex and diverse, therefore to comprehend these better, it is often required to link many of the datasets and make use of different tools to integrate these datasets. Several strategies are available and getting revised for the integration of these datasets. Over the past few years, building services with web interfaces have become a wildly used system for data and tool sharing that have resulted in accomplishing many bioinformatics projects. The interoperability problem of web services that used to resolve biology queries has led to the evolution of tools with web interfaces from HTML/web-based tools. Better tools and robust

realization of the multidisciplinary aspect of bioinformatics have enabled to cross the boundaries with respect to handling and integrating huge amount of the biological data. The tools use methods by exploiting experimental evidences as their foundation and predict the novel associations of biological entities.

Luscombe et al. (2001) pointed out two dimensions in bioinformatics to better understand the biological data. These two dimensions encompass both the breadth and depth of the data both for its organization and the understanding of the biological data. Biological sequence comparison by alignment has been processed by the increase in availability of data generated by NGS technologies.

Role of Artificial Intelligence (AI): AI has given much needed thrust to the bioinformatics and computational biology. Both the areas have provided the scientific community with way to handle enormous body of data that is getting added continuously. The delineation of the final outcome of the biological information rests on knowing the origin and evolution of species. That utilizes the molecular approach encompassing comparisons among the nucleotide sequences (DNA/RNA) and amino acids (peptides or proteins). This aspect helps to provide information w.r.t evolutionary relatedness of organism, genes, prediction functions, and structures besides other information (Manohar and Shailendra 2012).

With the availability of different types of AI algorithms, it has become common for the researchers to apply the off-shelf systems to classify and mind their databases. At present, with various intelligent methods available in the literature, researchers are facing difficulties in choosing the best method that could be applied to a specific dataset. Researchers need tools, which presented the data in a comprehensible fashion and annotated with context and estimates of accuracy and explanation.

With better tools and robust realization, the multidisciplinary aspect of computational biology has enabled to cross the boundaries to with respect to handling and integrating huge amount of the biological data. Retrieving the multitier genomic data and finding ways to link it to disease pathophysiology have enabled to reorient the approach for therapeutic intervention. As a result, it has now become far more possible to devise customized medical treatment. Advanced robust tools of the multidisciplinary aspect of computational biology have enabled to cross the limits for annotating huge amount of the biological data. Retrieving the multitier genomic data and finding ways to link it to disease pathophysiology will enable to reorient therapeutic interventions in a novel strategy. Extensive number of studies regarding these has been brought up (Ritchie et al. 2015; Schmidt et al. 2001; Zhao et al. 2010). This approach can be further harnessed for personalized genomics. Already, commercial players have come up wherein linking of different gene markers with related trait has now become possible. Futuristically, the genomic, proteomic, transcriptomic, and metabolomic data are going to be huge posing significant challenges, which can be addressed by computational and/or bioinformatics approach.

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Role of Artificial Intelligence in Diagnosis of Infectious Diseases

8

Vandana Kaushal and Rama Gupta

Abstract

The burden of infectious diseases on the world is overwhelming. The advent of novel technologies like machine learning (ML) and artificial intelligence (AI) has brought a paradigm shift in the manner in which the world is tackling these diseases. Clinical microbiology laboratories have undergone complete transformation—from manual processing of specimens to technology-driven automation and digitalization. We discuss here the integration of AI in clinical microbiology for image analysis of gram stains, automated digital culture plate reading, identification of bacterial isolates using matrix-assisted laser desorption-ionization/time of flight mass spectrometry (MALDI-TOF) data, and whole genome sequencing of microbial pathogens. We have also explored the application of AI in infection prevention and control especially in hospital acquired infections, surveillance, and epidemiology of diseases. Contributions of AI during the ongoing fight against Coronavirus disease (COVID-19) pandemic have been huge in the areas of surveillance, diagnosis, and treatment.

Keywords

Infectious diseases · Clinical microbiology · Machine learning · Artificial intelligence · Infection prevention and control · COVID-19 · Coronavirus

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8.1 Impact of Infectious Diseases

Infectious diseases are defined as disorders caused due to pathogenic microorganisms such as viruses, bacteria, fungi, and parasites that can spread from one individual to another. During the past, including the prehistoric era, these diseases caused epidemics and pandemics that led to high morbidity and mortality worldwide (Table 8.1). In the twentieth century, a large number of deaths occurred due to infectious diseases throughout the world. Today, we live in an ever more connected global village, and the effect of globalization is becoming more and more apparent in terms of transmission of infectious diseases. The blurring of borders due to increased international travel has affected global microbial traffic in such a manner that humans can reach almost any part of the earth today within the incubation period for most disease causing microbes. This had brought about a dramatic effect on the frequency of reemergence of many old and new infectious diseases. Emerging diseases include HIV, SARS-CoV, Influenza A/H1N1, MERS-CoV, dengue fever, West Nile virus, Zika virus, Ebola virus, Lyme disease Hantavirus, and coronavirus. Emergence of newer infections is also linked to various human factors such as population explosion, ecological factors like urbanization, climate change, and increased in land brought under agricultural use. Reemergence of many diseases that were once under control like tuberculosis, cholera, malaria, pertussis, influenza, etc. can be attributed to a myriad of factors like human behavior, over use of antibiotics, breakdown in public health measures, or appearance of new strains of known disease causing organisms.

8.2 New Technologies for Infectious Diseases

Humanity has faced several epidemics and pandemics responsible for ravaging human populations, which had changed history. The world has leant quite a few lessons from previous outbreaks. Advancements in information technology and digitalization of records have flooded the present era with big data where information is like an economic asset and novel and intelligent tools are gaining popularity in

Table 8.1 Historical account of major viral outbreaks and their effects in the past 150 years

Disease	Year of occurrence	Deaths
Russian flu	1889–1890	1 M
Spanish flu	1918–1919	40–50 M
Asian flu	1957–1981	1 M
Hong Kong flu	1968–1970	1 M
HIV/AIDS	1981–present	25–35 M
Swine flu	2009–2010	200,000
SARS	2002–2003	770
Ebola	2014–2016	1100
MERS	2015–present	850
COVID-19	2019–present	

tackling disease burden across the world. In the present times, we have the capacity to collect and analyze huge amount of information related to disease burden, distribution, transmissibility, progression, etc. Big data in the context of health care consists of billions of continuous computerized data entries regarding details of the patients, laboratory and radiological investigations, disease diagnosis, treatment (therapeutic drugs and surgical procedures), final outcome, etc. It becomes difficult to organize and analyze these incoming data streams using traditional computational algorithms. New age digital tools like machine learning (ML), artificial intelligence (AI), and Internet of things (IoT) have enabled scientists and public health agencies to analyze this big data in a meaningful way. Box 8.1 contains definitions and technical explanations of terms used in this chapter. World Wide Web has enabled raw data collection from a variety of smart devices, mobile phones, and sensors that are largely interconnected. This vast data when analyzed using AI powered tools provides significant information regarding the pattern or trends of emerging infectious diseases, which is helpful in monitoring, predicting, and preventing the spread of such diseases.

8.3 History of Artificial Intelligence

The term AI was first used by John McCarthy in 1956 to define the ability of computers to simulate cognitive human intelligence. AI enabled intelligent machines have an ability to think like human mind and perform functions such as learning and problem solving. AI was first applied in the field of medicine with the development of a tool called ELIZA in1964, which was a chat box that recreated the conversation between a psychotherapist and a patient (Weizenbaum 1976). In the year 1970, the Stanford University, USA, developed another system called Dendral where the scientists applied AI to analyze mass spectral data of organic molecules to identify unknown organic chemicals (Lindsay et al. 1980). In 1973, another artificial intelligence–based expert system called Mycin was designed for identification of pathogens responsible for severe infections like bacteremia and meningitis and to assist physicians in treating blood infections by recommending antibiotics dosages adjusted according to the body weight of the patient (Shortliffe and Buchanan 1975). Dendral and Mycin were written in LISP programming language. Scientists from the KAIST University in South Korea used a software called LUNIT in the year 2013 and developed an AI assisted algorithm for advanced image analytics for accurately identifying breast and lung cancer using mammography and x-rays images (Landau and Pantanowitz 2019). In 2018, James Kirby first demonstrated the use of deep convolution neural network (CNN) for automated identification and interpretation of gram stains from blood culture systems. Since then, the field of AI has grown and progressed incredibly (Smith et al. 2018).

Box 8.1 Definitions

Artificial intelligence is defined as a set of rules that enable a computer to make decisions and perform tasks, which are normally performed by a human brain. These rules are based on visual pattern recognition by automated systems followed by an action or conclusion, which is more reliable and efficient than humans.

Artificial neural network (ANN) is a collection of biologically inspired simulations of neurons that are performed on the computer configured to perform specific tasks like clustering, classification, pattern recognition, etc. by simulating the way the human brain analyzes and processes information. It is the foundation of artificial intelligence (AI).

Big data is the term used for extremely large amount of diverse ever growing data (structured and unstructured) that may be computationally analyzed for insights that lead to better decisions by revealing trends, patterns, and associations just the way human brain processes and analyzes the information.

Convolution neural network (CNN) refers to a deep learning tool that comprises of a series of algorithms interconnected just like the neurons of human optical cortex. These algorithms help in transforming images into analyzable data, which is then classified to give probabilities and trends.

Decision tree (DT) belongs to the family of supervised learning algorithm that uses a *tree*-like model of *decisions* and their possible consequence for solving classification and regression problems.

Deep learning refers to a specific type of ML where the computer uses large datasets analyzed by a series of algorithms that are interconnected like neural network of human visual cortex to make predictions about new data.

Internet of Things, or ***IoT***, is a term used for billions of computing and digital devices that are capable of collecting information and sharing the data through Internet.

K-nearest neighbors (KNN) algorithm is a machine learning supervised algorithm that can be used for solving classification problems and regression problems.

LISP or **list processing** is the name of a programming language, which is used for manipulation of datasets.

Logistic regression (LR) is a predictive analysis algorithm used to assign observations to a discrete set of classes or when the data has binary output.

Machine learning: The subset of artificial intelligence algorithms, which are adaptive and evolve over time, learning from datasets of past experiences and makes future predictions based on the new data is known as machine learning (ML). ML performs either discriminative tasks or regressive tasks. ML helps in both prognostic and therapeutic activities.

(continued)

Box 8.1 (continued)

Support vector machine (SVM) is another type of ML algorithm for unlabeled data represented as points on a graph. This support vector clustering classifies the data into various categories using regression analysis.

8.4 Clinical Microbiology and Artificial Intelligence

With the ever increasing burden of infectious diseases and the need for accurate and timely diagnosis for better treatment of patients and control of infections, there is a need of specialized clinical microbiology laboratories. Skilled microbiologists play a vital role in fast and accurate identification of pathogens using different staining techniques, for identification of microbial growth on a variety of culture media and their antimicrobial susceptibility pattern. This is a time-consuming manual process that requires skill, expertise, patience, and experience of a specialist microbiologist. During last two decades, there has been a lot of advancement in technology mostly due to integration of computers, robotics, and automation. With the advent of artificial intelligence and big data analytics, modern microbiology labs have transformed from manual processes to fully automated technology-driven arena focusing mainly on timely and accurate diagnosis. The way in which clinical laboratories look today is very different from how they looked 10 years ago. The world is moving toward automation at great pace making use of technologies like lab on a chip and cloud computing (Blaž and Luka 2019).

AI systems have existed in clinical microbiology laboratory for over a decade in the form of expert rules used by some automated systems for identification and susceptibility testing of microbes (Winstanley and Courvalin 2011). Antimicrobial susceptibility rules help in accurate reporting of antimicrobial combination most effective against a particular microorganism. The computer-assisted decision tree, based on rules of CLSI or EUCAST guidelines, simulates intelligent human behavior, thereby leading to more reliability, reproducibility, and efficiency in reporting. These nonadaptive AI algorithms have now paved the way to more adaptive AI algorithms that evolve over time as the machine learns.

Recent advances in medical image analysis and introduction of novel imaging biomarkers using artificial intelligence (AI) have been very helpful in timely and accurate diagnoses of diseases. Scientists have experimented with microscopes enhanced with AI that are highly proficient in accurate identification of bacterial images. This embedding of AI in microscopy was undertaken by training a CNN, a class of AI modeled on the mammalian visual cortex to analyze the freshly acquired visual data and comparing it with thousands of previously stored images in order to identify and categorize the new pathogen on the basis of its distribution, shape, and size.

Digitalization of microbiology laboratories coupled with AI advances has enabled faster reporting of negative cultures and accurate reporting of positive cultures using digital plate readers. This technology also helps in quick screening of pathogens growing on chromogenic agar plates without actual handling of culture plates by a microbiologist. Results are automatically transferred to patient's record on the Hospital Information System (HIS) enabling the treating physician to start appropriate antimicrobial therapy. The working of microbiology laboratories have been hugely impacted by these technological advancements as the skilled lab personnel are now able to devote their time to more difficult tasks.

8.4.1 Microscopy Image Analysis

AI was first applied for automated analysis of Gram stain from positive blood cultures by Smith et al. (2018). Morphological characteristics of bacteria like shape, size, distribution, etc. were selected to represent most common pathogenic bacteria. A pre-trained CNN platform called Google Inception version 3 was used to recognize several Gram stain images of unknown bacteria from positive blood culture bottles. These were classified into gram-negative rods, gram-positive cocci in chains, and gram-positive cocci in clusters etc. with a success rate of 95%. Later, the same group of researchers used a computer with more advanced graphics processing and analyzing unit and converted 100,000 image crops to create an algorithm that increased the classification accuracy to 99%. Software called Technologist Assistant (TA) has been combined with trained CNN to support technologists to evaluate selected cropped images on a computer screen enabling them to report from remote locations (Smith et al. 2020). Further advancements in microscopic analysis of bacteria include training algorithms to analyze and interpret new morphological types and to recognize these bacteria and fungi from Gram stained smears made directly from the specimens. Training software has been developed to identify and score bacteria that cause bacterial vaginosis based on Nugent classification (Nugent et al. 1991). Other smears that can be analyzed similarly include calcoflour white KOH smears, lactophenol cotton blue fungal slide preparations, and acid fast bacilli smears. Comprehensive training and supervised ML tools are required for such AI-based applications.

Microscopic examination of direct stool specimen or peripheral blood film is the gold standard for detection, characterization, and quantification of parasites. The identification of *Plasmodium* species and *Babesia* species in peripheral blood films is a time-consuming and highly specialized endeavor. AI has helped in making this process more efficient and accurate especially in a high volume laboratory or field hospitals where expertise is limited. A device for WBC differential count called CellaVision attached with automatic slide scanner has been cleared by FDA for screening of detection of intracellular parasites like *Babesia* in a Giemsa stained peripheral blood smear (Oscar 2007). The efficiency and accuracy of diagnosing malaria from peripheral blood films has been increased many fold with the development of an automated CNN model (Fuhad et al. 2020). A support vector machine

(SVM) has an ability to detect malaria parasite from microscopic images with 99.23% accuracy. This miniaturized mobile phone model that works through Internet has been validated for different mobile phones. Deep learning models used for automatic and quick detection of malaria pathogens from thousands of microscopic images significantly improve the efficiency of the clinical pathologists especially in rural settings (Fuhad et al. 2020). HRMAn (Host Response for Microbe Analysis) is a neural network-based ML software that has been used to analyze *Toxoplasma gondii* and *Salmonella enterica* infections in a variety of cell lines. Artificial intelligence and digital slide scanners have also been tried for detection of helminthes eggs and protozoan cysts from stool specimens (Holmström et al. 2017; Intra et al. 2016). This intriguing technology holds great promise for development of future diagnostic platform for accurate and speedy reporting of results. These images can be sent remotely and read by AI anywhere in the world, thereby helping the labs in resource poor settings.

8.4.2 Digital Plate Reading (DPR)

Digital plate reading software is used to interpret microbes growing on solid culture media by comparing the image with previously stored digital images of culture positive plates instead of directly viewing the plates. This technology is already being used in clinical microbiology laboratories further strengthening the automation process in these labs. The wave of automation in clinical microbiology laboratory has already replaced manual process like inoculation, incubation, and transfer of plates from workstation to incubator and vice versa. The current development efforts are primarily focused on automating the interpretation of primary cultures using AI, by developing efficient digital bacterial culture plate reading computer vision software (Glasson et al. 2016, 2017). As a consequence, a microbiologist can report the culture results while sitting in front of a computer screen. Full lab automation by Copan (Bresica, Italy), WASP lab, and BD sparks Kiestra have become popular for performing inoculation, incubation, and reading of petri dish images. This is known as total or full lab automation (TLA/FLA). Two upcoming systems in this field are Automated Plate Assessment System (APAS-LBT Innovations Ltd., South Australia) and i2a platform (Montpellier, France).

Digital Plate Reading in most advanced microbiology laboratories is presently performed by technologists. However, Copan has recently developed and marketed a software called PhenoMatrix, which is designed to capture and interpret images of culture plates (Faron et al. 2016, 2019; Van et al. 2019). Software called Optis has been developed by BD Kiestra for digital plate reading (Croxatto et al. 2017). DPR was first applied to segregate plates with growth from those showing no growth for miscellaneous specimens and to segregate urine culture plates into no growth, no significant growth, growth of mixed flora probably due to contamination, or pure growth suggestive of UTI pathogen. DPR also helps in screening of antibiotic-resistant bacteria by showing growth or no growth on chromogenic agar. Results of these studies have shown excellent sensitivity, comparable with nucleic acid

testing (NAT) for certain specimens (Van et al. 2019). Advanced AI systems are capable of characterizing the culture growth on the basis of colony characteristics, purity of culture, and colony count per milliliter of the specimen. A vast study undertaken by three centers for urine samples cultured on blood agar and MacConkey agar evaluated using COPAN software showed 99.8% sensitivity and 72.0% specificity (Faron et al. 2020). This efficiency would further increase with newer and advanced software. With the help of these fast evolving technologies, large volume of pyogenic cultures from sterile body fluids and urine can be screened for growth or no growth in very little time, thereby reducing the turnaround time (TAT) for culture reporting (Glasson et al. 2017).

8.4.3 Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDITOF MS)

MALDITOF MS is becoming a popular tool for fast and accurate identification of bacterial and yeast isolates. In fact, various studies have reported direct identification of bacterial pathogens and their antimicrobial resistance patters directly from the specimen (Florio et al. 2018; Doern and Butler-Wu 2016). This can be of immense help in predicting the resistance pattern of bacterial pathogens and strain typing especially during an outbreak. ML techniques using MALDI-TOF MS are able to differentiate vancomycin-intermediate *Staphylococcus aureus* (VISA) from vancomycin-susceptible *S. aureus* (Wang et al. 2018; Mather et al. 2016). Another group utilized a SVM model to distinguish between vancomycin-resistant *Enterococcus faecium* (VRE) from vancomycin-susceptible *Enterococci* (Griffin et al. 2012). They also performed hierarchical cluster analysis on 66 VRE isolates and found a cluster of four isolates, which were found indistinguishable when checked by pulse field electrophoresis. This technology has been found to be useful in strain typing of *Acinetobacter baumannii* that could potentially cause a nosocomial outbreak (Mencacci et al. 2013) and for capsular typing of *Haemophilus influenza* (Månsson et al. 2018).

8.4.4 Antimicrobial Resistance Prediction by Whole Genome Sequencing (WGS)

AI and ML can be applied to the field of clinical microbiology to predict antimicrobial resistance using WGS of commonly occurring pathogens. Sequencing can also be helpful in identifying fastidious bacteria, to detect virulent phenotypes and to investigate potential nosocomial outbreaks. In addition, WGS proves invaluable while investigating patients presenting with unusual disease presentation (Long et al. 2014; Olsen et al. 2015; Nasser et al. 2014). The data generated by WGS of bacteria is helpful in predicting sensitivity pattern of bacteria and identifying resistant and moderately resistant phenotypes based on their minimum inhibitory concentration (MIC) values (Long et al. 2017; Davis et al. 2016; Nguyen et al. 2018,

2019). Accurate prediction of antibiotic susceptible or resistant phenotypes of *Klebsiella pneumonia* against 16 antibiotics has been done using WGS data (Long et al. 2017). Another study has applied ML model to predict bacterial phenotypes and MIC of various antibiotics tested (Nguyen et al. 2018). Such ML predictions based on WGS are unlikely to replace traditional susceptibility methods due to inhibitory cost of WGS and due to constantly evolving resistance mechanisms. Still a timely predicted result based on ML can help in initiating appropriate and early therapy much earlier, thus leading to improved patient outcome. As WGS is gaining popularity, the cost is also decreasing, which will enable microbiology laboratories to introduce such ML models in their routine working.

8.5 AI in Infection Prevention and Control (IPC)

Another application of AI is in prevention and control of infections under these three areas identified by WHO—surveillance of nosocomial or healthcare-associated infection (HAI), accurate and quick laboratory diagnosis of these infections to facilitate preventive measures, and education and audit of hand hygiene compliance (Storr et al. 2017).

8.5.1 Surveillance of Healthcare Associated Infections (HAI)

Hospital acquired infections are those infections acquired by the patient during the process of health care. Surveillance of HAI is very important for implementing effective infection prevention and control of nosocomial infections. Surveillance done by conventional methods is time-consuming, expensive, and needs a lot of resources. In this advanced age of computers, newer information technologies and digital record keeping of patient data through HIS, surveillance of HAIs, has become partially automated and easy (Sips et al. 2017). Intensive data generated through electronic healthcare records has been analyzed through ML tools to predict outbreaks of influenza, methicillin-resistant *Staphylococcus aureus* (MRSA), and *Clostridium difficile* infection (Cusumano-Towner et al. 2013; Li et al. 2019; Wiens et al. 2014; Oh et al. 2018). Next-generation sequencing is now extensively used for identification of disease causing microbes, their antimicrobial resistance pattern, and strain typing (Deurenberg et al. 2017). Usefulness of ML tools in monitoring HAIs has been reviewed for surveillance of central line associated bloodstream infections (CLABSI), surgical site infection (SSI), Clostridium difficile infection (CDI), and sepsis (Scardoni et al. 2020). It has emerged from this review of 27 studies that surveillance conducted through ML-based tools resulted in early, accurate, and cost-effective prevention of HAIs as high risk patients could be identified at an early stage and timely infection prevention measures could be taken.

8.5.2 Improved Laboratory Diagnosis to Facilitate IPC Interventions

Deep learning through convolution neural networks (CNN) has helped to accurately classify tuberculosis on chest x-rays in resource poor areas where prevalence of TB is high. Automated detection of pulmonary tuberculosis based on chest radiography can be used to screen and evaluate a large number of patients (Lakhani and Sundaram 2017). CNN has also been used to accurately characterize bacteria isolated from blood culture specimen based on their Gram stain morphology (Smith et al. 2018). Direct microscopy coupled with AI tools offer innumerable possibilities for clinics and hospitals in remote areas that do not have a microbiologist to seek expert opinion by sending the images of their findings to a central facility and hence make the disease diagnosis. AI-assisted microscopy could also be applied to analyze Gram stains of sterile body fluids, CSF, and AFB smears for *Mycobacterium*, etc.

8.5.3 Hand Hygiene

AI has been applied to monitor compliance of hand hygiene among doctors and other healthcare workers. An automated gaming and training system commercially available by the name of SureWash has been implemented for education of healthcare workers (Higgins and Hannan 2013). Surveillance of hand hygiene compliance through AI-powered cameras installed near the sinks in surgical wards followed by an appropriate feedback by the infection control team resulted in improved compliance (Lacey et al. 2020). Though AI applications have a huge potential to control hospital acquired infections yet the need for strict policies and good governance remain crucial for improving IPC.

8.6 Disease Surveillance and Epidemiology

Web-based surveillance tools powered by AI are being used to predict epidemics in some countries (Orji and Moffatt 2018). Major tasks performed by these intelligent methods are risk assessment and timely detection of the outbreak; however, their widespread use is still lacking. Two groups of emerging viral pathogens, airborne viruses and arthropod-borne viruses, pose a constant threat to mankind. Out of these two, the airborne viruses are more dangerous as they spread very fast and in an uncontrolled manner especially due to increased international travel. The pandemic potential of such viruses has been seen during the outbreaks of SARS-CoV, influenza A/H1N1, and MERS-CoV (Fitzpatrick et al. 2020). With the emergence of new respiratory pathogen, there is a need for immediate action to diagnose new cases, for disease surveillance, contact tracing, and making policies for quarantine of infected persons. During the SARS-CoV outbreak, these mitigation strategies helped to eliminate virus from the affected area, but in the pandemic influenza A/(H1N1),

the virus quickly spread all around the globe leading to a pandemic. Since viruses that have a human and animal reservoir are very difficult to eradicate, there is pressing need for continuous surveillance. Dengue is the most widespread arboviral infection, and about 390 million people are infected every year (Liu-Helmersson et al. 2014). Zika virus can be transmitted in utero from a pregnant woman to her fetus, and it causes congenital neurological disorders and morbidity. In 2018, an outbreak of West Nile virus was reported by the European Centre for Disease Control (ECDC 2016) affecting both humans and animals (CDTR 2018). The threat posed by emerging arboviral diseases when they become epidemics emphasizes the need for a serious focus on their management and control as these viruses of sylvatic origin (usually a bird or small mammal) evolve rapidly, adapt to urban environments, and then spread the infection to human beings world over (Gould et al. 2017). Arboviral diseases are hard to diagnose as they present with nonspecific symptoms, and control of vectors is very important to prevent their spread.

Combining AI with modern simulation models based on machine learning, artificial neural network (ANN), decision tree (DT), logistic regression (LR), and support vector machine (SVM) can be very helpful for policy makers to assess complex layers like geographical, epidemiological, biological, social, cultural, and economic factors. Such systematic evidence-based assessment and multilayered analysis can help in the control and spread of such diseases.

Several teams from the United States, China, New Zealand, and South Africa have utilized autoregressive integrated moving average (ARIMA) model for predicting infectious diseases (Zhang et al. 2020).

8.7 Role of Artificial Intelligence in COVID-19 Pandemic

COVID-19, caused by SARS-CoV-2 (Zhou et al. 2020), was first discovered in Wuhan City of China in December 2019 and has since evolved into a global pandemic (Reeves et al. 2020). In order to address this global health crisis, ML and AI tools have been deployed to track the spread of virus and to identify the patients who are at a high risk of getting infected. These monitoring systems rely on real-time digital health data collected by each country.

AI technologies that have provided a vital role in helping humans in the ongoing fight against COVID-19 include supervised, unsupervised, and reinforcement learning. A mathematical model has been proposed for studying transmission dynamics of COVID-19 pandemic (Ndaïrou et al. 2020). Additional techniques like auto-encoders and recurrent neural networks are important components of many prominent natural language processing tools (Nguyen 2020).

8.7.1 Predicting and Monitoring the Infection

AI has been applied during COVID-19 pandemic by a Canadian company called BlueDot that specializes in forecasting of infectious diseases (Bogoch et al. 2020).

BlueDot links the data from centralized disease surveillance through artificial intelligence with the airline itineraries collected 24/7 to anticipate the forthcoming pandemics across our interconnected world. This company gathers data using an AI engine continuously for a large number of diseases from a range of different sources across the globe. It is worth mentioning that BlueDot was able to predict the onset of COVID-19 outbreak much before WHO alerted the world (Bowles 2020).

8.7.2 Early Detection and Diagnosis of Infection

Since radiological diagnosis is based on imaging data, it is most compatible for deep-learning techniques (Yu et al. 2018). Computed tomography (CT) chest images of COVID-19 patients predominantly show bilateral ground-glass opacification and consolidation (Wang et al. 2020). COVNet is a three dimensional automatic deep-learning software used for accurate and fast detection of COVID-19 as it takes less than 5 s to process each CT scan (Li et al. 2020). This method of radiological diagnosis is gaining popularity over the gold standard RT-PCR for COVID in case of critically ill patients where time is a big factor. Another similar AI system called InferRead CT Pneumonia can identify features of coronavirus infection in CT scans of patients having false-negative RT-PCR reports (Li et al. 2020). AI can thus assist in making quick triage and clinical decisions regarding patients with COVID-19 and hence facilitate the optimal use of hospital resources (Jiang et al. 2020). Hurt et al. (2020) used an AI-augmented system for plain chest x-rays to track and predict the progression of COVID-19 among hospitalized patients and to provide critical care to needy patients.

An artificial intelligence enabled cough-based screening system called AI4COVID that works through a smartphone has been developed (Imran et al. 2020). This app can easily distinguish between coughs of COVID-19 patients and several other types of coughs of non-COVID patients. It records the 3-s cough sound of a suspected patient, sends it to an AI engine running in the cloud, analyses the type of cough, and sends back the result within a timeframe of 2 min. Thus, it serves as an important screening tool.

8.7.3 Monitoring Treatment

Spread of this deadly coronavirus can be monitored using AI-based platform. A big database of X-rays and CT chest images can be created for proper monitoring of disease progression and treatment of COVID-19 patients (Haleem et al. 2020; Biswas and Sen 2020; Stebbing et al. 2020). Such tools capable of providing daily updates of patient's condition are extremely helpful during the pandemic. Another successful application of AI has been witnessed in the form of online training of doctors and paramedics for providing better care to COVID-19 patients and for control and spread of disease. Persons at risk of getting infection can be easily identified, and clinicians are better equipped to initiate timely treatment, thus

resulting in better outcomes. Jiang et al. (2020) developed an AI tool using several clinical parameters to predict the course of patient disease with good accuracy.

8.7.4 Surveillance

Web-based tools for surveillance called Google Flu Trends (GFT) was used to monitor influenza activity in real time (Christaki 2015). Drones have proven helpful in monitoring the movement of people during lockdown especially in containment zones and check those breaching the rules. AI-enabled CCTV cameras have been used to identify the mask violators. Robots have been used for dispensing medicines, giving hand sanitizers, and delivering educative public health messages for awareness. AI-enabled chatbot called SGDormBot has been used in Singapore for mass screening of COVID-19 symptoms in migrants (Chen and See 2020).

8.7.5 Real-Time Spread Tracking and Contact Tracing

A collaborative app for contact tracing was developed by two big tech giants, Apple and Google in April 2020 to trace COVID-19 patients. This app works on Bluetooth and is being used in several western countries. The Government of India in collaboration with private companies developed a similar app named Aarogya Setu in May 2020 to control the spread of COVID-19. This contact tracing app uses real-time location of its users via Bluetooth and alerts them about positive patients around them, the risk of getting the virus, and how to deal with it. Approximately 16.73 crore users have downloaded this app, thereby strengthening India's fight against COVID-19, and total of 173,111,694 samples had been tested for COVID-19 up till December 31, 2020. The Bluetooth contacts of positive patients are advised for taking precautions, get themselves tested, and quarantined depending on the extent of the exposure. This app identifies localities where clusters of positive patients could be anticipated and helps to undertake extensive testing in such areas, thereby helping in controlling the spread of COVID-19 infection.

8.7.6 Projection of Cases and Mortality

Ever since the start of COVID-19 outbreak in December 2019, several groups of scientists around the globe have come up with computational models to predict the spread of disease around the world (Duccio and Francesco 2020; Boccaletti et al. 2020). Such statistical models for disease forecasting are accessible to the public, and these intelligent mathematical tools give information regarding number of new cases, recovered cases, deaths, hospitalization needs, travelling restriction, and also emphasize on importance of social distancing, etc. The predictions using these models are sometimes variable due to vast and uncertain data and also due to differences in design of these models (Woody et al. 2020; Jewell Nicholas et al.

2020). The reason for such variations is nonavailability of data on this novel virus during the initial phase of outbreak and lack of authentic, complete, and reliable data from various geographical regions. Attempts are being made to resolve these issues in revised models, but errors in predictions are still high. Secondly, these models give predictions based on data of lab confirmed patients of COVID-19 who are symptomatic and/or need hospitalization. Complete data of asymptomatic patients is not available. Further scant information regarding predisposing conditions is available, thus limiting the reliability of such models. Consistent efforts and research are required to improve and standardize these models for making timely and accurate predictions based on information available.

8.7.7 Creating Chatbots

In order to face the challenge and threat posed by the growing pandemic of COVID-19, the Government of India launched an innovative app called MyGov. This helpdesk chatbot is helpful in establishing a beneficial partnership between government and citizens to provide accurate real-time information about the coronavirus to its two million users. MyGov chatbot has been created by Haptik (conversational Artificial Intelligence platform), and more than 7.8 billion people have used this chatbot till 31st December 2020.

8.7.8 Treatment Through AI

An AI powered tool for in silica screening can help to identify potentially effective therapeutic drugs from the pool of already existing drugs. This is known as drug repurposing. For example, ZINC15 has been found to be effective against COVID-19 using deep learning technology (Ton et al. 2020). A molecule transformer-drug target interaction (MT-DTI) NLP tool has been used to predict binding efficiency between antiretroviral drugs like Atazanavir and surface proteins of SARS-CoV-2 (Beck et al. 2020).

8.8 Summary, Conclusion, and Future Prospects

AI- and ML-driven technologies are extremely powerful and very promising in accurate and quick disease diagnosis and treatment. These advanced tools facilitate speed, consistency, and capability of handling large datasets. Integration of modern technologies with traditional tools has transformed the field of clinical microbiology.

Though AI tools look very promising in improving health care, it is very important to use these novel tools with great caution as the reliability of results generated by these techniques depends largely on the data collected from various sources. Therefore, challenge remains in improving the availability of high quality, accurate, and unbiased data so as to make reliable predictions and to enable better

decision making by healthcare authorities. Data integrity, appropriate data processing, algorithms, appropriate model selection, remodeling, continuous monitoring of models, and improved validation strategies must be used in order to achieve the maximum benefits of these futuristic tools. The constant mutating of diseases and viruses make it difficult to stay ahead of curve, but continued advances in AI promise in help people to live a longer and healthier life.

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DNA Digital Data Storage: Breakthroughs in Biomedical Research

9

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Abstract

Digital information is increasing continuously, and therefore long e-term storage is required to store data. Magnetic media and optical media have many disadvantages, which can be overcome by deoxyribonucleic acid (DNA), a natural mechanism of storing data. The latest technique has transform science fiction into reality. DNA has all the resources to replace the conventional hard disk store huge amount of data with in small volume. Although DNA has a massive potential to store data but some limitation such as very expensive costs, slow reading and writing mechanisms are required to solve till now. In this chapter, I highlight how DNA can store data in the future and limitation of using DNA as data storage.

Keywords

Digital information · Magnetic media · DNA · Optical media

9.1 Introduction

Biocomputers (BCs) are a computer made from living cell. BCs use chemical inputs and biological molecules such as proteins and DNA. BCs use two term "neural networks" and "genetic algorithms" to study and assess the given information. There are average 86 billion neurons in human brains that process and transmit information. Neural network works on the basis of the same principle. Genetic algorithms are based on genetic code sequences and computer retrieve information from code

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and give predicted outcomes or result in the production of a particular protein. These biocomputers are used for studying and reprogramming the living system. The scientists have already created a biological transistor inside *Escherichia coli* using DNA and RNA. These transistors are called transcriptors. The biological transcriptors control the flow of enzymes along a strand of DNA. These transcriptors could be used to reprogram living system such as gene expression. The biological transcriptors can also be used to cellular therapeutics. Scientists are trying to transmit genetic information between cells called the biological Internet. By using computational input inside the cell, researchers are trying to sense and report the presence of cancer. Such system will also help in designing of drugs. Artificial intelligence, brain–computer interface, optogenetics and molecular computers, and DNA computers are the recent technique related to BCs. BCs capable of performing task like the human intelligence are called artificial intelligence (AI) or machine intelligence. The point where BCs exceed human intelligence is called superintelligence. Brain–computer interface uses brain activity and converts it into artificial output that helps person to communicate their wishes that have brain disorder or are severely paralyzed.

Any innovation that inspired by nature is called biomimicry. Biomimicry is something like the leaf, bird wings, etc. and tries to figure out better resource from them. Biomimicry is based on nature principle because nature always operates in economical and efficient manner with no waste generation. DNA as a data storage is also a type of lesson that has been learnt from nature, living cell, and molecular biology to tackle the computational challenges faced by the silicone-based hardware. Nowadays, data is stored in an electronic devices using binary code 0 and 1, and to retrieve this data, applications are run to extract and process this binary code and present the information in its original form. But these technologies have limitations, especially when required to design small devices. To tackle this problem, scientist tried to make alternate mechanism for data storage using biomolecules such as DNA and synthetic chemicals to develop inevitable approaches to data storage and computation. Storing data in DNA was first demonstrated in 1988 (Davis 1996). After that, scientists have tried many experiments to store data using biological material.

9.2 Why Scientist Selected DNA for Data Storage System

DNA is a natural mechanism of data storage; it contains all the genetic instructions required for the body to grow and function. If DNA can store all biological data, is it possible to store digital information too. Researcher found that DNA can also store digital information. Scientists are using genetic DNA to create data storage system.

Humanity has generated huge data in past year. In the study conducted by Google by using company Doma software in 2018 on 3.88 million searchers, it was found that in every minute people watched 4.33 million videos on YouTube, tweeted 473,000 times, and sent 159,362,760 e-mails. From this study, it was estimated that 1.7 megabytes data will be required for storage per person per globally. Vast

Table 9.1 Properties of information data storage devices

Storage devices	Data reserve time (years)	Data holding capacity	Access time
Hard disk	>10	Terabyte	Seconds
Flash memory	>10	Gigabyte	Seconds
DNA	100–1000	Zetabyte	Hours

majority of our data is stored in magnetic media like hard drives and optical media such as CD and DVD. Therefore, optical or magnetic data storage system cannot hold data of 7.8 billion world population for more than century. With the passage of time, the data storage problems become more serious. Table 9.1 shows data storage capability of different devices. One of the major limitation magnetic and optical media is it is not robust, has low information density, and each media required special device to read and write data. These problems can be overcome by using DNA as data storage. The problem of robustness in magnetic media is that hard drive is unreliable with life and is 1–3% died in first year and in the subsequent year that rise to over 10% death. For example, in 1991, mummy of Otzi iceman was found in Alps. He was died in 5000 years ago, and yet we are still able to extract DNA, read it, and found to be lactose intolerance and still have living relatives in Austria today. If Otzi have been carry hard drive with him, we were not able to extract data after 30 year. Facebook cold storage data center is 5700 m^2 in size and can store 1 exabyte data. As huge amount of data is generated in 2018, hence storage of such data required some novel or natural storage material. DNA is the best answer to this problem, as we can store data of 1 zettabyte per gram DNA. In this way, only 5 g of DNA is required to store whole data of Facebook cold storage data center. On the basis of theoretical calculation, DNA can encode 455 exabytes per gram of ssDNA. Human have huge DNA storage capsules, as 37 trillion cells are normally found in human body. If human genome is 750 MB in size per cell of body, and then it contains 750 $\times$ 37 trillion MB data storage capacity.

9.3 How Data Can Be Stored in DNA

Traditionally, we stored data in binary code 0 and 1. Like binary code, DNA stores genetic information through the sequence of nucleotide base adenine (A), guanine (G), cytosine (C), and thymine (T). With four possible base units, data normally required two number of binary that can be stored in one nucleotide base. This means information can encode twice the amount of information in DNA than as in binary. In hard drive 0 is represented by area of the disk, which is magnetized. DNA has four nucleotide base A, T, G, and C. This mean we have four distinct value A, T, G, and C in a set of two 0 and 1. Therefore, we have to revise with binary file. Instead of storing each 0 or 1 individually, we store in comparison of two like 00-A, 01-T, 10-C, and 11-G (Fig. 9.1). Once we have this setting, we can encode data into synthetic DNA. This is exactly like the real stuff but only the difference is that synthetic DNA is not stored in cell. Data stored inside DNA can be read out by DNA

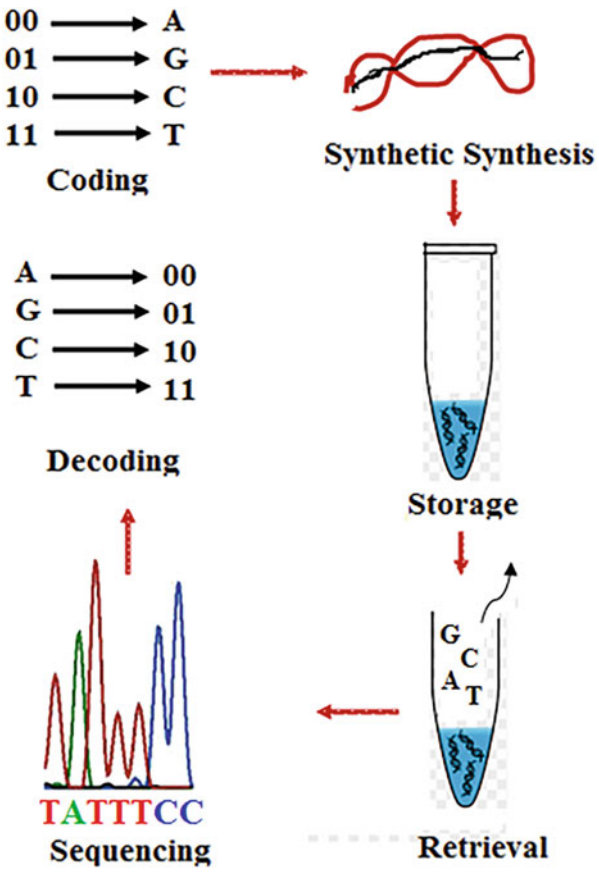

Fig. 9.1 Steps involved in developing DNA as data storage

sequencer. However, this process is not perfect, and read error may occur. The sequencer may read T instead of G or not able to sequence if DNA became damaged. In organic DNA, change in nucleotide may results in mutated proteins or even kills the cell. But in synthetic DNA, we don't have protein, and therefore, this problem can be overcome by adding error correcting codes into data that stored in DNA. How this error correcting codes works? It's simple, imagine we want to store three digits 2, 8, and 3, and you are able to recover all three codes even if one is missing. This is possible if you add these three digits ($2 + 8 + 3 = 13$) in data, e.g., if 3 is missing, it can be recovered by $13 - 2 + 8 = 3$. Now, DNA data can also be copied by using polymerase chain reaction (PCR).

So far, scientists have enabled to store entire books and music videos in DNA sequences. This digital books and audio files are converted into binary code that is translated into specific sequences in nucleotides. A strand of DNA, which is then freeze dried and once freeze dried this DNA, the information it contain can last for thousands of years. When the time comes to access this stored data, the DNA strands are sequenced and can be converted back to binary code or any other base

representation. Besides DNA storage, scientists are developing new molecular storage strategies using the wide varieties of small molecules, including small the molecules that make up metabolome. Metabolome is the complete set of all small and macromolecules in the biological system. Its diverse chemical composition makes incredible information dense. Complex processes occur in our body at molecular level; the makeup of metabolome is the informational basis for all these events. Based on this idea, scientists are creating extensive libraries of synthetic molecules that like metabolome can be used to encode information stored and even execute complex data computation. With the mixture of these complex molecules dissolved in solution, scientist can retrieve information by analyzing the mixture with special instrument. The read out from instrument indicates the presence or absence of specific molecules. This information can be converted to base representation system code like binary, which computer can interpret. For example, the presence of molecule "A" in sample can equated to 1 while its absence is equivalent to 0 in binary. By referencing the preestablished library, the presence or absence of the molecule in a mixture represents one bit of information. In a 4×4 well plate, with 16 unique mixtures, four molecules can then encode 64 bit data. Imagine this process on much larger scale and you can get an idea that just how much data can be stored in this way. Traditionally, by understanding how these molecules were react in certain environments, scientist can apply different reaction conditions to change data storing molecules in a manner like traditional computation. In this way, complex manipulation and calculation can be performed on data in multidimensional spaces, while this store in a very small volume of solution. This would allow for sophisticated parallel computing, enhanced parallel recognition, and improved such algorithm used by popular web services today. Once we able to find storage computing power of small molecules like DNA, metabolites will be able to contain more information in less volume, in which electronic devices become even smaller than they are today. One of the major drawbacks of DNA as data storage is the cost and speed of reading DNA. For the synthesis of one megabyte, 3500 US dollar is required.

9.4 Related Work

The data encoding is big challenges for researcher. Earlier researchers were able to recover very short massages. Bancroft et al. (2001) used ternary encoding to translate text to DNA. They sequenced three nucleotides A, T, and C for 26 English characters and space characters, but these encoding results reduced consistency for longer messages. In 2010, Gibson et al. performed in vivo study and recovered 1280 characters from bacterial genome, but the applications are quite different in vitro study. Later on Church et al. (2012) and Goldman et al. (2012) recovered 643 kb and 79 kb message, respectively. But both studies have bits of error, 10 bits error, and 25 nucleotides are missing in Church et al. and Goldman et al. studies, respectively. Finally, Grass et al. recovered 83 kb message without error. Yazdi et al. (2015) developed the first DNA-based storage architecture that facilitates random access to

data blocks and rewriting of information stored at arbitrary locations within the blocks. The CRISPR-Cas gene editing not only is a recent technique known for assisting scientists to treat genetic diseases but also has potential to be used in BCs. Shipman et al. (2017) encoded digital movie into the genomes of bacteria(s) using CRISPR-Cas, Cas1-Cas2 integrase system. They recorded the images of a human hand into the genome of *E. coli* with more than 90% accuracy. Recently, Kim et al. (2019) created CRISPR-Cas based core processors into human cells.

9.5 Conclusion

DNA-based storage has the capability to store data of world in future. Its durability and data holding capacity attracted researcher for use as storage devices. However, synthesis of DNA and reading and writing is expensive process, but advancement in biotechnology will definitely overcome this problems in the future. Now, it's the right time for collaboration of biotechnology and computer-based industries to accept the challenges to combat with problems.

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Biomedical Applications Cloud-Based IoT

10

Masood Ahmad, Rajeev Kumar, Alka Agrawal, and Raees Ahmad Khan

Abstract

The IoT (Internet of Things) is a mechanism of connecting biomedical and implantable medical devices by the network and sending the data over the network without any human interference. IoT is a novel technology in the biomedical that allows the network to reach out to the existing world of touchable objects. This technology is growing very fast, and most of the healthcare systems are now enabled by IoT. The best examples of this can be seen in the context of smart city, agriculture, health care, transport and logistics, etc. Cloud computing plays an important role in the IoT processes like data processing, securing the data, and other tasks. However, as the biomedical data is being transferred over the network, there are possibilities of breach in the security of the biomedical data. For solving and improving the security issues, an inventive approach is to integrate cloud and the IoT. For the integration of IoT and cloud, the key factors to reckon are: validation methods and IoT framework, which must be reliable and secure. This chapter is an exhaustive analysis of the IoT, Cloud, Cloud model, integration of Cloud with IoT, and its applications in biomedical. The study also enlists the various challenges that the practitioners are contending within the framework of IoT and Cloud. The chapter attempts to posit workable solutions that would be an effective reference for future research and adaptation in the domain of securing the biomedical data exchanged through Cloud and IoT.

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Keywords

Internet of Things · Security · Privacy · Cloud · Services · Biomedical applications

10.1 Introduction

In the digital world, the Internet of Things (IoTs) is a revolutionary technology, especially in terms of the biomedical, social, as and financial flexibilities that this technology offers. The IoT has captured most of the engineering fields like computer science, biomedical engineering, electrical and electronics engineering, and other technical and nontechnical fields. Like the other networked devices, biomedical IoT is also a networked device, which is used to communicate with other implantable devices. The use of IoT is predicted to reach approximately 22 billion till 2025 (Lueth 2018). Figure 10.1 tabulates the increasing span and rate of IoT networked devices from 2015 to 2025. Biomedical IoT devices are gaining popularity because of the ease of communication, which they afford over the Internet. However, these networked devices are facing security and monitoring issues (Alam 2017a). Web-based and mobile-based biomedical IoT control technology is popular, and numbers of networked devices are increasing by the day (Alam 2017b). Implantable IoT devices transmit the data to other device connected through the Internet (Alam and Benaida 2018).

Biomedical (implantable) IoT contains the sensor, which senses the signal and other object and store the data. Biomedical IoT devices are connected to each other through the wireless network and send the data to the cloud for the preprocessing (like, storage, computation, and many more) (Tiwari and Matta 2019). Biomedical IoT devices use heterogeneous technologies for data transmission and protocol for communication like "Bluetooth," "Zigbee," RFID, and near-field communication (NFC). For the worldwide data transmission, IoTs used mobile network services like GPRS-Edge, 3G, 4G, and 5G. IoT sensors automatically work as a machine to machine communication. Application of IoTs is too broad and ranges from

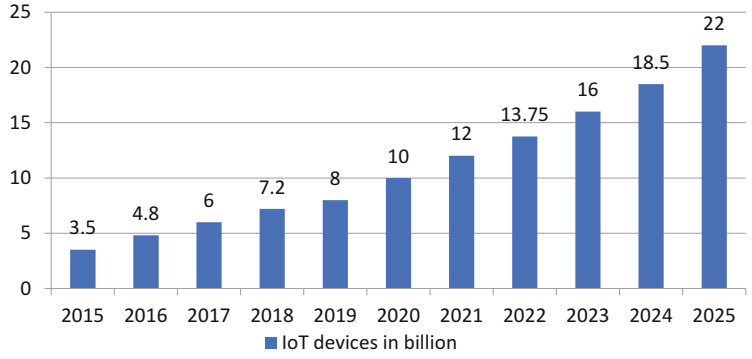

Fig. 10.1 IoT devices in billion

developing smart home, smart city, environment forecasting, healthcare management, energy management, and agriculture. All these sectors generate a large amount of data that needs to be easily and frequently accessed (Baskar et al. 2017). Managing large amount of data that is created by the heterogeneous devices requires flexible network. But the performance of biomedical IoTs decrease by the increased use of heterogeneous devices (Olleros and Zhegu 2016). The reasons for this include limited power, bandwidth availability, and memory. The best solution for overcoming these limitations can be the integration of Cloud and IoT. Cloud can provide on demand the storage space and application development platform (Misri et al. 2019). Cloud IoT concept evolved from MIT's Auto-ID labs, and this concept was created by the integration of two heterogeneous biomedical IoT devices and Cloud platform. The biomedical IoTs face the problems of limited computation power and storage space and limited power backup. Yet another drawback is that biomedical IoT operates by the energy, and in some cases, IoT consume more energy than required. This may cause extra heating in the device and reduce its efficacy and damage the organs of the patient. These issues also lead to security flaws and breach in the privacy of the biomedical IoT (Shah and Bhat 2020). Cloud IoT can ensure efficient power and resource management and provide a far more expansive platform for biomedical and different application areas. Cloud IoT may process the data in real-time application in a far more secure and reliable fashion. Clients (health care) can use the services of the Cloud IoT on *pay per use* basis or on subscription. Cloud IoT provides the countless services by Cloud-based application like Software as a Service (SaaS), Platform as a Service (PaaS), and Infrastructure as a Service (Iaas). Quality of Service (QoS) is also maintained by the Cloud (Mehetre et al. 2018).

Few characteristics of the Cloud IoT implementation include: a virtually large storage space and fast computational for biomedical IoT, processing of images, efficient and effective resource management, and quality of services among several others. Reliability of the data, secure transmission, and data storage are other solutions of the Cloud IoT. Hence, Cloud IoT technology can be a highly effective virtual means for transmitting the biomedical, economic, and business data. However, even Cloud IoT is vulnerable to cyber-attacks as data predators are continuously poaching on highly sensitive data for commercial exploits. Hence, the developers should focus on the safety, confidentiality, privacy, integrity, availability, and authenticity (Mehetre et al. 2018). For the authenticity and data encryption, the cryptography technique is used, to ensure that the biomedical (implantable) IoT is *tamper-proof* and has not been damaged by overheat (Pawar and Vanwari 2016). When two different technologies are integrated, then all the services should be flexible, unstoppable, and provide biomedical data under surveillance (Kosba et al. 2016). In this chapter, we have discussed the security issues, which affect the biomedical IoT and vulnerabilities, which are added with Cloud.

10.2 Related Work

Since biomedical (implantable) IoT support different protocols, it is difficult to maintain the adaptability, reliability, accessibility, and authenticity of the data being transferred through it. Integrated technology of Cloud IoT provides reliable communication, storage, and computation (Sandeep 2018). "Cloud" would provide support to the IoT by increasing the adaptability to interconnect with the real-world appliances (Ahanger and Aljumah 2019). In this league, we analyzed several research studies that explore the advantages and features of integrating Cloud and IoT in biomedical engineering. The relevant literature is enunciated below:

Pawar and Vanwari 2016 presented a detailed review on Cloud and IoT integration. In this paper, the authors find out different characteristics of cloud and IoT and also identify the prime factors that are responsible for the integration of Cloud and IoT.

Xu et al. (2000) explained the steps required for the practical implementation of integrated Cloud IoT.

Khan and Salah 2018 designed sensor-based cloud infrastructure and explained the architecture and implementation. This infrastructure was based on converting a physical sensor into virtual sensor over the cloud.

Al Fuqaha et al. (2015) implemented open source cloud-based integrated platform known as the IoTCloud, which permitted the users to create flexible and proper sensors for the IoT applications. Future Grid Cloud test bed was used for the performance and empirical analysis.

Qui et al. (2018) presented the latest principal representation for intercloud architecture and also helped in the common and different range of management based on the nonfunctional requirements of the heterogeneous cloud. However, this study does not elaborate the cloud relation with IoT.

Pustišek and Kos (2018) implemented a Media Edge Cloud framework, which contained storage space, CPU, and graphics processing unit. The Media Edge Cloud provides the parallel and common computing and also maintains the QoS. However, the empirical cost analysis has not been verified in the study.

Khattak et al. 2019 proposed a novel framework of communication for integrated vehicular ad hoc network, cloud and IoT, known as VCoT, through the vehicular clouds and IoT services for new applications. This framework is designed for particular application like smart city. It is operated and controlled by the LoRaWAN vehicular ad hoc network.

Tanweer Alam (2019) introduced the integrated framework Cloud-MANET-IoT for solving the data transmission security issue and increasing the performance. The main objective of this study was to create a communication system between the embedded Cloud and IoT. This chapter defines the Cloud IoT paradigm and integration elements and also points out the important issues in Cloud IoT along with their feasible solutions.

10.3 Internet of Things Model

IoT communicates over the Internet by using the divergent protocols with the heterogeneous devices. Devices connected from other devices in the network are also known as IoT or Things. IoT contains short range of communication and low power consumption devices like radio-frequency identification (RFID), Bluetooth, Zigbee, and many more.

Main elements of IoT like Internet, Processing, and Things are shown in Fig. 10.2.

10.3.1 Internet

Internet provides the connection among the devices by the Internet protocols.

10.3.2 Processing

In the IoT, processing on the received data is done through the sensors with the help of microcontroller.

10.3.3 Things

Things include sensors, appliances, and other instruments, which observe their surrounding by the RFID. Things act as an actor and perform the task after sensing the processed signals.

10.3.4 Hierarchical Architecture

This is an era of smart devices in which the device is connected to the Internet and the information is transmitted over the network. The model depicted in Fig. 10.3 is

Fig. 10.2 Elements of IoT

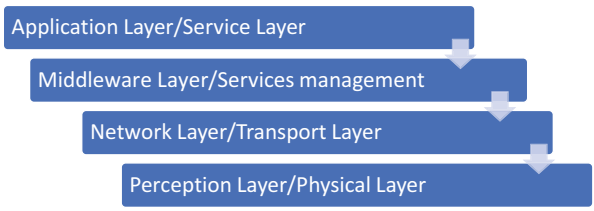

Fig. 10.3 Hierarchical IoT architecture

divided into four layers in a hierarchical manner. These layers are denoted as: the Application layer, Middleware layer, Network/Transport layer, and Physical layer. All the layers have their own defined function and services.

10.3.4.1 Physical Layer

First layer of the architecture is physical layer, which uses the sensors, like RFIDs, barcode reader, and many more. Main function of this layer is to take inputs and then send the information to the processing system. After processing, the information is sent to the upper layer for operation or control.

10.3.4.2 Network Layer

Network layer is used for data transferred from initial source to the goal source.

10.3.4.3 Middleware Layer

Middleware layer works as an intermediary between the application and network layer. The entire data management task is performed in this layer. Thereafter, the processed data is sent to the upper layer.

10.3.4.4 Application Layer

Application layer is the user-friendly layer that contains many application functions. Application layer promotes different application areas like the Smart industry system, Smart health care, and Smart city and traffic.

10.3.5 Cloud Model

Cloud provides the on demand service to the users like application development, computational power, homogenous database, and data analysis. Most of the IoTs choose Cloud service for its economic feasibility, reliable performance, flexibility, scalability, and enhanced security. Cloud differs from traditional processing systems because of various advantages that include: the feature, on demand services, broader network access, collection of multiple resources, and the elasticity of resource.

Figure 10.4 demonstrates the Services of Cloud in different stages. These stages are: Infrastructure as a Service (IaaS), Software as a Service (Saas), and Platform as a Service (Paas).

10.3.5.1 Software as a Service (SaaS)

SaaS provide the unlimited access to the software and database through the subscription for that service. Cloud provides the security for the users' data, and the data are not lost due to hardware failure. We can access Cloud anywhere and anytime from any geographical location.

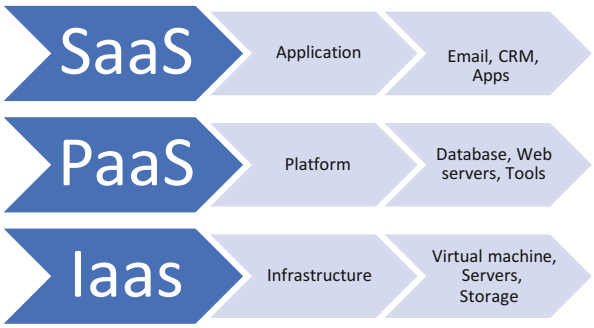

Fig. 10.4 Cloud service model

10.3.5.2 Platform as a Service (Paas)

PaaS provide multitasking on the work stations, and different users can work simultaneously on it. By using the interface, the users can manage, develop, test, and deliver apps to clients.

10.3.5.3 Infrastructure as a Service (IaaS)

IaaS provide the virtual space and resources to the users for purchasing the services. IaaS is scalable according to users' needs. IaaS provide the services like the billing system.

Cloud services are categorized into four entities which are:

1. **Public Cloud**
   Best explained by the term Public, the Public Cloud makes the resources available for public access through the Internet. Service providers charge the users according to the services accessed. Examples include: Google cloud, Amazon Elastic Compute Cloud (EC2), IBM Blue Cloud, and Sun Cloud.
2. **Private Cloud**
   Private Cloud is serviced by the company or organization for their use, for example, Google Drive. This is also referred to as the Mobile Cloud.
3. **Community Cloud**
   This service is hired by a particular community and organization for personal tasks; this is also like a private Cloud.
4. **Hybrid Cloud**
   Hybrid Cloud is a combination of all three Clouds: the Private, Public, and the Community Cloud services.

10.4 Integrated Cloud with IoT (Cloud IoT)

With the ubiquitous use of smart biomedical devices, the dependability on Internet is increasing. Since the interconnectedness among different *Things* is also on the rise, the rate of data generation by the things is also increasing voluminously. However, the biomedical IoT have limited storage space, and the data generated on the Things

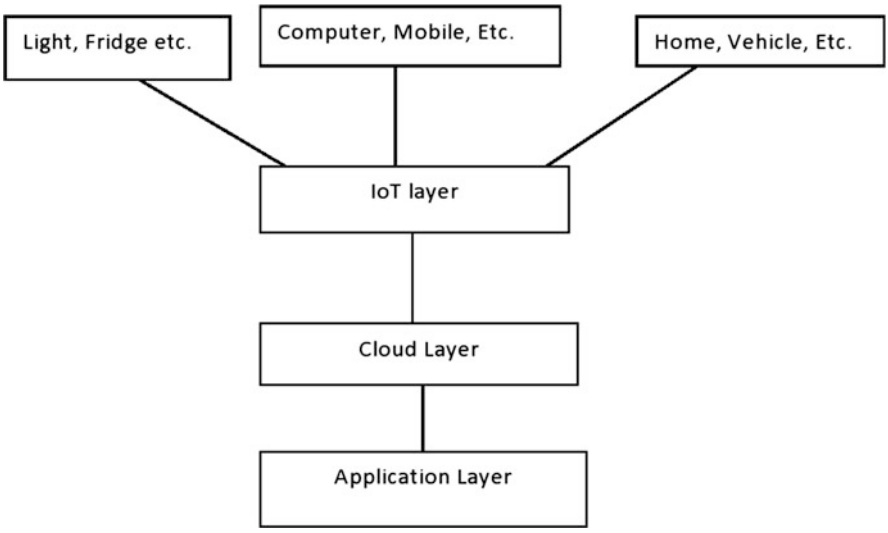

Fig. 10.5 Integrated Cloud IoT

cannot be stored in this capacity. In the biomedical IoT, the sensors send the data to mainframe system (apps), which needs infrastructure and resources for computing. Data storage on the mainframe system is costly, and the process of an application running in the mainframe is also times consuming. Besides this, if the biomedical IoT fails, then the whole system (device) stops functioning. The present day demand of the patients is biomedical IoT devices at low cost but with more accurate and computational power. Cloud IoT is an apt solution as it is cost-effective, robust, and tangible in nature (Farooq et al. 2015). In the integrated Cloud IoT, biomedical IoT sensors takes inputs data of patients and sends the data over the Internet while Cloud provides the space and resource for the processing on the data taken by the IoT implemented on the patient (Khan and Salah 2018). Figure 10.5 explicates the working of the integrated Cloud IoT. In the figure, the data from the biomedical IoT device is sent to the biomedical IoT layer, and this layer sends the data to the Cloud for the storing and preprocessing on the data. After the data has been processed, the users can access the data through the application layer.

10.4.1 Features of Integrated Cloud IoT

10.4.1.1 Storage Space

Biomedical IoT generates large amount of patient data. The generated patient data that comes in the semistructured and nonstructured form is called the *big data* (Dhaliwal et al. 2010). The biomedical IoT devices cannot capacitate the big data. However, Cloud can effectively and efficiently manage big data. Patient data is stored in the Cloud and processed on the patient data through different data

analytical tools. Further, required data can be accessed for extracting fruitful information. Multiple cryptography techniques are applied for securing the patient data from trespassing.

10.4.1.2 Computing Power

Every biomedical IoT has limited power of computing, and this debility impedes the data processing operation of the IoT. For removing this issue, the patient data is sent to the powerful and scalable computational Cloud.

10.4.1.3 Communication

Though biomedical IoT provide reliable data share over the network, the communication through the Internet is costly and is not economically feasible. But the patients can avail similar and more efficient services through Cloud. Moreover, these services are economical and can also be accessed at any given hour from any geographical location.

10.4.1.4 New Security Model

Maintaining the adaptability, validity, accessibility, and authenticity between biomedical IoTs and protocols is a very difficult and costly process. This issue can be solved by Cloud, because Cloud provides authenticity, easy access, and economically feasible distribution of patient data.

10.4.2 Cloud IoT Applications

Integrated Cloud and IoT communication technologies are like: Machine to Machine (M2M), Human to Human (H2H), and Machine to Human (M2H). The real-world application has been displayed in Fig. 10.6.

10.4.2.1 Smart Health Care

Digital health care is a rapidly growing application driven by Cloud IoT today. Healthcare monitoring system contains (1) sensors and implantable devices to monitor and record the patient's data; (2) healthcare devices use wireless body area network for communication, this permits the doctors to access the data even from remote zones; (3) and Cloud server for data storage and analysis. Health care produces huge data on a daily basis; the data are stored over the cloud. For the processing on the data, many data mining tools are applied for the decision making. Thus, the integration of Cloud and IoT technology is highly recommended for maintaining the efficacy of the Smart Healthcare.

10.4.2.2 Internet of Robotic Things (IoRT)

IoRT is emerging technology of AI and big data. IoRT is used for the surgery and other medical work. IoRT can be controlled by programs, manipulates, and transport materials in different types. IoRT is used to minimize the risks, better surgical results with low cost.

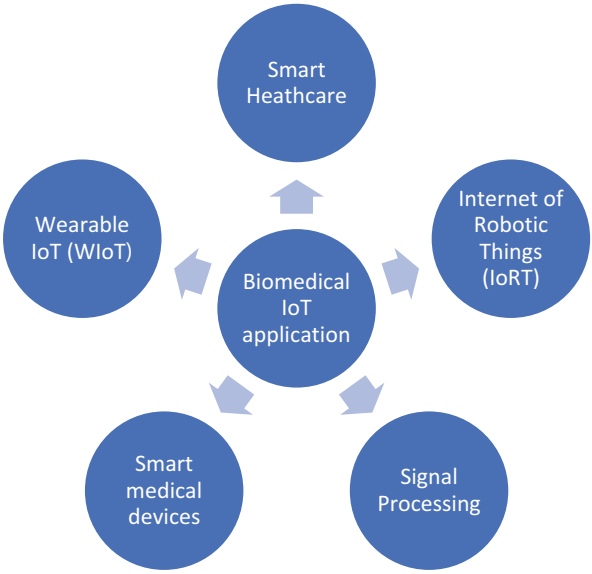

Fig. 10.6 Cloud-based IoT biomedical applications

10.4.2.3 Signal Processing

IoT used in biomedical for medical data processing. Biomedical IoT implant in the human body and capture the signal and send to the mobile and cloud for the processing of the data for the better treatment.

10.4.2.4 Smart Medical Devices

IoT is making the medical devices smart by the connected with the Internet. Biomedical devices take the patient information and directly transmit the data for the processing over the Internet and reduced the interference of human and save time and money.

10.4.2.5 Wearable IoT (WIoT)

Wearable device generates the huge amount of the personal health data. Cloud, Fog computing, and big data play an important role in the WIoT. All these services manage the huge healthcare data and improve the biomedical process at remote and local servers.

10.4.3 Open Challenges in Integrated Cloud IoT

It is clear that tangible benefits will add to the integration of Cloud and IoT. However, this technology needs to be secured from the data invaders who are constantly working on inventive mechanisms to hack and pilfer the sensitive data of the users. Hence, the researchers and the security practitioners need to eke out effective safeguards against cyber-attacks.

10.4.3.1 Privacy and Security

In the Integration of Cloud and IoT, the privacy and security is the most important concern. Privacy checking methods are required to check the patient data on the network. Moreover during the storage time also, the integrity and authenticity of data must be maintained because the privacy of patient data is necessary for the performance of application and framework. Thus, there is a need to develop secure framework for heterogeneous technique information sharing among the biomedical IoT and Cloud.

10.4.3.2 Need for Standard Architecture of Protocol

There is yet no standard architecture of protocol available for communication for biomedical IoT. All the protocols are available for heterogamous devices only. Even the homogenous devices in network use the heterogeneous communication protocol. It is possible that at the time of data aggregation gateway, due to incompatibility, the protocol does not support the device. Incompatibility issues become more serious when they are connected or integrated with Cloud. If service providers want to ensure secure and seamless integration of Cloud and IoT services, it is imperative to develop standard protocol.

10.4.3.3 Efficient Power Consumption

Power consumption is a very important issue in integrated Cloud IoT, because all biomedical IoT devices contain batteries. All these batteries do not have the requisite backup, and if video surveillance is in process, then problem becomes more serious. There is an urgent need to harness the natural resource (wind and solar power) for power generation, and inventive models can be designed in this context (Khari et al. 2016). Other solution to this problem is to write the program for the energy saving. Program routinely checks the biomedical devices and ensures that when the action occurs, then the biomedical device is in operation mode or in the sleeping mode.

10.4.3.4 Limited Bandwidth

Limited bandwidth of biomedical IoT devices bars the performance of biomedical devices, retarding their ability to give optimal efficiency. Therefore, there is a compelling need for high bandwidth so as to optimize on a biomedical device's performance.

10.4.3.5 Delayed Services

Cloud has various services and countless computing resources, but these services cannot be utilized and will not be beneficial if they are delayed. Thus, for removing the delay, fog computing must be enlisted along with the Cloud and IoT. The fog computing is used for achieving low latency and preventing delay for the sensitive applications.

10.5 Conclusion

Integrated framework developed in the twenty-first century has various advantages in biomedical data processing. Integration of Cloud and IoT (Cloud IoT) provides the services for powerful computing, unlimited data storage space, and availability of network any time to medical data. However, this technology needs more inventive interventions to ensure that the privacy and integrity of users' data is not infringed upon. The chapter chronicles several lacunae and possible solutions for the same in the integrated Cloud IoT in biomedical engineering. This discussive study is an attempt to engage the attention of the present day research investigations in domain of Cloud IoT in biomedical engineering.

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Next-Generation Sequencing in Cancer Research

11

Pushpinder Kaur

Abstract

Recent technological advances in next-generation sequencing (NGS) methods have made significant achievements in the field of cancer genetics. These technologies have helped to reveal diverse genomic heterogeneity landscape across various tumor types. The most promising aspect of NGS technologies is the identification of cancer-causing genes and actionable genomic alterations that could help in selecting patients for targeted therapies. This chapter focuses on applications of NGS in cancer research in solid and liquid biopsies, discusses NGS-related methods, reviews progress in clinical oncology, and summarizes challenges.

Keywords

Cancer · NGS · Cancer therapy · Clinical oncology · Genomics

11.1 Introduction

In recent years, the rapid progress in next-generation sequencing (NGS) technologies has profoundly impacted our understanding of cancer genomics. To date, NGS-based technologies have been comprehensively applied in many fields such as genomics, epigenomics, transcriptomics, metabolomics, and proteomics. These systems also have the potential to characterize the single-cell genome. Many large-scale projects such as the Cancer Genome Atlas (TCGA) (Weinstein et al. 2013), International Cancer Genome Consortium (ICGC) (Hudson et al. 2010),

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and American Association for Cancer Research (AACR) GENIE (Genomics Evidence Neoplasia Information Exchange) (Gulbahce et al. 2017) are using these high throughput technologies to characterize individual tumors and a various cancer types and subtypes. The application of NGS technologies into these large-scale sequencing projects has revealed insights into the mechanisms underlying tumorigenesis in various anatomical sites (Weinstein et al. 2013). In addition, these NGS-based assays are now being introduced into the clinical practice that enables physicians to provide patients with more personalized care. For instance, the identification of cryptic actionable variants uses whole genome sequencing in cancer susceptibility genes (Welch et al. 2011; Link et al. 2011).

NGS has been gaining more attention in recent years as an important diagnostic tool in the field of liquid biopsies, such as circulating tumor cells (CTCs), circulating tumor DNA (ctDNA), or cell-free DNA (cfDNA), with more sensitivity and specificity. The application of NGS in liquid biopsies has revealed insights into tumor heterogeneity and clonal evolution (Aparicio and Mardis 2014; Shi et al. 2018). Many physicians are using NGS liquid biopsy tests such as Guardant360, Guardant Health, Foundation Medicine for the detection of activating and resistant genomic alterations in actionable genes and to also determine the tumor mutation burden. More importantly, the genomic/transcriptomic/proteomic profiling at the same time from the same cell provides a more comprehensive understanding of the cellular processes that occur in tumor cells (Macaulay et al. 2015, 2017; Angermueller et al. 2016; Dey et al. 2015). Although the application of NGS in solid and liquid biopsies has expanded the translational research, however, the clinical implementation of NGS in both areas is challenging because of the complexities of the assays and requires validation before implementation in a clinical diagnostic laboratory.

This chapter aims to provide a concise picture of the recent technological advances in NGS and their applications in cancer research and will further discuss challenges and future research directions.

11.2 Overview of the Experimental Approach for DNA/RNA Sequencing Platforms

Below are the NGS technologies used to identify genomic alterations in solid and liquid biopsies:

11.2.1 Whole-Exome Sequencing (WES)

WES is routinely used for the identification of rare and common disease-related genetic variants in humans (Oliver et al. 2009; Bolze et al. 2010, 2013; Byun et al. 2010; Bamshad et al. 2011; Tennessen et al. 2012; Koboldt et al. 2013; Casanova et al. 2014). This technology targets approximately 1% of the whole genome and detects up to 85% of disease-causing mutations. There are two main methods of exome capture technology: solution-based and solid-phase hybridization capture or

array-based capture method. In the solution-based hybrid selection method, the DNA samples are first sheared and followed with basic steps of library preparation including the end-repair and A-tailing and adaptor ligation. The amplified fragments were then subjected to hybridization with biotinylated oligonucleotide probes specific to the region of interest. The magnetic beads are used to separate the biotinylated molecules, and non-hybridized molecules are washed away. The samples are then sequenced with any standard exome sequencing platforms such as Illumina, Nimblegen, and Agilent before doing computation analysis. The solid-phase hybridization capture method is similar except the probes that are bound to a high-density microarray. The array-based method requires less input DNA and is more efficient than a solution-based method (Asan et al. 2011; Bodi et al. 2013). However, the solid-based approach is less scalable, time-consuming, and requires additional equipment.

11.2.2 Whole-Genome Sequencing (WGS)

WGS enables the identification of the full spectrum of genomic alterations including noncoding mutations in cancer cells (Nakagawa and Fujita 2018). This sequencing technology provides a comprehensive picture of the entire genomic DNA sequence by capturing both large and small genomic alterations that would be missed when applying a targeted sequencing approach. Many studies have shown that WGS is more powerful than WES in detecting exome variants (Belkadi et al. 2015; Lelieveld et al. 2015; Meienberg et al. 2016). However, WGS provides less coverage than WES. The library preparation does not require any enrichment step, thus introducing less bias in sample preparation procedure and sequencing. The DNA is first mechanically or enzymatically fragmented, and then barcodes are attached to the fragments. The barcodes are 6–8 unique nucleotide tag sequences to allow the pooling of different samples into a single pool. The pool can be sequenced in a single lane of a flow cell.

11.2.3 Targeted Panel Sequencing

The NGS-based deep targeted cancer panel assays allow for the detection of most common genomic aberrations. The number of genes included in each cancer panel ranging from a small to larger number. For instance, the US Food and Drug Administration (FDA) has recently approved the NGS-based FoundationOneCDx test of 315 genes and MSK-IMPACT panel of 410 genes that identifies clinically relevant alterations in cancer-related genes and can guide therapeutic decisions. In comparison with WES, targeted panels offer deep coverage and high sensitivity for the identification of rare and low-frequency variants (Gulbahce et al. 2017; Weinstein et al. 2013). Since these panels included genes that have a known association with cancer, they have a very high probability to detect a variety of somatic mutations and low probability to detect unknown variants (Kluk et al. 2016;

Froyen et al. 2016; Gulbahce et al. 2017; Weinstein et al. 2013). However, targeted panels have lower sensitivity in detecting copy number alterations (CNAs) and complex genomic rearrangements. There are two methods used for targeted sequencing: amplicon-based or hybridization capture-based NGS. The amplicon sequencing method uses PCR to produce DNA sequences called amplicons. The barcodes unique to each amplicon are added so they can be identified and amplicons are then pooled. Before pooling, libraries are prepared from each sample and target enrichment was performed via PCR amplification. In comparison with the amplicon-sequencing method, the hybridization capture-based NGS approach targets a larger number of genes (>50) and comprehensively profiles all variant types. For the library preparation, the DNA is fragmented by the mechanical or enzymatic shearing method. The targeted region of interest is then captured using biotinylated oligonucleotide probes. Streptavidin magnetic beads are then used to separate biotinylated molecules, and nonhybridized molecules are washed away. Samorodnitsky et al. (2015) evaluated both approaches and found that the hybridization capture-based approach performs better with more uniform sequencing depth and better coverage (Samorodnitsky et al. 2015), while the amplicon-based method utilizes a smaller amount of DNA and had higher on-target rates.

11.2.4 RNA Sequencing (RNA-seq)

This technology is used to characterize the whole transcriptome. RNA-seq not only is used to identify the genes that are differentially expressed in different biological conditions but also allows detecting gene fusions, splice junctions, noncoding RNAs such as microRNAs and long noncoding RNAs (Oliver et al. 2009). In addition, this platform also identifies post-transcriptional modifications, RNA-editing, and mutations/SNPs. The RNA library preparation workflow involves the conversion of RNA into the first-strand cDNA followed by second-strand cDNA synthesis using reverse transcriptase and random primers. The cDNA is fragmented by mechanical method or enzymatic digestion and followed by end repair to generate blunt ends and adapter ligation. The library molecules are then amplified with PCR to create the final cDNA libraries for sequencing (Fig. 11.1).

11.2.5 Single-Cell DNA/RNA Sequencing

Tumor heterogeneity is the biggest hurdle in precision cancer therapeutics. To understand the dynamics of tumor heterogeneity and its association with genomic instability, it is essential to sequence the entire genome or transcriptome at the single-cell level. Single-cell sequencing technologies enable to characterize heterogeneity and provide insights into the cell's phenotype and genotype. Single-cell DNA seq technologies have high sensitivity and specificity for the detection of minority clones and mutually exclusive or cooccurring alterations (González-Silva et al. 2020), which can be used to select appropriate targets for therapy. Single-cell

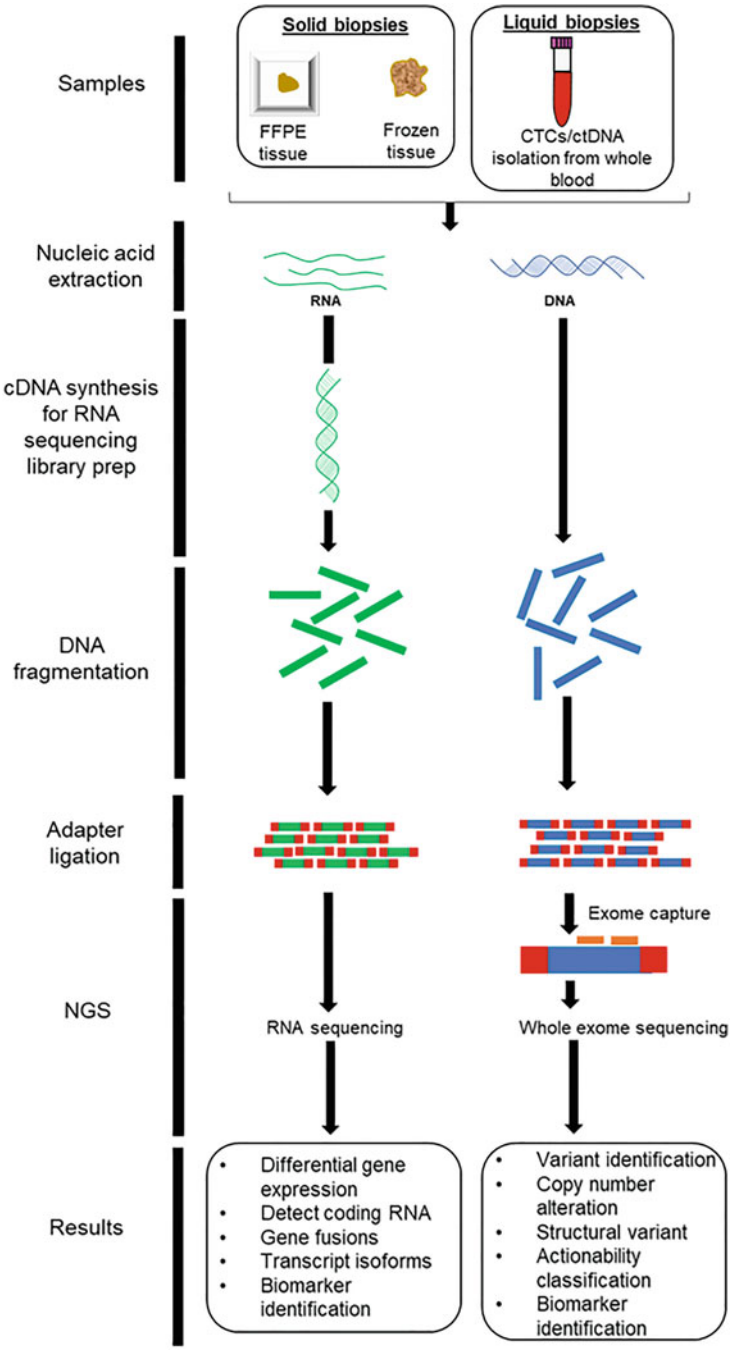

Fig. 11.1 A summary of NGS workflow. The process involves nucleic acid isolation from biological and clinical samples. The next step involves the main steps of NGS workflow, i.e., library prep, sequencing, and data analysis

transcriptomics is used to profile gene expression between individual cells and to detect rare cell types within a heterogeneous cell population. This technology is also employed in immunological research and provides insights on key processes in cell development and differentiation. There are many single cell RNA-seq (scRNA-seq) platforms such as Drop-seq, InDrop, MARS-seq, SMART-seq2, Fluidigm C1, and 10× Genomics chromium. Some of these methods use fluorescence-activated cell sorting (FACS) technology, which involves labeling cells with fluorescent markers or micromanipulation equipment for single-cell isolation. The 10× genomics chromium system is automated equipment that encapsulates single cells using a gel bead in emulsion (GEM) approach. The processing of single cells for library preparation are having simple steps including reverse transcription, cDNA amplification, and library construction for sequencing (Fig. 11.1).

11.3 NGS Utility in Clinical Oncology in Solid Tumors

Rapid progress in the development of NGS technologies in recent years has enabled clinicians to provide patients with more personalized care. As an alternative to single gene analysis, NGS provides a more comprehensive catalog of genomic alterations and a better understanding of the mechanism of drug resistance. These sequencing technologies can help the clinicians in different ways including diagnosis, prognosis, cancer subtype classification, identification of actionable mutations, and selection of targeted therapy or personalized medicine, switch to a second line inhibitor if resistance occurs or treatment fails. The cancer diagnosis is generally performed using small presurgical biopsies or aspirations and an accurate diagnosis is difficult to obtain. Nikiforov et al. (2014) found that the fine needle aspiration cytology method results in unclear diagnosis in 20–30% of the thyroid cancer cases in comparison with the thyroid cancer-related genetic markers (ThyroSeq v2) NGS-based assay. This panel performed with 83% of the overall positive predictive value and 92% accuracy for cancer diagnosis in thyroid nodules in 143 fine needle aspiration samples. Similarly, in another study of fibrolamellar hepatocellular carcinoma patients, DNAJB1-PRKACA chimeric transcript was identified in 15/15 (100%) patients using RNA-seq, suggesting the promising role of this sequencing technology in tumor pathogenesis (Honeyman et al. 2014). The NGS-based panels are also suitable in identifying genomic alterations that may be targeted by certain targeted specific drugs or provide prognosis to guide treatment decisions. A study of metastatic colorectal cancer patients who had other RAS mutations, in addition to KRAS mutations in exon 2, predicts poor response and negative treatment effects to inappropriately targeted therapies (Douillard et al. 2013), suggesting the applications of these technologies in targeted therapy. Jänne et al. (2015) found that resistance to EGFR-targeted therapies in NSCLC patients can possibly be overcome by switching to second-line treatment. The mutations in GNAS and KRAS identified using targeted deep sequencing approach provides valuable information regarding prognostic biomarkers in intraductal papillary mucinous neoplasmic lesions (Tan et al. 2015). Similarly, Kriegsmann et al. (2014) observed the high frequency of druggable

molecular alterations in PI3K pathway and their association with clinical outcomes in triple negative breast cancer cases using breast cancer-specific ultradeep multigene sequencing.

Many studies on various cancer types have shown that NGS can be utilized in identifying drug targets and drug-resistance mutations (Malapelle et al. 2015; Muller et al. 2015; Tinhofer et al. 2016; Preusser et al. 2015). The Ion Torrent AmpliSeq panel identified mutations in KRAS, NRAS, and BRAF that are associated with anti-EGFR treatment resistance in metastatic colorectal cancer patients (Malapelle et al. 2015). The NGS technology can be used as a screening strategy in selecting patients for genomic-based clinical trials that can utilize personalized treatment plans based on patients' tumor molecular profiles. The individual cancer therapy (iCat) study evaluated the feasibility of identifying actionable alterations in pediatric solid tumors using Oncopanel NGS-based assay. The authors found that 43% of high-risk, relapsed patients had potentially actionable alterations (Harris et al. 2016). Another example is the NCI-MPACT trial (National Cancer Institute-molecular profiling-based assignment of cancer therapy) where the therapy is selected according to the mutations detected using NGS technology in various tumor types (Lih et al. 2016). In MOSCATO-01 (Molecular Screening for Cancer Treatment Optimization) trial, patients with solid cancers underwent multiplatform profiling (array-based comparative genomic hybridization (CGH), RNA-seq, WES). The potentially actionable target was identified in 49% of the patients and 24% of them received a matched targeted therapy that resulted in improved clinical outcomes (Massard et al. 2017).

Taken together, these studies suggest that the implementation of NGS technologies in solid biopsies can aid in the prognosis and choice of therapeutic strategies.

11.4 NGS Utility in Clinical Oncology in Liquid Biopsies

Compared with conventional tissue biopsy, liquid biopsy is less invasive, more feasible, and provides a more comprehensive picture of tumor heterogeneity (Siena et al. 2018). Liquid biopsy could be used for all stages for cancer diagnosis, prognosis, recurrence-risk prediction, tumor mutational burden, and drug response (Kidess and Jeffrey 2013; Cai et al. 2015). Nowadays, NGS technologies are gaining enormous attention in the field of liquid biopsies in terms of detection sensitivity and clinical implications for personalized therapy. The NGS analysis of liquid biopsies has been applied in multiple tumor types such as breast, prostate, cervix, lung, leukemia, and other cancers (Gulbahce et al. 2017; Lohr et al. 2014; Ramsköld et al. 2012; Yu et al. 2012).

Many studies have used liquid biopsies to detect actionable genomic alterations. For example, the application of NGS technology in cfDNA analysis in patients with a variety of cancers identified potentially actionable alterations in 40% of the patients (Schwaederle et al. 2016a). Kaur et al. (2020) identified the complete repertoire of somatic alterations (mutations, CNAs, and structural variations (SVs)) in CTCs and matched formalin-fixed paraffin-embedded (FFPE) specimens from metastatic

breast cancer patients using WES approach and also highlights the intrapatient genomic differences between CTCs and metastases. Marchetti et al. (2014) study has shown that deep sequencing NGS technology can detect circulating EGFR mutations involved in acquired resistance to tyrosine kinase inhibitors in non-small cell lung cancer CTCs.

The NGS profiling of liquid biopsies may help in stratifying patients for basket trials (enrolls patients with any cancer type) and umbrella trials (enrolls patients with one cancer type). For example, ctDNA molecular profiling for 54 cancer-related gene panel in patients with diverse cancers identified 58% of the patients that had a molecular alteration and 71% of them had an alteration tractable by an FDA-approved drug (Schwaederle et al. 2016b). Moreover, the NGS technology can also identify patient-specific DNA rearrangements that can be used to design specific assay to monitor changes in tumor burden in plasma samples (Olsson et al. 2015; Leary et al. 2010; McBride et al. 2010; Russo et al. 2016).

In summary, the implementation of NGS technologies in liquid biopsies can reliably reveal the molecular signature of the patient's tumor and can also be used for the detection of the emergence of treatment resistance and to guide therapy selection.

11.5 Challenges of NGS Technologies

Although the advent of NGS technologies has enabled the identification of genomic alterations that could help in the selection of targeted therapy, there are some challenges for implementing these approaches in the clinical setting and the data interpretation. The NGS technologies are capable of producing hundreds of gigabytes of base calls and quality score data in a single sequencing run. To analyze the NGS data, high-performance computer clustering is required so that different algorithms and pipelines can be directly compared. Although some open-source tools and commercially available software are available, however, smaller labs cannot afford the cost and advanced network infrastructure. Another challenge arising in the analysis of multiple datasets is to identify consistent and reproducible data from sequencing technologies and laboratory platforms. Kaur et al. (2019) identified inconsistencies in the genomic alterations in the GENIE and TCGA datasets, suggesting validation steps are required for both experimental work and data analysis of NGS-based assays independently by the clinical laboratory before implementation. Another limiting step is the interpretation of the genetic variants identified through NGS that requires validation before implementing in clinical settings, particularly that contributes to disease pathogenesis. The interpretation of variants of unknown significance (VUS), which account for about 40% of total variants, represent a problem and VUS use in the clinical settings is challenging. This creates confusion in the patients' genetic data when receiving reports of undefined variants. Another major caveat is in the personalized cancer treatment field when filtering out driver mutations from passenger mutations, especially when both may change during tumor development.

11.6 Concluding Remarks

This chapter discusses the NGS methods used in solid and liquid biopsies and summarizes their potential applications and limitations in terms of their implementation in clinical practice. The advent of NGS technology in oncology has revolutionized our fundamental understanding of biology and opened up new frontiers of research from identifying mutations to a comprehensive analysis of the patient's genome to personalized cancer therapy. Although the utility of these technologies in cancer research have proven remarkably in uncovering cancer-causing alterations, several challenges remain to overcome in clinical settings. To achieve long-term success in the clinical domain, more research is needed to overcome the challenges associated with the translation of genomics into the clinical arena. The availability of user-friendly bioinformatics tools will provide novel insights into tumor biology, which may lead to the development of effective therapeutic strategies.

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CRISPR: Genome Editing and Beyond

12

Aiana and Kashmir Singh

Abstract

Ever since the DNA double helix was discovered, DNA-manipulating technologies have enabled significant advances in biological sciences. One such tool that mediates genome modification with high precision is the use of targeted nucleases such as site-directed ZFNs (zinc finger nucleases) and TALENs (TAL effector nucleases) which work on the principle of DNA-protein recognition. The advent of RNA-programmable CRISPR-Cas9 technology has brought about a transformative phase in the field of precision genome engineering. CRISPR-Cas9 is a multiplexable genome editing tool that has enabled the precise manipulation of specific genomic elements. This system consists of a programmable CRISPR RNA (crRNA) and a Cas9 nuclease, which cleaves the DNA by generating double-stranded breaks at specific target sites. Desired changes occur at the target sites when subsequent cellular DNA repair process takes place. This chapter discusses how the CRISPR sequences were discovered in bacterial and archaeal genomes, the basis of their classification, the molecular mechanism that helps in genome modification, the applications of CRISPR-Cas9-mediated genome editing, and the challenges that this technology needs to overcome in the future.

Keywords

CRISPR-Cas9 · TALENs · ZFNs · Archaeal genome

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12.1 Introduction

Phages are the most abundant entities on planet earth and exhibit a constant danger to prokaryotic life. Several innate defense strategies such as restriction modification of phage genome, bacteriophage adsorption suppression, and abortive infection have been developed by prokaryotes to withstand phages. The revelation that an adaptive and heritable immune system named CRISPR (clustered regularly interspaced short palindromic repeat) is present in prokaryotes was one of the most exciting discoveries. This adaptive immune system memorizes past infections, and when reinfection occurs, it uses RNA-guided nucleases to silence specific sequences of phages, plasmids, and transposons. CRISPR loci has many noncontiguous direct repeats which are separated by variable sequence stretches called spacers (corresponding to captured plasmid and viral genome sequences) and are often flanked by cas (CRISPR-associated) genes. A large, heterogeneous protein family is encoded by cas genes, including functional domains that are typical of helicases, nucleases, polynucleotide binding proteins, and polymerases. CRISPR-Cas system is formed when CRISPR is combined with Cas proteins. Cas proteins guide the three phases of CRISPR-based immunity, namely, adaptation, crRNA biogenesis, and interference. Apart from adaptive immunity, the slow evolution and high conservation suggested the possibility of other important nondefense functions of this system such as pathogenicity and regulation of collective behavior. CRISPR-based immunity has many underlying aspects that provide its prospects in industrial applications such as driving viral evolution, using its hypervariability for typing purposes, executing natural genetic tagging of various strains, and regulating viral resistance in case of domesticated microbes. CRISPR-Cas systems have the ability to inhibit particular phage or plasmid DNA sequence transfer into a host, which might help in genetic engineering for the prevention of undesirable genetic element dissemination.

12.2 Discovery

CRISPRs were discovered by Yoshizumi Ishino in 1987 while analyzing alkaline phosphatase isozyme conversion causing gene *iap* in *E. coli* K-12 cells. When *iap* gene containing fragment of DNA was sequenced, it was observed that different clones had the same repetitive sequence downstream of iap gene (Ishino et al. 1987). However, a definite function of this sequence was not known due to the absence of advanced sequencing technologies. After a while, similar sequences were observed in other strains of *E. coli* as well as other bacteria like *Mycobacterium tuberculosis* (Hermans et al. 1991), *Shigella dysenteriae* (Nakata et al. 1989), etc.

In 1993, Mojica and coworkers identified similar sequences in archaebacterium *Haloferax mediterranei* while studying regulatory mechanisms that help halophilic archaea to survive in environments that are highly saline. It was suggested that these sequences could be responsible for regulation of gene expression leading to double-stranded DNA transition from B to Z form to facilitate a regulator protein binding. Later, it was hypothesized that these sequences could be responsible for replicon

partitioning when similar sequences were found in *Haloferax volcanii* (Mojica et al. 1995). These unusual sequences were later detected in multiple archaeal and bacterial genomes when automated DNA sequencing was invented during the 1990s. Various scientists described these sequences using different terms such as SPIDRs (spacers interspersed direct repeats), SRSRs (short regularly spaced repeats), and LCTRs (large cluster of tandem repeats) (She et al. 2001). It was found that the replication origin of archae *Pyrococcus horikoshii* and *Pyrococcus abyssi* had two sets of symmetrically located LCTRs on each side which again implied a role in replicon partitioning (Zivanovic et al. 2002). The relatedness between these sequences in archaeal and bacterial genomes was first identified by Mojica et al in 2000. The confusion caused by different names for these sequences was impeded when Jansen et al. coined the term CRISPR in 2002. Through comparative genomic analysis, it was established that CRISPR sequences contain numerous direct repeats interspersed with some sequences which are non-conserved and are situated in intergenic regions. Also, the repeat cluster has a several hundred base pair common leader sequence. When CRISPR sequences were found in both archaeal and bacterial systems, it was inferred that there is a possibility of these sequences having a more general function in life systems. However, no such sequences have still been found in eukaryotes.

Four conserved genes were found to be present next to the CRISPR region when CRISPR regions were compared in various genomes with the help of accumulated genomic sequences. These genes were named CRISPR-associated (cas) genes 1–4 (Jansen et al. 2002). Cas1 and Cas2 had no functional similarity with any known protein's functional domains. On the other hand, Cas3 encompassed seven motifs which are found in superfamily two helicases, and Cas4 was related to RecB exonucleases. This led to the assumption that Cas3 and Cas4 were involved in various DNA metabolism processes such as transcriptional regulation, DNA repair and recombination, and chromosome segregation. As Cas proteins were associated with CRISPRs, it was also proposed that these are involved in CRISPR loci genesis. It was also suggested that Cas proteins might have a role in a DNA repair system that is specific to only thermophilic organisms (Makarova et al. 2006).

The fact that thermophilic and hyperthermophilic organisms had more amounts of larger CRISPRs compared to mesophilic organisms indicated that CRISPR might have a role in adaptation to high temperatures (Jansen et al. 2002). But this suggestion turned out to be insubstantial when the availability of new sequences revealed the presence of CRISPR sequences in mesophilic organisms as well. It was established that CRISPR sequences work as a part of biological defense system upon the entry of foreign genetic elements just like the eukaryotic RNA interference system when Mojica and Pourcel, in 2005, detected spacer regions homologous to prophage, plasmid, and bacteriophage sequences between the repeat sequences and that plasmids and phages were not able to infect the organisms that had these spacers between CRISPR sequences. It was also proposed that pieces of the foreign DNA are captured which is triggered by CRISPRs so that a memory of past genetic invasions can be constituted.

In the same year, another group of scientists indicated that antisense RNA can be produced using CRISPR and that there is a correlation between the number of phage origin spacers and the degree to which the host resists phage infection (Bolotin et al. 2005). A year later, Makarova et al. analyzed Cas protein sequences in detail and tried to find the similarity between these sequences and the eukaryotic RNA interference system. They also emphasized on the resemblance between the memory component of the CRISPR-Cas system and the vertebrate adaptive immune system except the fact that the former is inheritable. The diversity, high mobility, and pervasiveness of the CRISPR-Cas system directed toward the possibility of this system emerging in ancient archaeal ancestors and horizontal spread to bacteria. The prospect of CRISPR-Cas systems being utilized in Cas protein encoding organisms for gene silencing was also put forward.

In 2007, it was experimentally proven that CRISPR-Cas functions like the acquired immune system when a phage sequence inserted into *Streptococcus thermophilus* CRISPR spacer region made the bacteria resistant to the phage, and when the same protospacer sequence was removed from the genome of that phage, the resistance disappeared (Barrangou et al. 2007). In 2008, it was demonstrated in *E. coli* that the CRISPR region, upon transformation, forms RNA molecules that work along with Cas proteins which were encoded by the genes present alongside the CRISPR region (Brouns et al. 2008). In 2011, heterologous protection was observed against phage infection and plasmid transformation in *E. coli* having reconstituted *S. thermophilus* CRISPR-Cas system expression (Sapranauskas et al. 2011). By 2013, it was proved that target DNA can be cleaved by purified CRISPR-Cas9 RNA complex in vitro and *Streptococcus pyogenes* CRISPR-Cas system was used for genome editing in mouse kidney and human nerve cells (Gasiunas et al. 2012; Jinek et al. 2012). All these observations led to CRISPR-Cas being known as the acquired immunity system in prokaryotes.

12.3 Classification

The classification of CRISPR-Cas systems and consistent annotation of Cas proteins becomes difficult because the genomic location of CRISPR-Cas loci varies highly and most cas genes evolve quite rapidly (Vestergaard et al. 2014). As the genome architecture and composition of CRISPR-Cas systems is very complex, it took a polythetic approach that involved structural, phylogenetic, and comparative genomic analysis (Makarova et al. 2011). Mainly there are two classes of CRISPR-Cas systems which can be easily distinguished by unique signature proteins: class I and class II.

12.3.1 Class 1

Originally, class I CRISPR-Cas system comprised of type I, type III, type IV, and 12 subtypes that can be differentiated by comparing loci organizations, sequence of

repeats, and effector protein sequence similarity clustering. Recently four new subtypes—III-E, III-F, IV-B, and IV-C—have been added to this class.

A ssDNA stimulated superfamily two helicase that can unwind RNA-DNA duplexes, and dsDNA is encoded by the signature gene cas3 which is present on all type I system loci. There are seven subtypes within type I systems which include I-A to I-F and I-U (later reclassified as I-G) (Makarova et al. 2011). In type I-A and I-B systems, cas genes have clusters of two or more operons, while in types I-C, I-D, I-E, and I-F, there is typically only a single operon that constitutes the Cascade complex subunit genes along with cas1, cas2, and cas3 genes (Vestergaard et al. 2014). All the subtypes of type I system have a specific combination of operon organization and signature genes. In I-F systems, cas2 and cas3 are fused with each other, while cas4 is missing in I-E and I-F systems. In the phylogenetic trees of Cas3 and Cas1, both I-E and I-F systems are monophyletic and have distinct signature genes. I-A, I-B, and I-C systems are derived from the same ancestral type I gene arrangement which is cas1-cas2-cas3-cas4-cas5-cas6-cas7-cas8 (Makarova et al. 2015). Subtype I-B has the same arrangement, while I-A and I-C have diverged arrangements with rearranged order of and differential loss of genes. However, these subtypes did not have any distinct signature gene. Instead of Cas8 protein, I-D systems have a Cas10d and a Cas3 variant.

The signature gene cas10 is present in type III systems which encodes a Palm domain containing multi-domain protein. To identify the loci of type III systems, many PSSMs are needed due to the large amount of variation in Cas10 protein sequences. Cas10 is generally fused with HD family nuclease domain that has a circular permutation of conserved domain motifs (Makarova et al. 2006). Type III systems have six subtypes, i.e., III-A to III-F. Subtype III-A has genes that encode a small subunit csm2, and most of its loci contain cas1, cas2, and cas6 genes, while these genes are absent in most subtype III-B loci (Makarova et al. 2013). It has been observed that both these subtypes target RNA and DNA co-transcriptionally (Hale et al. 2012; Samai et al. 2015). In subtype III-C, the sequence of Cas10 protein shows extreme divergence, and the cyclase-like domain of Cas10 is apparently inactivated. The Cas10 protein encoded by subtype III-D loci does not contain the HD domain. III-E systems have a putative Csm2-like small subunit fused with various Cas7 proteins in such a way that the effector module's crRNA binding part is compressed within a large multi-domain protein. Subtype III-F consists of a single Cas7-like protein with the Cas10-like large subunit fused to the HD domain which is supposed to cleave target DNA due to the presence of catalytic residues (Makarova et al. 2018).

Type IV CRISPR-Cas system loci do not contain cas1 and cas2 genes and are not present near a CRISPR array. Subtype IV-A contains the dinG gene, while subtype IV-B lacks this gene but has a separate version of the small subunit of the effector complex and encompasses ancillary gene cysH (Shmakov et al. 2018). IV-C system Cas5 and Cas7 homologues are quite similar to the IV-A and IV-B system proteins.

12.3.2 Class 2

A single subunit crRNA effector module is present in class 2 CRISPR-Cas systems. Types II, IV, and VI CRISPR-Cas systems are included in this class.

Type II CRISPR-Cas systems have the signature gene cas9, which codes for a protein that combines target DNA cleavage (Jinek et al. 2012) with the functions of crRNA effector complex and helps in adaptation (Heler et al. 2015). Cas9 is also the most studied multi-domain effector protein. Type II loci also contain cas1 and cas2 and most of the loci encode tracrRNA which is somewhat complementary to the respective CRISPR array repeats (Chylinski et al. 2013, 2014).

The effector proteins of type V systems have a different domain architecture compared to type II systems. These effector proteins of type II systems (cas9) have two nuclease domains, HNH nuclease and RuvC-like nuclease, each cleaving a strand of the target DNA. On the other hand, type V effectors (cas12) have only one domain, RuvC-like nuclease, which causes the cleavage of both strands (Strecker et al. 2019). Effector proteins of type VI systems (cas12) are entirely different from type V and type II systems, containing two HEPN domains which target invading DNA genome transcripts. These effector proteins have nonspecific RNase activity which makes the virus-infected bacteria dormant.

The uncomplicated design of class 2 effector complexes makes these CRISPR-Cas systems an easy choice for genome editing.

12.4 Molecular Mechanism

Cas9 and all the other effector protein nucleases generate targeted breaks in the DNA which leads to DNA damage response, which in turn stimulates various endogenous repair mechanisms. The double-stranded break (DSB) caused by cas9 in target DNA can be repaired through either nonhomologous end joining or homology-directed repair. Eukaryotes largely use nonhomologous end joining (NHEJ) pathway for DSB repair, which is quite error prone as it induces numerous small insertions or deletions (indels). This repair mechanism is useful in cases where large targeted gene knockouts are required. Alternatively, homology-directed repair (HDR) is error-free but has a lower efficiency compared to NHEJ-mediated repair. HDR uses cas9 to deliver a repair template that is homologous to the target site so that a specific change such as a DNA segment insertion or a point mutation can be introduced.

The recognition and cleavage of foreign DNA by CRISPR-Cas systems occurs in a sequence-specific manner. There are three stages that take place during the CRISPR-Cas defense mechanism: (a) spacer acquisition or adaptation, (b) crRNA biogenesis, and (c) target interference.

12.4.1 Adaptation

The invading mobile genetic element has a distinct sequence called protospacer which gets incorporated into the CRISPR array and generates a new spacer. This leads to the host organism being able to display this system's adaptive nature by memorizing the foreign genetic material (Barrangou et al. 2007). The spacer acquisition process involves Cas1 and Cas2 as they are present in most of the CRISPR-Cas systems. However, some CRISPR-Cas systems further require other proteins such as Cas4, Cas9, Csn2, etc. for the same process. The target sequence selected for integration into CRISPR locus is specific. The protospacer adjacent motif (PAM), which is a short sequence located adjacent to the protospacer, plays an important role in acquisition and interference. Protospacer selection occurs with the help of PAM-recognizing domain of Cas9. After the selection is done, Cas1, Cas2, and Csn2 are recruited by Cas9 so that the new spacer can be integrated into the CRISPR array (Swarts et al. 2012).

12.4.2 crRNA Biogenesis

Immunity is enabled when CRISPR array gets transcribed into pre-crRNA, a long precursor crRNA which is processed into mature guide crRNAs that contain memorized invader sequences (Carte et al. 2008). The processing of pre-crRNA is carried out by Cas6 in type I and type II systems and by Cas5d in type I-C systems. In type II systems, tracrRNA is involved in pre-crRNA processing as its anti-repeat sequence facilitates the formation of RNA duplex with pre-crRNA repeats, stabilized by Cas9. This duplex is processed by RNase III of the host creating a crRNA intermediate form, which ultimately forms a mature small guide RNA (Deltcheva et al. 2011). Type V-A systems have Cpf1, which alone is sufficient for maturation of crRNA. Aside from processing of premature crRNAs, Cpf1 cleaves the target DNA by using this processed crRNA (Fonfara et al. 2016) (Fig. 12.1).

12.4.3 Interference

The mature crRNAs are now used as guides that help in interfering with the invading nucleic acids. In class 1 systems, target degradation is achieved by Cascade (CRISPR-associated complex for anti-viral defense) like complexes, while in class 2 systems, one effector protein is enough for target interference (Zetsche et al. 2015; Bhaya et al. 2011). Self-targeting is avoided by specific recognition of PAM sequences located upstream (types I and V) or downstream (type II) of the protospacer (Jiang et al. 2013; Semenova et al. 2011). Target degradation occurs in type I systems when Cas3 nuclease is recruited after the invading DNA is localized by Cascade in a crRNA-dependent manner (Brouns et al. 2008). Cas3 generates a nick on the target DNA and degrades it. The tracrRNA-crRNA duplex in type II systems guides Cas9 to create a double-stranded break in the invading DNA.

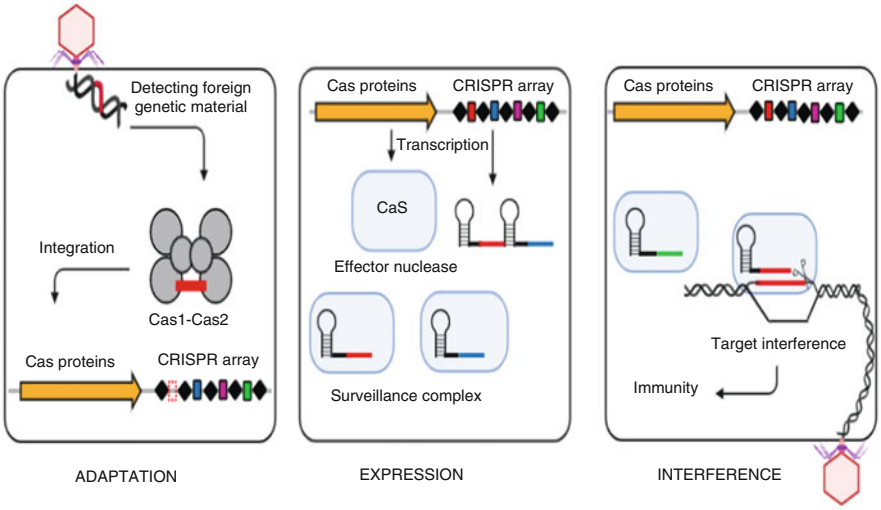

Fig. 12.1 Three stages of CRISPR immunity: A part of foreign DNA is selected by Cas1-Cas2 complex and is integrated into CRISPR array of the host during adaptation. crRna maturation involves the transcription of CRISPR array into pre-crRNA, which is then processed by Cas proteins, and a surveillance complex is formed. During interference, foreign genetic elements that are complementary to crRNA are targeted by Cas effector nucleases which leads to target interference and immunity

Type III systems have Cas10-Csm and Cas10-Cmr complexes which can target both RNA and DNA. Cas10 subunit cleaves DNA, while Csm3 and Cmr4 cleave the transcribed mRNA (Tamulaitis et al 2014; Staals et al. 2014).

12.5 Applications

12.5.1 Genome Editing

Cas applications have a very extensive scope, but precision genome editing still remains at the forefront of this technology. Genome editing can be induced by RNA-guided nucleases such as Cas9 and Cas12a by initiating double-stranded break repair at a specific DNA site. Cas9 has been used to disable defective genes that cause neurological diseases like Huntington's disease (Staahl et al. 2017) and amyotrophic lateral sclerosis (Gaj et al. 2017). Using Cas9, an entire chromosome has been eliminated in human pluripotent stem cells (Zuo et al. 2017); an endogenous retrovirus has been inactivated in pigs (Dong et al. 2017). T cells have been engineered as a lead up to the development of advanced immunotherapies for targeting cancerous cells (Rupp et al. 2017). Apart from somatic cell editing, correction in human embryo genetic mutations is also possible (Ma et al. 2017).

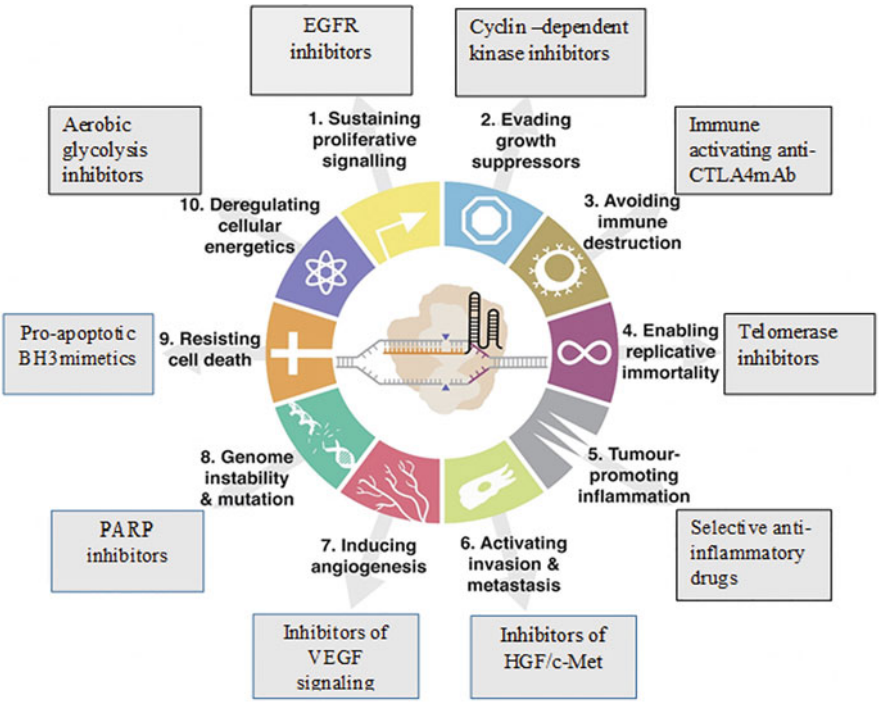

Fig. 12.2 A collection of hallmarks that characterize the cancer phenotype. (Proposed by Hanahan and Weinberg)

However, creating heritable changes in the human germline remains ethically questionable (Fig. 12.2).

12.5.2 Transcriptional Regulation

Catalytically deficient Cas9 (dCas9), which is a functional scaffold that recruits RNA or protein components to a certain locus to disturb transcription without permanently changing DNA, is created by mutating nuclease domains of Cas9 which leads to the decoupling of enzymatic activity from DNA binding (Kampmann 2018). Use of dCas9 has enabled rapid, specific, and multiplexed genetic knockdowns in various cell types like neurons and immune cells (Bak et al. 2017; Zheng et al. 2018). This shows that genomic perturbation is feasible without risking damage to DNA, which is a very important attribute when it comes to therapeutic development studies. A modified dCas9 target gene activation system has been successfully implemented in gain-of-function studies to treat acute kidney injury, type 1 diabetes, and murine muscular dystrophy (Liao et al. 2017).

12.5.3 Post-transcriptional Engineering

The transcriptome can be transiently perturbed through direct RNA targeting by applying Cas effectors. A programmable RNA-targeting system, created by engineering SpCas9 with the help of PAM-presenting oligonucleotide, helps in RNA targeting with Cas9 (RCas9) (O'Connell et al. 2014). Pathogenic RNA foci can be eliminated, and mRNA splicing defects can be rescued by targeting RCas9 to RNA (Batra et al. 2017). Cas9 also helps in achieving site-specific RNA modifications by fusing with single-base RNA modifiers. Cas13a has been used in plant and mammalian cells for specific knockdown. RNA editing and RNA interference is also possible in mammalian cells with the help of programmable RNase activity of Cas13b enzymes (Smargon et al. 2017; Cox et al. 2017).

12.5.4 Programmable DNA and RNA Imaging

The function of mRNAs, noncoding RNAs, and specific genomic loci necessitates correct spatiotemporal localization. Repetitive genomic loci has been imaged in live cells using fluorescent reporter fused dCas9 (Knight et al. 2018). As dCas9 is very stringent in PAM recognition, it can be used for high-resolution CRISPR live cell imaging of DNA loci (Maass et al. 2018). However, a low signal-to-noise ratio limits widespread localization studies of specific genomic loci at non-repetitive genomic sequences. RNA-targeting Cas9 has helped in visualizing repeat expansion-containing transcripts that are clinically relevant by tracking RNA in live cells. Catalytically deficient Cas13a (dCas13a) also helps in RNA imaging through RNA-guided RNA targeting (Abudayyeh et al. 2017).

12.5.5 Nucleic Acid Detection and Diagnostics

Cas12a and Cas13a can be used in innovative nucleic acid detection tools as they possess RNA-guided nuclease activities. Multiple turnover nuclease activity is activated by a target nucleic acid by correctly base-pairing with the guide RNA in both Cas12a and Cas13a. Out of a pool of RNA, the detection of target RNA transcripts of interest can be done by detecting its RNase activity with the help of the switchable nuclease activity of Cas13a (East-Seletsky et al. 2016). Multiple CRISPR-based diagnostic platforms such as SHERLOCK (Specific High-Sensitivity Enzymatic Reporter UnLOCKing), SHERLOCKv2, DETECTR (DNA endonuclease-targeted CRISPR trans reporter), etc. have been developed by exploiting the nuclease activity of Cas proteins. CRISPR-Cas-based detection of a specific transcript facilitates rapid and accessible diagnostics for future use (Gootenberg et al. 2017, 2018; Chen et al. 2018).

12.6 Future Prospects

A rapidly increasing interest can be seen in the area of CRISPR-Cas in recent years. With the help of CRISPR-Cas-based tools, the pace of research in understanding genetics of unstudied organisms and discovering disease contributing genes has vastly accelerated. But many facets of this fascinating system still require further insight. One such aspect is the accomplishment of immunization by incorporating new spacers in CRISPR array and the biochemical basis behind it. Further research can be conducted on CRISPR-Cas functions that are unrelated to immunity such as various regulatory processes by studying Cas protein interactions with DNA repair and recombination pathway components. Despite the considerable potential of CRISPR-Cas9 technology, further research needs to be done so that this system can be made a safe and applicable tool for approaches that are therapeutically useful. Another major concern is off-target cleavage by Cas9 which is a major issue that needs to be addressed as it affects the precise remodeling of genomic content in eukaryotic cells. This technique needs to have higher specificity as some cases have seen higher frequencies in off-target genetic alterations compared to desired mutations. Also, CRISPR-Cas systems have a relatively large size, and its packing is not suitable for viral vectors. Efficient genetic engineering requires a smaller-sized CRISPR system. The applications of Cas9 get limited because a PAM site is required at the target sequence which arises the need for a multiple PAM site selection system so that target scope can be increased. Knock-in homologous recombination also needs to have increased efficiency. New strategies that inhibit endogenous NHEJ activity or CRISPR-associated transposase application should be considered. Substantial advancements have been made in the last few years, but many aspects of this system still need to be discovered. It is anticipated that a holistic understanding of the multifariousness of CRISPR-Cas biology can be unearthed in the near future.

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Omics in Tuberculosis Diagnosis: Today and Tomorrow

13

Indu Verma and Khushpreet Kaur

Abstract

The term "omics" refers to collective high-throughput approaches that include genomics, transcriptomics, proteomics and metabolomics. Primary focus of OMIC technologies includes identification of genes and genomic variants (genomics), mRNA expression levels (transcriptomics), proteins (proteomics) and low molecular weight metabolites (metabolomics) in cell or tissue type. Omics technologies may lead to detection of the novel molecular signatures (gene/protein/metabolites) which are specific to disease and may serve as a promising candidate for early diagnosis, prediction of therapeutic response and prognosis of disease. Individually, these technologies have contributed significantly towards medical advances. Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (Mtb), is one of the deadly diseases, and worldwide millions of people suffer from TB and die every year. Diagnostics constitutes the most important component of TB control programme, and in recent years considerable progress has been made in the field of TB diagnosis. However, a rapid, cost-effective point-of-care (POC) diagnostic test for different forms of tuberculosis is still lacking. In this context, recent advancements utilising the multidimensional omic approach including genomics, transcriptomics and proteomics provide an improved platform for discovering the key molecular signatures to facilitate TB diagnostics and predicting treatment response. Utilising advanced and integrated omics technologies, recently there has been a tremendous progress in the field of biomarker discovery for TB diagnostics to achieve the goals set by the World Health Organization (WHO) End TB Strategy and the Foundation for Innovative New Diagnostics (FIND) Strategy for tuberculosis. This chapter is focused on

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various TB diagnostics based on various omics approaches with main emphasis on genomics and proteomics.

Keywords

OMICS · Metabolonomics · *Mycobacterium tuberculosis* · Transcriptomics

13.1 Introduction

As per the WHO report 2020, 4000 people died every day, and nearly 30,000 fell ill with this disease during 2019 (WHO 2020a). India being the most populated country of the world has nearly one fourth of global TB burden. The End TB Strategy aims to end the global TB epidemic, with targets to reduce TB deaths by 95% and to cut new cases by 90% between 2015 and 2035 (WHO 2015), and these targets can be achieved only by effective tools for TB diagnosis and treatment.

Following exposure to Mtb through inhalation of aerosol droplets, tubercle bacilli enter into the lungs and taken over by host innate immune system. However, if innate immunity fails, Mtb start multiplying inside alveolar macrophages in the lung. The intracellular multiplication of Mtb triggers the host adaptive immune response resulting in the control of bacterial replication in 90–95% of cases without any overt signs or symptoms of disease, and this stage of disease is known as "latent TB infection (LTBI)". It is a stage where host innate immune system maintains a dynamic relationship with tubercle bacilli with the containment of infection in an organised structural collection of immune cells known as granuloma (Schwander and Dheda 2010). In contrast, in some individuals, Mtb takes over the adaptive immune response resulting in the granuloma rupture and replication and dissemination of bacilli thus resulting in "active TB" with clinical symptoms, radiographic abnormalities and/or microbiologic evidence (Fig. 13.1). In some individuals, there is a stage of "subclinical TB" which is before the appearance of classical symptoms of "active TB". About one-third of the world's population has latent TB, and individuals with LTBI may develop active TB in the near or remote future, a process called TB reactivation (Fig. 13.1) with 5–10% lifetime risk of reactivation within the first 5 years after initial infection. However, the risk is considerably higher in the presence of predisposing factors, such as HIV infection or any event that weakens cell-mediated immunity (Gengenbacher and Kaufmann 2012). Another significant aspect of TB is the ability of Mtb to infect almost every organ in the human body. The most common form of TB is pulmonary tuberculosis (PTB) affecting the lungs; however, with advances in diagnostic tools, it is being understood that extrapulmonary TB (EPTB) also contributes significantly to morbidity and mortality associated with TB infection. Extrapulmonary tuberculosis has a broad spectrum of clinical manifestations as any organ system including bone, joint, genitourinary tract and central nervous system can be involved (Fig. 13.1).

Conventionally, bacteriological confirmation in biological samples via sputum smear microscopy and culturing acid fast bacilli either using solid or liquid culture

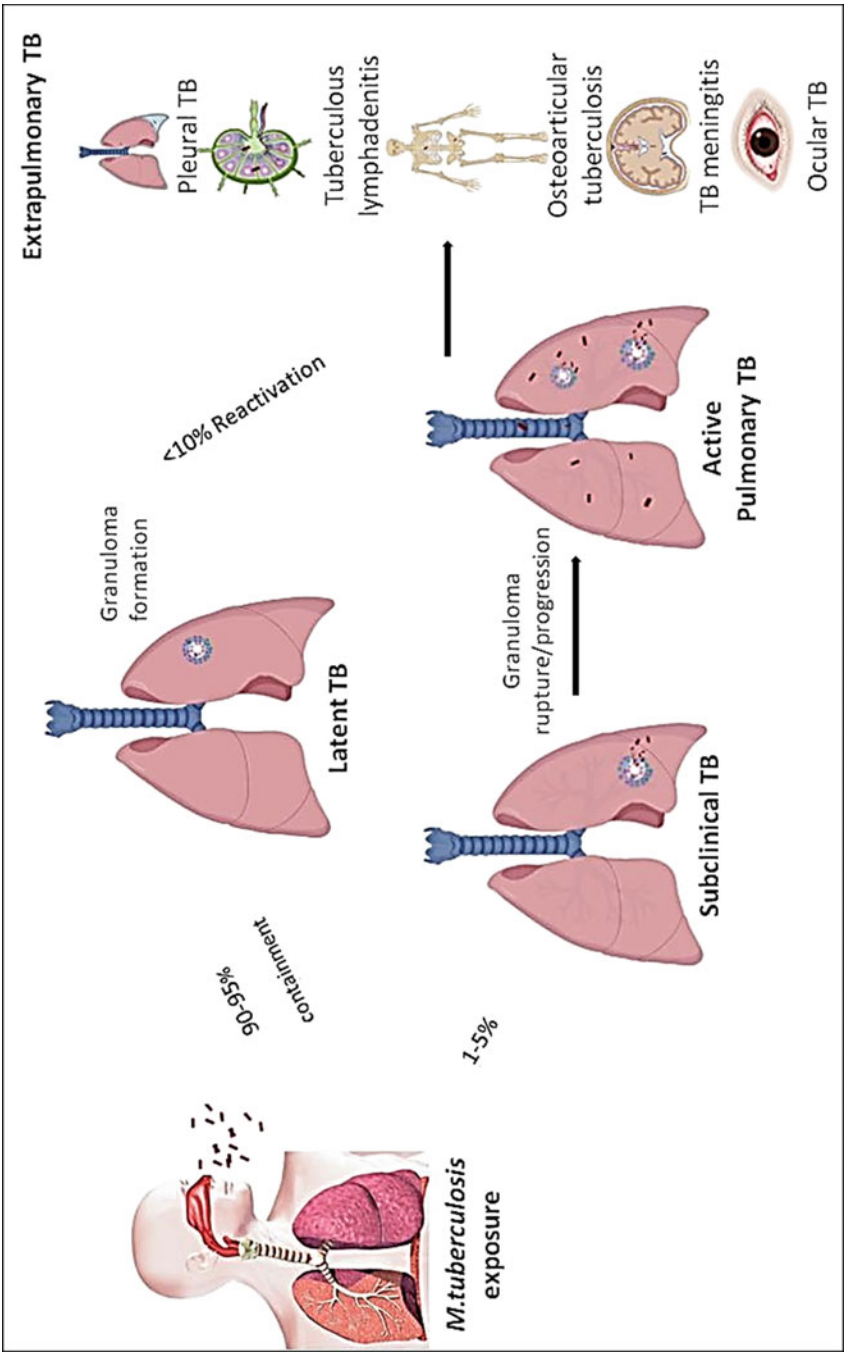

Fig. 13.1 Disease spectrum of tuberculosis

have been used as gold standards for TB diagnosis. These diagnostic tests have their own limitations since it depends on mycobacterial abundance in the biological specimen. Smear microscopy shows suboptimal sensitivity, while culturing Mtb improves the sensitivity, but it takes several weeks or more (Kidneya et al. 2013). Further, speciation of Mtb requires more skillful techniques like matrix-assisted laser desorption ionisation-time of flight mass spectrometry (MALDI-TOF) to differentiate Mtb from non-tuberculous bacteria (NTM) making it unsuitable for resource-limited settings and cannot be implemented as point-of-care test. Even though introduction of Xpert MTB/RIF, a fully automated nucleic acid amplification test (NAAT) based on pathogen-specific DNA markers has eased the early, rapid diagnosis of TB, but it is more expensive than existing diagnostics. The only point-of-care test is lateral flow urine lipoarabinomannan strip test developed by Alere Determine™ TB LAM Ag, based on the detection of mycobacterial lipoarabinomannan (LAM) glycolipid antigen in urine of active TB patient only in HIV patients. In 2015, the WHO approved the limited use of LF-LAM to assist TB diagnosis and screening of active tuberculosis in patients with HIV/AIDS thus limiting its utility as diagnostic test for large number HIV-negative TB subjects (WHO 2019).

Thus, TB diagnostics field is still evolving particularly with the introduction of advanced omics tools including genomics, transcriptomics and proteomics that has led to discovery of the molecular signatures to facilitate TB diagnostics and predicting treatment response. Various new diagnostic modalities have been developed and approved by the WHO that are currently being used for TB diagnosis (Figs. 13.2) or at different stages of recommendation process by the WHO (Table 13.1), while others are still at the stage of laboratory research (Branigan 2020).

13.2 Genomics in TB Diagnosis

The study of the genome termed as “genomics” forms the basis of prevention, diagnosis and management of many communicable and non-communicable diseases. With the recent advances in various genomic technologies, research directed at pathogen genomes has led to increase in our understanding of disease transmission and virulence mechanisms thus enabling the development of new classes of diagnostics, vaccines and therapeutic agents. There is growing evidence that a better knowledge of the genomics of pathogens and their vectors is likely to play a major role in the prevention and treatment of infectious disease. Since the sequencing of the first Mtb genome in 1998 (Cole et al. 1998) further aided by various genomic tools, there has been considerable progress in the diagnosis, treatment and control of TB. Recently, several new technologies have emerged based on the detection of mycobacterial DNA resulting in the timely and specific diagnosis of tuberculosis as well as providing the information about the type of infecting strain and pattern of anti-TB drug resistance which is very pertinent to treat the patients effectively. The development and implementation of automated nucleic

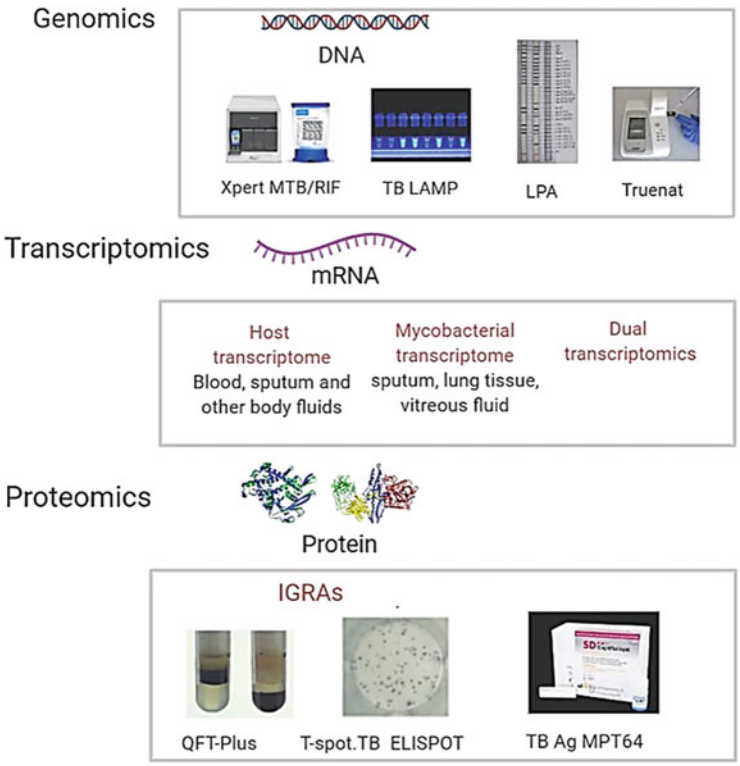

Fig. 13.2 Application of omics in tuberculosis diagnosis

acid amplification tests (NAATs) provides a significant impact on early TB diagnosis and initiation of treatment. Further, recent advances in whole genome sequencing (WGS) technology promise a rapid, efficient and accurate platform for TB diagnosis with a potential to revolutionise the clinical management of tuberculosis. Though it is fairly expensive particularly for setting up the WGS facility, sequencing costs are coming down at astounding rates.

13.2.1 TB Diagnostic Tests Available in the Clinics Based on Genomics

Following negative recommendation of the WHO for the commercially available serodiagnostic tests in 2011 (WHO 2011), there has been an advancement in the TB diagnostic field particularly using various molecular biology tools. Some of these tests are already being used in the clinics for patient care (Fig. 13.2) based on the WHO recommendations released time to time, whereas some tests although available in commercial formats are still not approved by the WHO for clinical use.

Table 13.1 Test of TB diagnosis based on various omics technologies at different stages of the WHO recommendation

S. no.	Type of test		Test/tool (manufacturer)	Format	Omics tool used for the test development	Time of results	Stage of development/ WHO review
1	TB diagnostic test	a.	GeneXpert Omni (Cepheid)	Rapid molecular PCR	Genomics	<90 min	WHO review: 2021
		b.	Stool processing solutions (KNCV Tuberculosis Foundation; FIND/ Rutgers)	Rapid molecular PCR/sample processing	Genomics	Not yet available	WHO review: 2021
2	Drug susceptibility test	a.	Xpert MTB/XDR (Cepheid)	Rapid molecular PCR	Genomics	<2 h	WHO review: 2020
		b.	RealTime MTB RIF/INH Resistance (Abbott)	High-throughput molecular PCR	Genomics	~3 h	WHO review: 2020
		c.	BD MAX MDR-TB (BD)	High-throughput molecular PCR	Genomics	<4 h	WHO review: 2020
		d.	Cobas MTB-RIF/INH (Roche)	High-throughput molecular PCR	Genomics	3 h	WHO review: 2020
		e.	FluoroType MTBDR Version 2.0 (Hain)	High-throughput molecular PCR	Genomics	≤2.5 h	WHO review: 2020
		f.	Genoscholar PZA TB II (Nipro)	Molecular LPA	Genomics	6 h	WHO review: 2020
3.	Treatment monitoring test	a.	Xpert MTB HR (Cepheid)	Host blood RNA response/PCR	Transcriptomics	Not yet available	Early development; expected launch in 2023
		b.	RISK6 signature assay (QuantuMDx)	Host blood RNA response/PCR	Transcriptomics	<30 min	Early development/ projected year of WHO review: 2022
		c.	RTT TB (Lophius Biosciences)	Host blood immune response/ PCR	Transcriptomics	18–32 h	Early development; expected launch in 2022
4	Latent TB test	a.	Access QFT (Qiagen)	Immunoassay blood test/IGRA	Proteomics	<20 min	WHO review: 2021

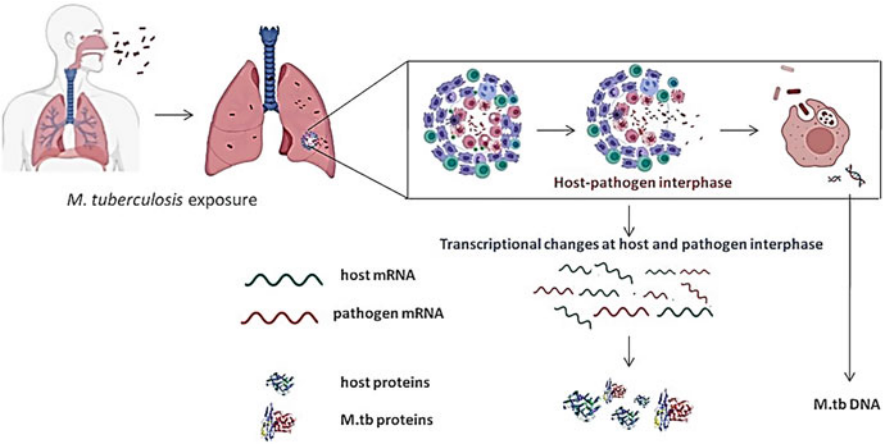

Fig. 13.3 Steps involved in Xpert MTB/RIF (nucleic acid amplification) test

Xpert MTB/RIF (Nucleic Acid Amplification Test): In 2010, the WHO approved the use of Xpert MTB/RIF for active TB diagnosis and rifampicin sensitivity (Fig. 13.3). Xpert MTB/RIF relies on the amplification of gene encoding mycobacterial RNA polymerase β subunit (rpoB) which is specific to *Mycobacterium tuberculosis* complex (MTBC) and also determines the rifampicin resistance-associated mutations in rpoB gene by molecular probes. Although the sensitivity of Xpert is lesser than culture with a limit of detection (LOD) of ∼131 bacterial colony-forming units (CFU)/mL compared to 1–10 CFU/mL in culture, it is comparatively higher than smear microscopy. Further, to overcome these limitations, Xpert MTB/RIF Ultra (Ultra) has been developed that incorporates two additional targets, i.e. IS1081 and IS6110, to rpoB with LOD of Ultra being decreased to 16 bacterial CFU/ml. The accuracy of TB diagnosis is significantly higher with Xpert Ultra in terms of sensitivity and specificity for both pulmonary tuberculosis and extrapulmonary tuberculosis (Jiang et al. 2020; Zhang et al. 2020). The use of Xpert MTB/RIF assay was assessed in various biological specimens and found to have varied sensitivity and specificity. The pooled sensitivity of Xpert MTB/RIF was 23% (95% C.I., 16–32%) in abdominal TB (Sharma et al. 2020), 30% in pleural TB (95% C.I., 17–44%), 53% in TB meningitis (95% C.I., 28–79%) and highest in TB lymphadenitis with 90% (95% C.I., 86–94%) as reported by Tadesse et al. (2019). The use of Xpert MTB/RIF in paediatric population for EPTB diagnosis has extensively been studied. The meta-analysis studies have been performed by Seo et al. (2020) to assess the accuracy of Xpert MTB/RIF for detecting EPTB using all type of samples in children and reported the sensitivity of 71% (95% C.I., 0.63–0.79) and specificity of 97% (95% C.I., 0.95–0.99) (Seo et al. 2020). Further the advent of Xpert MTB/XDR assay provides the quick and universal access to drug susceptibility testing (DST) and effective treatment of resistant TB strains. Xpert MTB/XDR sets new standards, simplifies the process of DST and detects mutations in the genes

associated with resistance towards first-line anti-TB drugs like isoniazid and fluoroquinolones and second-line injectable drugs, i.e. amikacin, kanamycin, capreomycin and ethionamide, in a single run, and results are available within 90 min of time. It showed the sensitivity of 94–100% and a specificity of 100% for all drugs except for ethionamide when compared to sequencing (Cao et al. 2020).

Line Probe Assays (LPAs) Line probe assay is based on the principle of target amplification using PCR followed by hybridisation on a strip immobilised with specific oligonucleotide probes. Earlier, in 2008 the WHO approved the use of LPA as a rapid test for detecting MTBC and resistance to rifampicin and isoniazid in sputum smear-positive specimen and in culture isolates (World Health Organization 2008). GenoType MTBDRplus ver. 2.0 (Hain Lifescience GmBH, Germany) detects MTB complex directly in smear-positive specimen or culture and its resistance to rifampicin (mutations in the rpoB gene) and isoniazid (mutations in katG and inhA genes). LPAs offers quicker and safer procedures when compared to conventional slow and complicated methods of DST testing. Meanwhile, newer version of LPAs for detection of multidrug resistance has been developed named as second-line line probe assays which were further endorsed by the WHO in 2016 to detect mutations in second-line anti-TB drugs (WHO 2016). MTBDRsl (v1.0) detects mutation within DNA gyrase, a gene (gyrA) associated with fluoroquinolone resistance; 16s ribosomal RNA encoding gene (rrs) associated with streptomycin resistance; and embB encoding for mycobacterial arabinosyl transferase, a target for ethambutol resistance. MTBDRsl (v2.0) further detects additional mutations associated with DNA gyrase B gene (gyrB) associated with fluoroquinolone resistance and eis (enhanced intracellular survival gene) promoter region associated with kanamycin resistance. Additionally, Genoscholar NTM + MDRTB II detection kit (Nipro, Tokya, Japan) has also been approved based on its ability to distinguish between mycobacterial species and resistance to rifampicin and isoniazid based on gene mutations (WHO 2016). These DST assays are useful but cannot be implemented at all health facilities as they require an established laboratory infrastructure, molecular testing facility and trained personnel.

TB-Loop-Mediated Isothermal Amplification (TB-LAMP) LAMP is a simple and cost-effective nucleic acid amplification test that doesn't need any sophisticated equipment and infrastructure. Amplification is performed using specifically designed primers under isothermal conditions, and results can be interpreted visually under UV light. A commercial molecular assay based on LAMP technology (TB-LAMP), i.e. Loopamp MTBC Detection Kit, has been developed targeting the gyrB and IS regions by Eiken Chemical Company Ltd. (Tokyo, Japan) for the detection of MTBC. In 2016, the WHO recommended the use of TB-LAMP in peripheral health settings as replacement test for sputum smear microscopy to detect MTBC directly in the sputum samples (WHO 2016). The pooled sensitivity of TB-LAMP was higher than sputum smear microscopy, i.e. 78% vs. 63%, respectively. TB-LAMP increased the sensitivity of TB detection in sputum smear-negative cases by 42% in adult patients (WHO 2016). The overall pooled sensitivity

and specificity of this assay was observed in the range of 76–80% and 97–98%, respectively, in different settings (Shete et al. 2019). Furthermore, LAMP has good sensitivity and specificity not only against culture (85.71% and 88.89%) but also with respect to XpertMTB/RIF (88% and 86.67%) in EPTB specimens (Singh et al. 2019).

Truenat Assays Truenat assays based on PCR technology can be run on portable, battery-operated device and function with minimal user inputs making them suitable and affordable for use in low-resource primary health-care settings. Three truenat assays are commercially available developed by Molbio Diagnostics Pvt. Ltd. India, Truenat MTB and Truenat MTB Plus used for Mtb detection targeting nrdB gene (ribonucleoside-diphosphate reductase large subunit) and multiple copies of IS6110 region, respectively, and truenat MTB-RIF Dx for rifampicin resistance detection targeting rpoB core region. Multi-centric studies conducted by FIND reported that accuracy of truenat assays has been comparable to that of MTB/RIF Ultra and Xpert MTB/RIF. Recently in July 2020, truenat assays have been endorsed by the WHO for TB diagnosis and rifampicin resistance detection in adults and children (WHO 2020a).

13.2.2 Recent Advances and Future Prospective in Genomics for Tuberculosis Diagnosis

In addition to the above mentioned WHO-recommended molecular tests for tuberculosis diagnosis, several other tests are also available in the market from various manufacturers (Table 13.1). However, these tests still have to undergo various validation processes for their recommendation by the WHO (2020b). These tests include Real Time MTB RIF/INH Resistance (Abbott), Cobas MTB-RIF/INH (Roche), BD MAX MDR-TB (BD), FluoroType MTBDR Version 2.0 (Hain) and Genoscholar PZA TB II (Nipro). Further, next-generation sequencing for detection of drug resistance, i.e. Deeplex Myc TB, a targeted sequencing assay, is also on the path to the WHO review. Whole genome sequencing (WGS) is also being used for clinical use; however it is still not recommended by the WHO, and results need to be interpreted in context of clinical presentation of the patient.

Real time PCR (RT-PCR) based assays developed by Abott, RT MTB targeting IS6110 and PAB (protein antigen B) genes for detection of MTBC while RT MTB RIF/INH employed for identification of resistance targeting rpoB for rifampicin resistance detection and katG and inhA for isoniazid resistance showed fairly high sensitivity and specificity for TB diagnosis and resistance detection (Wang et al. 2019). Cobas MTB-RIF/INH is an automated real-time PCR-based qualitative test used for detection of resistance to RIF/INH targeting 18 rifampicin resistance-associated mutations in rpoB gene and 7 isoniazid resistance-associated mutations in katG gene and ihnA gene. BD MAX MDR-TB developed using multicopy genomic targets of IS6110 and single copy target of IS1081 has also shown to have high sensitivity and specificity for detection of *Mycobacterium tuberculosis*

complex and rifampin and isoniazid drug resistance. Furthermore, FluoroType MTBDR Version 2.0 developed by Hain Lifesciences and Genoscholar PZA TB II (Nipro) for detection of pyrazinamide (PZA) resistance are also under the WHO review process. Kohli et al. (2020) in the meta-analysis of data utilising these assays reported the sensitivity of >91% and specificity ranging from 97 to 100% for MTBC detection in the respiratory specimen, while sensitivity of about 92% and 70–91% has been observed for rifampicin and isoniazid resistance, respectively.

The next-generation sequencing in TB diagnosis has been successfully applied for characterisation of strain and drug susceptibility testing. In clinical practice, it may further facilitate the delivery of personalised treatment to patients affected by TB and drug-resistant TB. High cost associated with sequencing turns out to be a hurdle for its implementation as a diagnostic tool in poor and low-middle-income countries (LMIC); however, the prices nowadays are coming down due to its implementation in clinical use. Next-generation whole genome sequencing provides potential benefits towards TB control, transmission and MDR/XDR TB treatment. In the high-income countries like Europe and the USA, WGS of Mtb has been routinely used in health-care settings for diagnosis, Mtb speciation and drug resistance profiling. Jajou et al. (2019) reported the consistency of WGS in drug susceptibility testing and demonstrated the use of WGS as a replacement for conventional DST as it accurately detects mutations associated with the low-level resistance in 90% of the isolates. Though culture-based WGS takes 21 days to predict resistance to TB drugs, Soundararajan et al. (2020) has efficiently reported the use of WGS directly on uncultured sputum samples to detect Mtb and identify its drug resistance profile. Apart from determining the drug resistance profiling of Mtb strain, WGS proves a valuable tool in understanding the international TB outbreak, defining the epidemiological links between the TB cases and identifying genetic diversity in strains (Vander Werf and Kodmon 2019; Meehan et al. 2019).

Thus, genomics-based tools can positively influence the TB care from lab to clinics resulting in improved diagnostics as well as new outbreak management strategies. Considering the remarkable progress, it is expected that tuberculosis will be one of the infectious diseases for which a complete genomics approach can be implemented even in health-care settings. The first pilot project using WGS for diagnosis, antibiotic susceptibility determination and epidemiology was recently launched by the Public Health England, an executive agency sponsored by the Department of Health and Social Care, UK. That day is not far off when TB clinic and laboratory will rely on genomic analyses of the pathogen and perhaps even the host to give a complete understanding of an individual's infection.

13.3 Proteomics in TB Diagnosis

Proteomics enables the study of system biology at different molecular levels including functional aspects of proteins, signalling pathways, interaction of various proteins, etc. Development of advanced analytical proteomic technologies for protein purification, state-of-art mass spectrometry instruments and bioinformatics

paved the way for newer tools in disease diagnosis and treatment. Considering the significant involvement of proteins in biological systems, proteomics is expected to play a substantial role in the management of diseases at diagnostic as well as therapeutic levels and may find its place in clinics in future. Identification, quantification and characterisation of protein biomarkers in the biological fluids became a viable approach with the emergence of newer techniques in proteomics. In the field of tuberculosis, in recent years, there has been tremendous progress to explore the mycobacterial proteome for the identification of protein markers for the development of rapid diagnostic tests for tuberculosis. As per the Tuberculist database (a database for the genome sequence annotation of *Mycobacterium tuberculosis* H37Rv, the reference strain commonly used in the study of TB), 4018 proteins are encoded in the genome of Mtb H37Rv strains (Jena et al. 2016). In the past, several diagnostic kits and ELISA-based serological assays have been developed for TB diagnosis utilising the commonly known immunodominant and abundantly produced mycobacterial proteins employing low-resolution proteomics tools. These studies limited the analysis of mycobacterial proteome to only a few hundred proteins (Jakhar et al. 2020), insufficient to provide a signature array for specific diagnosis of tuberculosis. However, serological diagnostic kits for tuberculosis based on the results of these earlier studies were banned by the WHO in 2011, and it was advised to rely on accurate WHO-recommended microbiological or molecular tests only for TB diagnosis. At the same time, the WHO policy strongly recommended further research to identify new/alternative point-of-care tests for TB diagnosis and/or serological tests with improved accuracy (World Health Organization 2011) Hence, TB diagnostic field has evolved a lot in recent years both for the characterisation of mycobacterial proteome for biomarker discovery and identification of host immune markers following interaction with mycobacterial proteins. Recent advances in protein mass spectrometry and functional proteomics have contributed significantly to diagnostics development in tuberculosis.

13.3.1 TB Diagnostic Tests Available in the Clinics Based on Proteomics

(a) *Interferon-γ release assays (IGRAs)*: It is an in vitro test to measure the cell-mediated immune response to Mtb antigens, viz. ESAT-6 and CFP-10. IGRAs have been widely utilised and FDA approved in diagnosing latent Mtb infection and screening of high-risk group individuals who are at high risk for progression to active TB. Two types of IGRA tests are commercially available, i.e. Quantiferon-TB Gold which measures the amount of IFN-**γ** and enzyme-linked immunospot (ELISPOT)-based T-spot TB indirectly measuring the number of IFN-γ-producing peripheral cells. Few studies are available demonstrating the ability of IGRAs for diagnosis of extrapulmonary tuberculosis and childhood tuberculosis (Shin et al. 2015; Lombardi et al. 2019).

(b) *MPT64 TB Check (Mtb and NTM differentiation)*: MPT64 (28 kDa Mtb-specific secretory protein) antigen being highly specific protein to Mtb complex is

employed for diagnosis of tuberculosis and species differentiation. TBCheck MPT64 (Hain Lifesciences GmbH, Germany) is commercially available and used in routine diagnostics that allows rapid identification of MTB complex in positive liquid culture and used for discrimination between MTB complex and NTM. Various studies have reported the use of MPT64 as a promising marker for EPTB diagnosis with fairly higher sensitivity (Hoel et al. 2020; Grønningen et al. 2019).

13.3.2 Recent Advances and Future Prospective in Proteomics for Tuberculosis Diagnosis

13.3.2.1 Pathogen Proteomic Biomarkers as Candidates for TB Diagnosis

Proteomic profiling has been done in several studies and explored for the diagnostic potential for different stages of tuberculosis as well to monitor the treatment response as reviewed by Haas et al. (2016). Antigen 85 complex, a mycobacterium-specific 30–32 kDa family of three proteins (Ag85A/Ag85B/Ag85C) with enzymatic mycolyltransferase activity, is one of the most explored mycobacterial proteins in the earlier studies for its diagnostic potential in tuberculosis (Bentley et al. 1999; Kashyap et al. 2007). Besides, diagnostic potential of several other mycobacterial proteins like HBHA and HspX in various serodiagnostic assays has also been earlier evaluated to improve the TB diagnosis (Meier et al. 2018). Tripathi et al. (2019) reported that patients with active disease presented with high levels of IgG antibodies against CFP-10, ESAT-6 and PE3 mycobacterial proteins expressed during active infection. Recently Wang et al. (2020) reported a novel and culture-free ultrasensitive diagnostic method for detection of MPT64 in sputum with high sensitivity. This ultrasensitive ELISA demonstrated the sensitivity and specificity of 86.9% and 92.0%, respectively.

In addition to ELISA-based techniques to detect mycobacterial proteins in biological fluids of TB patients, recently nucleic acid aptamers have also been utilised in Aptamer Linked Immobilised Sorbent Assay (Aptamer ALISA). Aptamers are short sequences of either single-stranded DNA (ssDNA) or RNA which are selected via systematic evolution of ligands by exponential enrichment (SELEX) process. Aptamers are sensitive, highly specific and bind with great affinity to target sequences. Sypabekova et al. (2017) identified MPT64-specific DNA aptamers through SELEX technology that showed strong binding affinity with high sensitivity and specificity in the sputum samples of TB patients. Aptamers for another mycobacterial antigen HspX have also been used for the efficient and direct detection of HspX antigen in PTB (Lavania et al. 2018) as well as in pleural TB, a type of EPTB (Kumari et al. 2018).

Several studies have reported the use of various advanced proteomic tools for the development of diagnostic assays in tuberculosis. Mehaffy et al. (2020) carried out an advanced proteomic tool multiple reaction monitoring mass spectrometry (MRM-MS) assays for the detection of mycobacterial peptides in the extracellular

vesicles isolated from serum samples of subjects exposed to TB infection and having latent TB patients. This study led to identification of 40 mycobacterial peptides derived from 19 proteins in latent TB subjects and also reported that peptides derived from mycobacterial proteins involved in nitrogen metabolism might be candidates for pathogen-specific biomarkers for detection of latent TB infection. In urine also, proteomics offers important tools for the discovery of diagnostic and/or prognostic biomarkers. Young et al. (2014) reported the identification of tuberculosis protein biomarkers in human urine samples and concluded that 3 of 10 mycobacterial proteins identified in the urine of TB patients are promising biomarkers for point-of-care diagnostic testing for TB.

13.3.2.2 Host Proteomic Biomarkers Candidate for TB Diagnosis

In addition to pathogen-associated protein biomarkers, in recent years, efforts are being made by the researchers to identify disease-specific host proteomic biomarkers in various biological fluids for tuberculosis. Serum proteomic approaches have been used to identify protein biomarker for differentiating the active TB from latent TB and also for the progression of active TB. Proteome microarray analysis of serum from active TB patients exhibits differential protein profile when compared with latent TB. In 2006, Agranoff et al. identified a combination of four biomarkers (serum amyloid A, transthyretin, neopterin and C-reactive protein) that could identify active TB cases with a sensitivity and specificity of 88 and 74%, respectively, by ELISA. With more and more studies in this field, it is being realised that no single protein can provide the level of sensitivity and specificity required for an optimum diagnostic test. Achkar et al. (2015) reported excellent diagnostic accuracy of two host protein bio-signatures for active TB in both HIV-negative and HIV-co-infected TB patients. Mateos et al. (2019) for the first time reported the comparative proteomic profile of saliva and sputum from active TB patients and their healthy contacts. There results suggest the importance of protein signatures involved in complement activation and inflammation. In another study by Penn et al. (2019), two biomarkers based on the combinations of either 3 or 5 proteins were identified that could predict the TB 1 year before the appearance of symptoms to be diagnosed by currently available TB diagnostic tools. Similarly, Garay-Baquero et al. (2020) used a quantitative proteomics discovery methodology to study nondepleted plasma of active TB and healthy controls. They identified novel host protein markers along with validation in two independent cohorts thus leading to a 5-protein biosignature with potential to improve TB diagnosis. With further development, these biomarkers have potential as a screening test to be used as POC test in clinics. Recently, Peng et al. (2020) reported a combination of 15 host serum protein biomarkers that could differentiate active TB from latent TB with a sensitivity of 85.4% and specificity of 90.3%.

Host proteomic has also been extensively studied in various biological fluids of extrapulmonary tuberculosis. Tuberculous pleurisy is a common type of tuberculosis (TB), but its diagnosis is challenging.Pan et al. (2020) studied proteomic profiles of tuberculosis pleural effusion (TPE) and malignant pleural effusion (MPE) and identified 14 proteins with significant difference between TPE and MPE and

reported a signature combination of three proteins that could provide a sensitivity of 73.0% and specificity of 89.4% in discriminating TPE from MPE. In another study reported by our group, vitreous samples from patients with tuberculosis of the eye, i.e. tuberculous uveitis (TBU), with non-TBU and without uveitis were analysed by shotgun proteomics using liquid chromatography tandem mass spectrometry (LC-MS/MS). Pathway analysis of differentially expressed proteins (DEPs) provided not only basis for the molecular mechanisms of TBU pathogenesis but also lead to identification of vitreous protein biomarkers that can be further explored for the differentiation of TBU from non-TBU (Bansal et al. 2020).

Proteomic tools have also been utilised for the study of drug resistance in tuberculosis and use them as biomarkers for the treatment response. Yang and Wu (2019) studied the differentially expressed peptides in the blood samples from TB patients showing multidrug resistance (MDR-TB) as compared to drug-susceptible tuberculosis (DS-TB) patients using LC-MS/MS and ingenuity pathway analysis (IPA) and proposed potential proteomic biomarkers for the diagnosis of MDR-TB with a caution for further investigations in this field. In another study recently carried out by Chen et al. (2020), mass spectrometry and parallel reaction monitoring was used to detect and validate differential serum proteins in MDR-TB and DS-TB patients. These authors developed a diagnostic algorithm using three potential candidate biomarkers such as sCD14, PGLYRP2 (peptidoglycan recognition protein 2) and FGA (fibrinogen alpha chain) resulting in sensitivity of 81.2% and specificity of 90% to diagnose MDR-TB.

Thus, in spite of recent surge in the studies for the identification of several putative biomarkers employing proteomics in tuberculosis, translation of these biomarkers from bench to bed is full of challenges including wide spectrum of disease hampering the appropriate stratification of the patients for validation studies and poor performance of gold standard microbiological tests particularly in some types of extrapulmonary tuberculosis and paediatric tuberculosis.

13.4 Other Omics Technologies for Potential Use for TB Diagnosis

Besides genomic and proteomics, various other tools involving transcriptomics, metabolomics, epigenomics, lipidomics, etc. are also being explored by various researchers for their potential use for the rapid and specific diagnosis of tuberculosis. Transcriptomics employing the mRNA expression profiling using whole genome microarrays and RNA sequencing has proved to be a promising tool for identification of key molecular signatures. These signatures provide potential benefits for the diagnosis and prognosis of TB and for monitoring of drug treatment (Singhania et al. 2018;Lee et al. 2016; Berry et al. 2010). Several studies have documented the perturbation in host transcriptome associated with active tuberculosis when compared with latent infection or healthy controls, and key molecular signatures have been identified to differentiate the active tuberculosis from latent infection and predict the onset of tuberculosis and response to treatment (Zak et al. 2016; Suliman

et al. 2018; Roe et al. 2020; Penn-Nicholson et al. 2020). A major limitation of these studies is that the diagnostic accuracy of identified signatures has been validated in limited number of datasets, and clinical utility of these studies is yet to be documented.

Warsinske et al. (2019) compared 16 gene signatures using 24 independent datasets with >3000 transcripts to investigate whether multiplexing of 1 or more transcripts has the potential to identify patients with active tuberculosis with the desired sensitivity as set by the WHO target product profile. It was found that only two gene signatures reported by Sweeney et al. (2016) and Sambarey et al. (2016) satisfy the WHO target product profile criteria for a non-sputum-based screening test. There are 3 transcripts in the gene signature identified by Sweeney et al. (2016), i.e. GBP5, KLF2 and DUSP3, and 10 transcripts identified by Sambarey et al. (2016), i.e. FCGR1A, HK3, RAB13, RBBP8, IFI44L, TIMM10, BCL6, SMARCD3, CYP4F3 and SLPI, demonstrating the highest accuracy in distinguishing the active TB patients from healthy controls, patients with latent TB infection or other diseases. Cepheid's Xpert MTB HR test currently in diagnostic pipeline is based on the Sweeny 3 signatures, while another test developed by QuantuMDx is also in pipeline and based on RISK6 RNA gene signature (GBP2, FCGR1B, SERPING1, TUBGCP6, TRMT2A and SDR39U1) (Penn-Nicholson et al. 2020).

M. tuberculosis transcriptional profiling at the site of infection can also be utilised to identify new biomarkers. In vivo, pathogen is in constant contact with its host and environment, and it develops various virulence mechanisms for adaptation and survival and evasion of host defence barriers and manipulates immune responses. These molecular changes happening in vivo at host-pathogen intersection offer better understanding of bacterial survival mechanisms under different environmental conditions and development of new diagnostics and therapeutic targets. In some recent studies, specific mycobacterial transcripts that are expressed at different stages of infection and determine the disease progression have been identified. Sharma et al. (2017) performed the transcriptional profiling of mycobacterial genes using whole genome microarrays in sputum samples of pulmonary TB patients. Dysregulated mycobacterial gene expression profile in the in vitro model of intraocular tuberculosis (IOTB), TB of the eye, has also been reported (Abhishek et al. 2018, 2019). Further, the presence of these signatures in the vitreous fluid of IOTB provides the evidences to utilise these for diagnosis. Wildner et al. (2018) carried out gene expression profiling of bacilli in sputa samples during the course of anti-TB treatment. Transcriptional profiling between drug-susceptible and drug-resistant strains has also been well studied and provides evidences that mutations acquired in drug-resistant strains lead to transcriptional changes (De Welzen et al. 2017). Gomez-Gonzalez et al. (2019) carried out an integrated omic analysis to understand how variations in genome and methylation pattern effects the gene expression levels and ultimately the Mtb pathogenesis. Tang et al. (2020) performed the transcriptional profiling of MDR strains compared to reference laboratory strain of Mtb using RNA seq and found unique transcriptional features relevant to both drug resistance and virulence. From the available literature, it is evident that host transcriptomic

signatures are relatively much more explored as compared to mycobacterial transcripts for their potential for a TB diagnostic test, and more studies are required in this direction.

Metabolomics is the relatively new branch of "omics" to monitor the small molecule metabolites in the biological system that are closely associated with diagnosing disease status, determining progression and monitoring of treatment responses based on biochemical profiles. Recently, several studies are coming up in context to tuberculosis exploiting the emerging field of metabolomics. Cho et al. (2020) reported the use of metabolome approach for the diagnosis of active tuberculosis and identified high serum levels of glutamate, aspartate and sulfoxy methionine in active TB patients compared to LTBI. Vaquer et al. (2019) also reported the differential metabolome profile associated with active TB compared to household contacts of TB patients. Dutta et al. (2020) performed the integrative metabolomics and transcriptomic analysis and identified N-acetylneuraminate, quinolinate and pyridoxate metabolites that responded to drug treatment. TB drugs induce changes in the host metabolome as evident from the urinary metabolome that changes over the time during the course of treatment (Combrink et al. 2019). Further, validation studies of these metabolite markers in larger cohorts need to be done to investigate their role as diagnostic biomarkers and in monitoring treatment responses.

13.5 Concluding Remarks

The WHO End TB Strategy aims to end the global TB epidemic, and to achieve this goal, the WHO along with FIND developed a set of target product profiles (TPPs) to guide and develop new diagnostic tools for tuberculosis diagnosis. The criteria set for these TPPs include detection of TB at different stages of disease including LTBI, subclinical and active TB as well as to differentiate between drug-susceptible and drug-resistant TB which are applicable for different health-care settings. In recent years, by using the combination of different omics technologies, several tests have been developed, and some of them are either in the late stage of test development or under review by the WHO for recommendations for clinical use (Table 13.1). Although in the last few years, TB diagnostics field has shown significant advancement, diversion of resources from TB to COVID-19 during 2020 has proved a roadblock in this process and may affect the goals of the End TB Strategy.

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Advances in Proteomics Approaches and Chronic Kidney Disease

14

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Abstract

Chronic kidney disease (CKD) is a serious health burden affecting approximately 16% of the world population. Chronic kidney disease is associated with gradual loss of kidney function and ultimately kidney failure. In CKD, there occurs loss of the ability to release waste electrolytes, whose buildup may hamper normal metabolism. The glomerular filtration rate and serum creatinine are main diagnostic measures in this condition. However, there is an intrinsic problem of poor diagnosis during early stages. This problem can be resolved by the use of proteomics analysis, which provides a complete profile of different proteins under a specific condition. There are various factors which influence occurrence of chronic kidney disease. Increased release of inflammatory cytokines from the adipose and kidney tissues may be responsible for renal injury. The perturbations of the protein biomarkers may occur decades prior to clinical identification. Proteomic analysis involving mass spectrometry coupled with chromatography can provide better resolution to understand the changes of biomarkers of CKD. Next-generation advancements in proteome analysis can facilitate better diagnosis, analysis, and treatment of chronic kidney disease. In this article, the recent advances in proteomic approaches are discussed, which can influence the diagnosis, etiology, and treatment of chronic kidney disease.

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Keywords

Chronic kidney disease · Inflammation · Proteomics · Mass spectroscopy · Proteome · Diagnosis

14.1 Introduction

Chronic kidney disease (CKD) has become a serious health problem worldwide affecting more than 15% of the world population, and the burden is expected to increase because of modern lifestyle (Jha et al. 2013). This disease is associated with gradual loss of kidney function. There are several risk factors associated with CKD like lifestyle, age, climate, gender, race, genetics, and metabolic factors. Thus, CKD is multifactorial and complex disorder. In this disease, the waste filtration capability of the kidney decreases, and subsequently, dangerous levels of uric acid, electrolytes and metabolic wastes tend to build up in the body, ultimately leading to kidney failure. The diagnostics of CKD is based on measurement of serum creatinine concentrations and assessment of the glomerular filtration rate (GFR). However, there is a problem of poor diagnosis during early stages of kidney disease. In this condition, proteomic analysis pose a great potential for diagnosis of CKD. The proteome analysis of a cellular system provides information about the influence of environmental factors on the cellular system in association with hidden genetic signature. Further, proteomics is very valuable tool in the diagnosis, analysis, and treatment of CKD. The perturbations of CKD-related biomarkers may occur decades prior to clinical identification. These clinical biomarkers can be identified along with serum parameters and GFR assessment to diagnose the early onset of CKD. Proteomic analysis can provide details about characteristic proteins and biomarkers associated with the disease.

The proteomic analysis with MS coupled with chromatography techniques provides a high degree of resolution to understand the changes in the various proteins and biomarkers of CKD. Next-generation advancements including matrix-assisted laser desorption ionization-time of flight (MALDI-TOF), surface enhanced laser desorption ionization-TOF (SELDI-TOF), laser capture microdissection-mass spectroscopy (LCM-MS), and iTRAQ in proteome analysis along with powerful high-throughput methods can provide better diagnosis and analysis and treatment of CKD. There are new dimensions being explored focusing on nephropathy antigen, urinary signature peptides, etc., which may help in the prognosis in chronic kidney disease. The high-throughput technologies of genomics and proteomics are continuously improving and thus provide a new platform to deal with human health problems like CKD.

14.2 Historical Background

Kidney disorders have been a common problem of mankind for years. The common problem is the poor diagnosis. The early detection of the disease is not possible in most cases because of overlapping symptoms, and sometimes the patient might ignore symptoms. The symptoms of the kidney disease sometimes are like those of some other illness, and these non-specific symptoms make patients reluctant, and they delay visiting to a specialist. The untreated state for months makes the situation worse leading to chronic disease. Various physiological conditions can be responsible for occurrence of CKD. These conditions include obesity; high blood pressure; diabetes; inflammation of the kidney, glomeruli, tubules, and surrounding structures; occurrence of kidney stones; malfunctioning of the urinary tract; and associated cancers. Increased release of inflammatory cytokines from the adipose and kidney tissues may cause renal injury in obesity. Type 2 diabetes associated with impaired glucose metabolism and insulin resistance may increase the risk of kidney disease. Studies have revealed an increased probability of kidney problem in patients with diabetes mellitus. The major complications associated with CKD include fluid retention leading to edema and swelling in arms and legs, high blood pressure, cardiovascular problems, imbalance in the body solutes like potassium levels in your blood, increase in fragility of the bones, weakened central nervous system, and weekend immune response. Therefore, almost every part of the body is affected by CKD. Long-term diseased condition can be irreversible and lead to end-stage kidney disease eventually requiring either dialysis or kidney transplant.

The analysis of cellular mechanisms with changes in metabolisms and protein levels was being done from many years. However, it was in 1994 when Dr. Marc Wilkins, Australian scientist, coined the term "proteome" and put forward the concept of proteomics. Since then various researchers have put new concepts and tried to explore the potential of proteomics. Proteomics can provide solutions to the unanswered questions of the genomics analysis. The use of proteomics is wide spectrum, and it can be used to understand the pathogenesis and etiology of various diseases like cancer, obesity, and CKD. The diagnosis of kidney disorders and CKD can be done by monitoring some metabolites, cytokines, chemokines, growth factors, etc. The simplest approach of two-dimensional difference in gel electrophoresis (2D-DIGE) provides insights about protein profile and can be used for understanding the disease progression. Since every protein is important and can play important role in modulation of disease, each component of proteome needs to be analyzed. A small factor can change disease progression and manifestation. The comparison is used to be performed with respect to healthy controls.

The advancements in the identification of biomarkers at rapid pace are resulting in early diagnosis of CKD. The development of genetic tests can be very useful for rapid diagnosis. There have been continuous improvements in resolution capability of proteomic analysis over the period. The mass resolution helps in an increase in the mass range for better proteomic analysis. Mass spectrometry (MS) and its variants have evolved rapidly with advancements in engineering and technology. The advanced MS analysis provides speed and accuracy to the diagnosis, management,

and treatment of CKD. The few important aspects related to this subject have been discussed in the following section.

14.3 Proteomics Advancements and Chronic Kidney Disease

14.3.1 Advancements in Proteomics Analysis

The proteomic analysis may be performed using 2D-DIGE, MS, and their variant methods. The 2D-DIGE analysis provides insights about protein profile, and comparative analysis with healthy controls can provide details about the disease progression. Another important advancement is the introduction of mass spectrometry technology. Now, MS is a fundamental technology which is used in proteomic analysis in recent time. MS analysis can provide information about proteins and native protein complexes, and it can help in the study of whole cells to understand the folding of proteins in proteomes (Makarov et al. 2006; Snijder et al. 2017; Kaur et al. 2018). The workflow of the proteomics analysis by mass spectroscopy is outlined in Fig. 14.1. There are continuous advancements in the technology leading to improvements in the analysis. These improvements have enhanced the capability

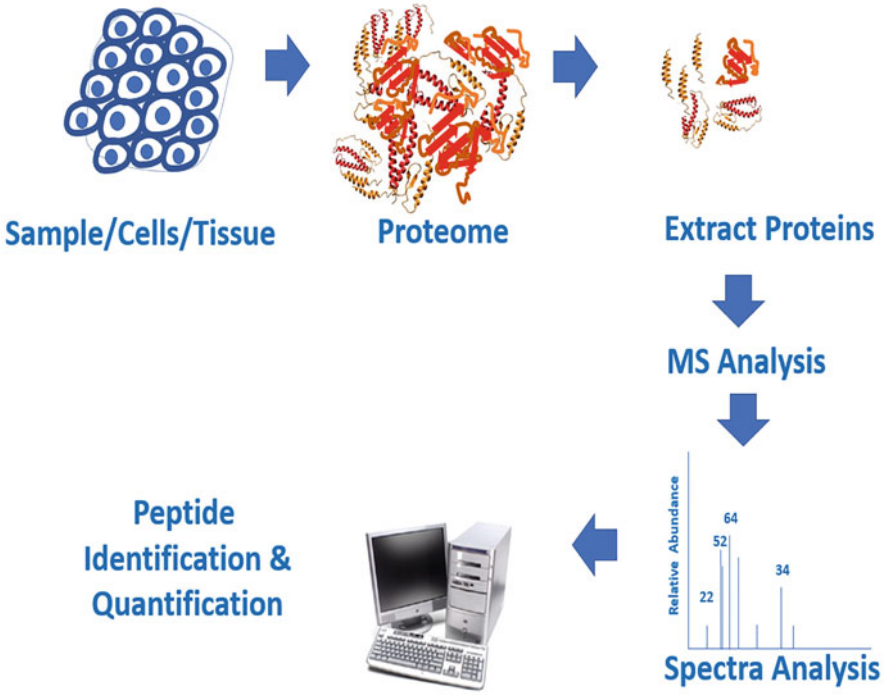

Fig. 14.1 The workflow of the proteomics analysis by mass spectroscopy

and spectrum for protein profile and proteomic discovery (Aggarwal et al. 2019). The increase in the identification capability of peptides and post-translational modifications (PTMs) has led to development of high-end instruments with greater resolution and mass accuracy like Orbitrap and time-of-flight (TOF) mass analyzers (Hu et al. 2005; Yates et al. 2006; Marshall and Hendrickson 2008). The advancements in the technology have made the proteomics cost-effective and relatively robust. Earlier use of high-field magnets for ion cyclotron resonance MS of intact proteins was costly (Macek et al. 2006; Cai et al. 2017; Rose et al. 2012). The current variants of this technology are comparatively cost-effective than previous ones.

There is prerequisite to characterize intact proteins to verify sequence and PTMs (Fornelli et al. 2012; Ehkirch et al. 2018; Chen et al. 2018). These PTMs govern the functionality of the proteins and enzymes. Hence, such analysis can provide support for protein-based therapeutics. In MS analysis, the fragmentation of amide bond of a peptide is comparatively easier. There is a need of robust methodologies to perform fragmentation of the amide bonds in intact proteins (Rosati et al. 2012). Electron transfer dissociation (ETD) and ultraviolet photodissociation (UVPD are two important advancements to achieve more efficient fragmentation of intact proteins. These can be used in combination for better results (Cleland et al. 2017; Brodbelt 2016; Cannon et al. 2014). These advancements make the top-down MS analysis easier. The technological advancements help to combine different analyzers and mass spectrometer to form a "hybrid instrument," for example, triple quadrupole mass spectrometer. In this instrument, one quadrupole was used to select m/z values, the second was used as a collision cell, and the third quadrupole can perform analysis of ions. Another hybrid instrument is the Orbitrap Fusion Lumos Tribrid mass spectrometer, which comprises of five different ion separation devices (Senko et al. 2013). Technically this is a more advanced hybrid instrument, in which a quadrupole mass filter and an ion routing multipole direct ion to ion trap for collision-induced dissociation. Orbitrap provides high resolution, and it is apt for high-mass accuracy analysis. The peptide ion generation and fragmentation occur via collision-induced dissociation. The advantage of ion routing multipole is that multiple experiments can be performed parallelly. The use of this approach reduces overall time required for the analysis. Therefore, the use of such instrument enhances the speed and throughput of the analysis of samples resulting in identification of larger number of peptides and proteins.

In the last decade, there has been continuous improvements in proteomic analysis, focusing on ion mobility spectrometer (IMS), which can improve the ion separation capabilities. A typical IMS instrument use high-pressure gas and constraining electric fields to increase the ion separation (Shvartsburg et al. 2013). The increase in the ion separation is critical as it can facilitate improved resolution of the mass spectrophotometer. The use of ion mobility separation with a quadrupole time-of-flight (TOF) mass spectrometer has been performed to analyze protein samples (Hoaglund et al. 1998). With time, the use of IMS proteomic approaches has increased. There has been a switchover to new-generation instruments in comparison to traditional mass spectrophotometer.

These next-generation instrument focuses on ion mobility resolution using an electrical wave at lower pressure to force ion movement through a gas. In another improvement called trapped IMS (TIMS), electric and radiofrequency fields for ion separation and improved resolution are used (Silveira et al. 2014). The use of TIMS with parallel accumulation-serial fragmentation (PASEF) technology provides further improvements in proteomic analysis. In PASEF, there happens mass selective release of peptide ions from the TIMS device for further analysis by MS/MS (Meier et al. 2015).

It is evident that protein correlation profiling is a useful method in proteomics, and pulldown immunoprecipitation of target protein to obtain protein complexes may act as a good way to validate these methods (Huttlin et al. 2015). These methods can provide useful information about the protein-protein interactions. The affinity pulldown experiments are widely performed to analysis protein interactions in order to decipher the process or pathway. However, there is a limitation of requirement of higher affinity before pulldown experiments. Another advanced method called "spatial proteomics" may solve this problem. This recent method determines proteins in the region around a bait even without higher affinity between interactors. The only prerequisite for spatial proteomics is that interactors should be within a defined region. This method can provide valuable information about the protein-protein interaction especially histone modifications and gene regulation. The introduction of the labels into two different samples is done, and interaction can be monitored. However, there should be accurate control over reaction to avoid error-prone interactions, mixing errors, and mass balance errors resulting in false positive results during proteomic analysis.

It is noteworthy that the proteome shows different pattern across different body organs and tissues. The proteomic analysis of the liver, muscle, kidney, and blood would give different observations. There is heterogeneity within various tissues in the kidney. The glomerular, endothelial, and tubular cells are different from each other in protein expression profile. Therefore, it is advised to have proteomic analysis for better understanding of the protein expression profile across tissues in clinical condition. The diseased phenotype is directly related to the genotype. But the influence of the external factors and stress conditions can lead to the change in the metabolism and protein profiles (Srivastav et al. 2019). Thus, proteomic analysis can provide better understanding of the system. Therefore, the focus is shifting from genomic to proteomic analysis. However, the comparative analysis would be elucidating the detailed dynamics of the clinical condition like CKD. In such situations, albuminuria/proteinuria analysis of the urine may provide information about the specific pathway affected. In another situation, it can be just non-specific release of protein from the glomerular filtration unit. The proteomic analysis is also dependent on consideration of external factors and age, sex, diet, and diurnal variations. The correlation of these variations with the proteome analysis can provide a complete picture of the clinical condition (Nikolaeva et al. 2016; Shao et al. 2019; Rebholz et al. 2019). GFR substantially put influence on the proteomics chronic kidney disease (Christensson et al. 2017; Sekula et al. 2016). The analysis of the GFR can provide interesting insights about the pathological changes. However, GFR

does not completely elucidate kidney functions, and there may be non-correlation in statistical analysis (Rhee et al. 2013). A toxic molecule would if nephrotoxic can hamper GFR negatively. This correlation is negative when the molecule is nephrotoxic. The proteomic analysis can provide information about various biological processes including metabolism, inflammation, growth, and differentiation. In last decades, there has been a continuous improvement in the technology and engineering, which has improved the capabilities of genomics and proteomics approaches (Huttlin et al. 2017; Srivastav and Suneja 2019). It is expected that future advancements in proteomics approaches would expand the horizon of analysis for better understanding of biologics and improve the diagnosis and treatment of diseases like CKD, cancer, obesity, etc.

14.3.2 Chronic Kidney Disease and Proteomics

Prognosis of CKD can help in the better treatment and management of disease. The metabolite, enzymes, chemokines, cytokines, and hormones can provide accurate information about the disease pathogenesis. The use of multiple biomarkers can provide accurate prognosis when there is high variability expected from use of single biomarker. Researchers have suggested several biomarkers from plasma, urine interleukins, and chemokines for CKD, for example, IL-18, IL-10, IL-6, MCP-1, and FGF-23 (Gentile and Remuzzi 2016).

There are multi-factors, which can modulate the chronic kidney disease (Hirschhorn and Daly 2005). The chances of occurrence of kidney diseases and nephropathy are higher in diabetic condition. Nephropathy is a condition where damage to the blood vessels and nephron occurs. In nephropathy, inflammation takes place, and many proteins associated with inflammation act as mediators of nephropathy. Interestingly, deposition of calcium can occur in vessel wall, and it can be patchy, diffuse, or concentric depending upon the location and lead to diseased condition (Moe and Chen 2008). Calcification associated with chronic kidney disease is generally in the medial smooth muscle, is concentric, and has a diffuse distribution (Amann 2008). It is also seen that immunocompromised conditions or chemotherapy increases susceptibility for CKD. Even inflammation of the membrane of the heart (pericardium) and pregnancy complications can contribute to the development of CKD. The inflammatory proteins may act as mediators of inflammation. Also, proteins can act as modulators of the cell-crystal interactions and hence nephrolithiasis process (Sharma et al. 2016; Narula et al. 2016, 2018). The renal tubular injury occurs in stepwise manner subsequently leading to CKD. It has been observed that proteins like NGAL, KIM-1, TIMP-2, and IGFBP-7 can be used as biomarkers for early diagnosis of CKD (Carrero et al. 2009; Luczak et al. 2016; Jia et al. 2017).

In a study, Romanova et al. analyzed differential expression of cytokines and proteins by multiple reaction monitoring approach and quantified cytokines, chemokines, and growth factors using the multiplex Luminex xMAP technology in CKD patients. They found elevated serum concentrations of CUL5, antigen

KI-67, NKRF, CAPRI, IGSF22, APPBP2, CCD171, and CCD43 in CKD patients. The APOA4, AAT, VIL1, complement component, and cytokine concentrations increased in patients with renal disorders (Wen et al. 2013; Luczak et al. 2015; Glorieux et al. 2015; Peters et al. 2017). The finding correlated with ELISA suggests the importance of such analysis.

In another study, protein profile of more than 200 urine samples were analyzed by capillary electrophoresis-MS analysis (Good et al. 2010). There were about 273 urinary peptides observed that showed variations between test and controls. It is referred as CKD273 and has found worldwide approval. These CKD273 has significant potential in differentiation between chronic kidney patients and normal individuals and can act as good criteria for prognosis of CKD (Schanstra et al. 2015; Pontillo et al. 2017). CKD273 gives details about collagen fragments, blood-derived proteins involved in inflammation and tissue repair, which is a sign of kidney damage.

The chronic tissue inflammation can contribute significantly to the clinical condition. It is a potential risk factor in diabetic kidney disease. There has been observed association between circulating TNF receptors 1 and 2 and the risk of ESRD (end-stage renal disease) in diabetic kidney disease (Niewczas et al. 2012). The inflammatory proteins in circulation can act as potential biomarker for CKD. A group of researchers focused on these circulatory inflammation proteins, and they measure a panel of 194 proteins in diabetic kidney disease (Niewczas et al. 2019). In one comprehensive analysis related to diabetes, more than 200 subjects were investigated and identified a kidney risk inflammatory signature (KRIS). KRIS can be defined as a set of 17 inflammatory proteins, consisting mainly of TNF-R superfamily members. These TNF-R have strong association with CKD. The investigators suggested that non-kidney sources like leukocytes may play significant role in generating circulating KRIS proteins. The comprehensive proteomic database is generated by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) CKD Biomarkers Consortium across different age groups (Rhee et al. 2019). During an ideal analysis, as per the good availability of the high-throughput data, cardiovascular events, and cognitive development and other sub-phenotypes related proteomic data, need to be considered for comprehensive and relevant proteomics and diagnosis in CKD studies (Grams et al. 2017).

There is prerequisite to shift paradigms from genomics to proteomics and possibly an integrative analysis. The current proteomic capability is constantly evolving because of the technological advances. These advancements can improve the diagnosis, detection, treatment, and management of CKD. MS analysis has many variants with different capabilities for different analysis types. LC-MS instruments are widely used in clinical laboratories for diagnosis and analysis of diseases and toxicology analysis. MALDI-TOF MS profiling methods are also being used because of higher sensitivity and accuracy. It provides validation of data and analysis. The experimental design and analysis are optimized with respect to the clinical condition, target, and objectives in a proteomic study. Isobaric tags for relative and absolute quantitation (iTRAQ) is an advanced approach compared to 2D-DIGE in quantitative analysis with higher sensitivity and accuracy. In this

approach, isobaric tags can be put in proteins, and these labels help for relative and absolute proteomic quantitation by mass spectroscopy. This gives information about the total protein from different sources. However, the validation of altered proteins or change in protein profile can be performed by conventional methods like Western blotting.

There are various challenges associated with comprehensive proteomic analysis. There is a prerequisite for a proteomics analysis to characterize intact proteins to analyze sequence information and post-translational modifications (Fornelli et al. 2012; Ehkirch et al. 2018; Chen et al. 2018). The fragmentation of amide bonds is one of the major challenges. The use of robust methods for fragmentation of the amide bonds can provide an answer to this problem (Rosati et al. 2012). In proteome analysis, the characterization and identification of native proteins is also required. Sometimes the membrane proteins are difficult to isolate and characterize because of their transmembrane hydrophobic regions. There have been improvements in this context, where native protein complexes can be analyzed (Marcoux and Robinson 2013). The new technology involves surface-induced dissociation to fragment protein complexes. This modification helps to direct higher amount of energy into ion complex. The membrane proteins are difficult to handle because of their fragile nature and hydrophobicity. However, in this case, crosslinking can be performed, and this would avoid the dissociation of the membrane complex. The denaturant buffers should be avoided, but if the complex is crosslinked, then denaturant would not hamper much with the proteomic analysis (Larance et al. 2016).

14.4 Conclusion

There has been a huge progress in the field of genomics, and large-scale sequencing efforts have been made to measure susceptibility for multigenic diseases. However, the success rate is not very good. This may be because scientists were unable to understand the missing patterns of heritability for some diseases. The possible reason could be the post-transcriptional and post-translational modifications and role of external factors in controlling the epigenetics (Koch 2014). The worldwide burden of chronic kidney disease is about 16%, very significant, and it requires researchers and clinicians to focus more attention on this problem. The gradual loss of function of the kidney is major issue, where the disease is neglected during early-onset stages. This poses the problem of early diagnosis by the clinicians. The glomerular filtration rate (GFR) and level of serum creatinine do not alarm about early-onset of CKD. The use of advanced proteomics approaches can improve for early diagnosis and subsequent treatment of CKD. The perturbations of the chronic-related biomarkers may occur many years prior to clinical identification. Mass spectrometry analysis coupled with chromatography techniques provides a high degree of resolution to understand the changes in the various biomarkers of CKD. There are next-generation advancements in the field of proteome analysis, and mass spectrometry has revolutionized the biomedical field (Schaffer et al. 2019). It has provided better diagnosis, analysis, and treatment of CKD. The high-throughput technologies have

improved the way to deal with CKD. The focus of the research should be also on to determine the role of proteome and various metabolites in disease pathogenesis. The advent of new technologies and advancements in the study of post-translational modifications would enhance the pace of proteome analysis in CKD. The detailed pathway affected and genes and protein involved in CKD can be deciphered by integrated use of genomics and proteomics.

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3D Bioprinting of Tissues and Organs: A New Paradigm in Regenerative Medicine and Biomedical Engineering

15

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Abstract

3D bioprinting is the most popular additive manufacturing method, in which solid objects are constructed by depositing several layers in sequence for the fabrication of biostructures. Bioprinted structures, like tissues, organs, and Organ-on-chip, in 3D, are now widely used to study and understand the functions of the human body. The 3D bioprinted structures are closely similar to naturally occurring biologics systems, and studies performed on 3D biostructures can be more biologically relevant than in vitro studies conducted in 2D. 3D bioprinting has advanced over the years and is commonly used in tissue engineering and regenerative medicine. The fabrication of biomaterials like cells, tissues, and organs using 3D bioprinting is becoming an alternative and favorable tissue and organ transplantation approach. Bioprinting is a relatively novel method and holds great promise but has several challenges and limitations. To this end, this chapter summarizes the concept of 3D bioprinting, bioinks and their classification, different methods of bioprinting, and their applications in areas of health, pharmaceutical science, and biomedical engineering. The chapter also highlights the challenges associated with the clinical utilization of 3D bioprinting. 3D bioprinting and its applications in personalized medicine and tissue bioengineering are continuously growing. In the future, this technology could provide advanced tools to the researchers to develop targeted treatment and improve patient outcomes.

Keywords

3D bioprinting · Tissue engineering · Regenerative medicine · Bioinks · Pharmaceuticals · Medical devices · Organ-on-chip

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15.1 Introduction

3D bioprinting (3DBP) technology is defined as "the process of obtaining a three-dimensional object in a layer-by-layer fashion with computer-controlled designs" (Eshkalak et al. 2020).The ultimate aim of this technology is to design tissues and organs similar to naturally occurring biologics systems. In the conventional method, non-advanced tools or non-standard geometries are used to produce objects from the bulk substances, resulting in low-quality production (Campbell et al. 2011; Hwang et al. 2018). In contrast to the conventional method, 3DBP technology is more rapid and convenient to handle and automate customized processes (Peterson et al. 2014; Anciaux et al. 2016; Wang et al. 2016a). 3D bioprinting technology is based on 3D printing principles that provide an opportunity to obtain biological materials which include cells, tissues, and organs from biocompatible and biomimetic biomaterials. There are several kinds of 3D printing methods such as inkjet bioprinting, laser-based and extrusion-based bioprinting, and stereolithography bioprinting that are currently in use. Apart from organ printing, it is also utilized in preclinical studies to generate in vitro/in vivo models for developing and screening drug delivery systems. For pharmaceutical research, it offers faster means of drug testing at a low cost and can also have better biological relevance to humans than animal studies. It is a robust tool technology and offers several advantages such as enhanced safety profile and R and D productivity with high efficacy (Ngo et al. 2018). Tailored medication, personalization, quick disintegration, and mini dispenser unit are some of the advantages of 3D printing. However, there are certain limitations of 3DBP. Indeed, bioink formulation does not possess adequate self-binding property, precise viscosity of ink, and exact object printability during the end of the process (Ali et al. 2020). Technology has undoubtedly enhanced patient compliance with the development of scaffolds, tissues, organs, and novel drug carrier systems which are suitable for patient anatomy and specificity by optional and rapid production. The novel technology of 3D bioprinting holds great promise; it is a cost-effective and time-saving technique and is attracting immense attention, especially in healthcare, pharmaceutical sciences, and biomedical engineering (Koçak et al. 2020; Yan et al. 2018).

15.2 History

For the first time, Dr. Kodama from Japan discovered 3D printing technology in his name during 1980. In 1984, the stereolithography apparatus was invented, representing the birth of 3D printing. Moreover, the first use of laser technology illustrating 2D patterning of live cells was reported in 1999. In 2002, the first extrusion-based bioprinting method, which was known as "3D Bioplotter," was launched. Wilson and Boland developed the first inkjet bioprinter in 2003. In the year 2006, living cells were deposited by the application of an electrodynamic jetting. Scaffold-free vascular tissue was fabricated by using bioprinting in 2009. The coming years saw the rise of numerous new bioprinting products, for example, artificial liver and articular cartilage in 2012 and tissue integration with the vascular

system in 2014. In 2016, the cartilage model was manufactured by the Integrated Tissue and Organ Printing (ITOP) system. In the same year, Spritam (levetiracetam) tablets for oral use were manufactured using an inkjet printing method; the first 3D-printed drug approved by the Food and Drug Administration (FDA) in 2016 was developed by Aprecia Pharmaceuticals (Jose and Christoper 2018). In 2019, a cardioid structure was bioprinted in Tel Aviv University; the collagen human heart at various scales was engineered using FRESH technology (Gu et al. 2019).

15.3 3D Bioprinting Procedure

The procedure of 3D bioprinting is generally relied upon the principle of the exact layering of biomaterials. The initial step in 3D bioprinting is to generate a 3D model design using CAD/CAM software. Selection of biomaterial or bioink is also included in this step (Zhang et al. 2018). The second step involves converting the digital model into the STL file format, which stands for standard tessellation language or stereolithography that can be readable to the printer (Agarwal et al. 2020). The STL file contains polygons or triangles, which provide instructions about the designed surface for introducing a model to the printer. In the third step, the data is converted into G-code that encloses information for printing the material layer by layer with the help of slicer software that is placed in the 3D printer (Gross et al. 2014). After that, the extra printed parts are removed before the final step. Step five is a post-processing step, and it relies on different technologies for the maturation of the fabricated construct in a bioreaction to create a complete 3D object (Jamroz et al. 2018; Highley 2019; Papaioannou et al. 2019) (Fig. 15.1).

15.4 Bioinks

The most crucial step in the bioprinting strategy is to select and design the ideal bioinks. Bioinks are "a formulation of cells suitable for processing by an automated biofabrication technology that may also contain biologically active contents and biomaterials" (Groll et al. 2018). Characteristics of ideal bioinks include high mechanical, chemical, and rheological properties. Moreover, it should have good biocompatibility, biodegradability, nontoxicity, and non-immunogenicity, which aid in both cell viability and printability.

Two kinds of bioinks that are adopted in 3D bioprinting are as follows:

1. Scaffold-free bioinks.
2. Scaffold-based bioinks.

Scaffold-free bioinks are the ones in which living cells are directly printed into 3D constructs (Achilli et al. 2012; Mironov et al. 2009; Cui et al. 2017). Firstly, clusters or aggregates are produced from cells (like spheroids) and placed into suitable patterns, which further go for fusion and maturation into broad-scale functional

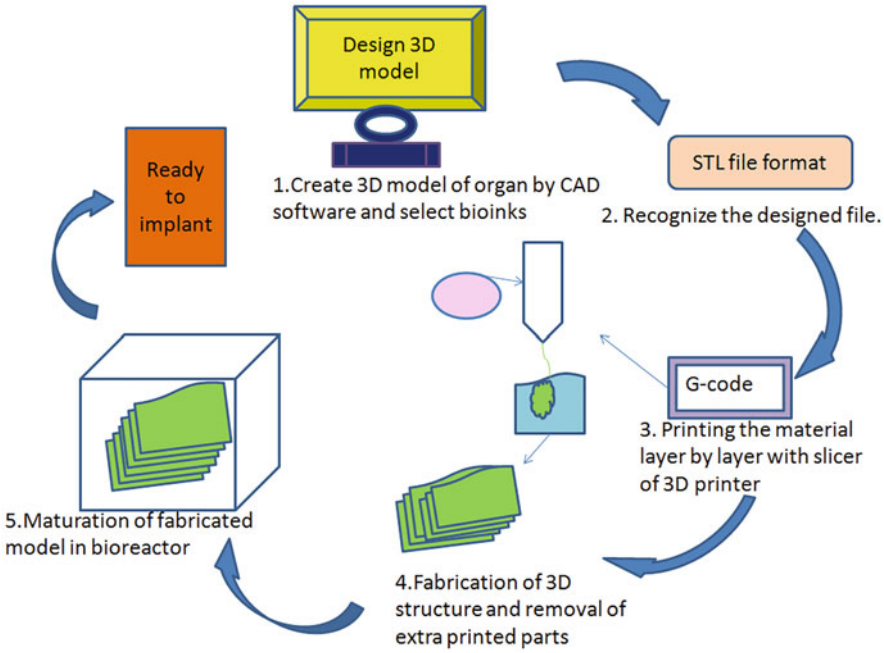

Fig. 15.1 Process of 3D bioprinting

tissues (Rezende et al. 2013). In the case of scaffold-based bioinks, polymeric hydrogels are used to fix the cells, and then the scaffolds are employed for printing with tissue-specific 3D architectures (Liu et al. 2017). In this case, when the degradation of the hydrogel scaffold occurs, the encapsulated cells begin to proliferate and grow into wide-scale tissues. The later bioink strategies are beneficial in manufacturing biomimetic tissues. However, it also exhibits certain drawbacks due to the immobilization of cells in the scaffold, which induce hindrance in cell proliferation, migration, and colonization. Furthermore, scaffold-free bioinks show close biomimicry of the generated tissue and improve functionality for a longer duration (Yipeng et al. 2017). Bioprinting technology consists of three essential components: polymer solution (natural or synthetic), viable cells, and 3D printers (Aljohani et al. 2018).

15.5 Hydrogels for Bioprinting

Hydrogel is a class of 3D network polymers formed through crosslinking methods to form gel-like structures and are essential components of bioink. It functions as a cell carrier, maintains its viability, and serves as substrate or scaffolds supporting its growth and proliferation. The hydrogel can swell in water but does not dissolve in it. Moreover, a few hydrogels exhibit permeability that is similar to the natural

extracellular matrix (ECM). The hydrogels are considered as an ideal material for cell bioprinting due to biocompatibility, high water content, and biodegradability (Wang et al. 2020). Electrospun fibers were utilized as scaffolds to replace the blood vessels, bone, cartilage, and other organs in earlier times. However, nowadays, it is substituted with many polymers as collagen, chitosan, polyanhydride, alginate, agarose, Matrigel, and fibronectin (Gu et al. 2015; Jia et al. 2014). There are two types of hydrogels applied for bioprinting of biomaterials: a natural polymer, synthetic polymer, or a combination of both natural and synthetic polymer-based biomaterials. Natural polymers are widely available from sources like animals, plants, and microbes and are the main component of extracellular matrices (ECMs). Before printing, natural polymers enclose the viable cells and biologically active agents and make them safe from printing stress during the process. After printing, semipermeable substrates are formed, and these substrates are permeable to nutrients and metabolites of a cell; through this, cells can exchange gases (e.g., oxygen). The natural polymers commonly used include hyaluronic acid (HA), gelatin, fibrin, laminin, collagen, and fibronectin. Apart from this, various other biopolymers such as chitosan, alginate, starch, and cellulose are also used as hydrogels (Liu et al. 2018a).

On the contrary, synthetic polymers are produced by several chemical synthesis processes. It is known to mimic the extracellular environment with the help of various native components of ECM. Natural polymers are usually modified to ameliorate the biodegradability, biocompatibility, thermal, physico-mechanical, biological, chemical, and conductive properties through synthesis of composites (Xu et al. 2012). Derivations in cross-linkable structures provide a better opportunity in tissue engineering and regenerative medicines for application purposes. Poly (ethylene glycol) (PEG), PEG-dimethacrylate (PEG-DMA) (Cui et al. 2012), poly (ethylene glycol) diacrylate (PEGDA), poly(ε-caprolactone) (PCL), polymethylmethacrylate (PMMA) poly(vinyl alcohol) (PVA), polyurethane (PU), poly (hydroxypropylmethacrylamide) (PHMMA), GelMA/gellan gum, and alginate hydrogel (Visser et al. 2013), and poly lactic-co-glycolic acid (PLGA) biomaterials are implemented to engineer desired organ through the scaffold-free cell printing technology (Shim et al. 2012; Guo et al. 2014). Among these polymers, PEG, PLGA, and PU are known as synthetic polymers, which exhibit excellent 3D printability, tissue compatibility, and stability properties (Zhang and Wang 2019).

15.6 Methods of 3D Bioprinting

15.6.1 Inkjet-Based Bioprinting

It is a conventional and commonly used method of printing. In this method, droplets acquiring cells and biomaterials are patterned layer-by-layer on a substrate (Shafiee and Atala 2016). Generally, bioink is filled into the cartridge, and then droplets are placed on the substrate with the assistance of computer-controlled thermal or piezoelectric (acoustic) actuators to build a 3D model (Zhang et al. 2016). The heat

required to generate bubbles is up to 300 °C for a few microseconds, and then picoliter-sized ink droplets from the nozzle spread onto the substrate (Arslan et al. 2016). Generally, the high temperature encompasses a minor impact on the viability of the cellular material (Xu et al. 2005). The piezoelectric crystal actuator generates electrical pulses to eject nanoliter or picoliter droplets at regular intervals (Park et al. 2017). Furthermore, this method offers high resolution and allows printing with high cell viability (Demirci and Montesano 2007). The benefits of inkjet bioprinting include the high printing speed (up to 10,000 droplets per second), cost-effectiveness, and wide availability. In addition, the repairment of skin and cartilage tissues is a common application of inkjet bioprinting where the high speed of printer enables direct ejection of cells and biomaterials onto skin and cartilage lesions. However, the major drawback is that the cells can sometimes form aggregation in between the cartridges and block the nozzles (Arslan et al. 2016).

15.6.2 Laser-Based Bioprinting

Laser-based bioprinting or laser-induced forward transfer bioprinting (LIFT bioprinting) is a method applied for printing living cells in the engineering of tissue structures. In this method, the high-energy light source is utilized for printing design (Moroni et al. 2018; Hospodiuk et al. 2017). During the process, repeated laser beam pulse is received by substrate at a controlled rate for printing the cells (Badylak and Gilbert 2008). This device is comprised of a focusing system, pulsed laser beam, bioink, laser absorbing ribbon, and a receiving substrate (Nguyen et al. 2017; Lorson et al. 2017). The resolution of laser bioprinting relies on various parameters such as surface tension, type of laser used, the wettability (substrate), viscosity (organic layer), and the air voids between the substrate and the ribbon (Liu et al. 2018b). Unlike inkjet printing, it has no nozzle, and as such high density of cells and viscous bioinks can be loaded without causing clogging. It has been used to print cellularized skin constructs, cells of the human dermal fibroblasts, and pulmonary artery endothelial cells. However, expensive installment and selection of bioinks make this method less preferable (Arslan et al. 2016).

15.6.3 Extrusion Bioprinting/Direct Writing

Extrusion bioprinting (EBB) is very common additive manufacturing method that is applied in the area of tissue engineering and biofabrication (Ji and Guvendiren 2017). This technique is broadly used on a large scale for bioprinting the cells in which the continuous filaments from the automatic nozzle extrudes the bioink on to the substrate (Ozbolat and Gudapati 2016; Hospodiuk et al. 2016). The biological materials are extruded and regulated by pneumatic or mechanical dispensing techniques. The viscosity ranges of hydrogel solutions for supporting cell materials lie from 30 to $> 6 \times 10^7$ mPa/s for extrusion-based bioprinting (Murphy and Atala 2014). This device has a dispenser (single ejector or multiple ejectors), an automatic

robotic system, and a stage controller. The significant advantages of EBB are its high printability speed, reduced risk of clogging, compatibility for printing materials, and high-density cell deposition (Wang et al. 2017; Yeo and Kim 2017).This technology is commonly used to manufacture scaffolds that mimic soft tissues and bone structures, which provide an opportunity for possible implants. Nevertheless, the pressure and shear stress influence deposited cells' viability in viscous fluids forming a structure with low fabrication resolution (~200 μm) by extrusion (Xiong et al. 2017). These drawbacks can be overcome by optimization and modification in the nozzle, syringe, or motor control systems to some extent (Yu et al. 2013; Dababneh and Ozbolat 2014).

15.6.4 Bioplotting

Bioplotting is type of 3D printing technique employed for tissue engineering and regeneration. Its adaptability provides new opportunities to extrude spheroids or tubes of materials from the syringe (Gloria et al. 2009). Generally, this printer system has multiple syringes and can be applied to numerous cell types enabling the fabrication of soft tissues. In this mechanism, deposition of materials is arranged in a bottom-up approach (layer-by-layer) and cured via UV radiation. Despite these advantages, challenges in selecting the bioprinting materials are known to exist (Mobaraki et al. 2020).

15.6.5 Fused-Deposition Modeling (FDM)

Among additive manufacturing techniques, FDM is one of the oldest processes involved in 3D printing. In the FDM printer, the head resembles an inkjet printer (Salentijn et al. 2017). However, during the heating process, beads are liberated from the print head, as it then constructs the object in fine layers. The procedure undergoes several repetitions until the complete structure of each layer is formed precisely. After that, each layer becomes hard, gradually developing into a solid object. FDM printers can use several polymeric materials, but it does not show similarity to formulate bioinks for bioprinting applications (Rahim et al. 2019).

15.6.6 Stereolithography (SL)

This method is based on photo-polymerization in which photosensitive material/s is cured to produce a 3D object (Kumar and Kim 2020). Firstly, the appropriate bioink is prepared by mixing cells in the solution of photosensitive prepolymer. The SLA device initiates the photopolymer's chemical reaction by using the digital mirror that induces the crosslinking of the bioink (Melchels et al. 2010). After repetition of the process, the parts of the object are fabricated in a layer-by-layer manner. Finally, curing is the essential step to improve mechanical integrity and to remove the

uncrosslinked portion of the bioink. SLA printers transfer the energy into a liquid photopolymerizable resin in the form of UV laser beam. However, certain resins have shown potential carcinogenic properties. This is the way the FDA-approved resin should be used for clinical applications (Wang et al. 2016b; Goole and Amighi 2016). SLA-based technologies are broadly applicable in the biomedical field to build prosthetics, custom implants, bone, or cartilage tissues. Although the SLA is a rapid printing process, it is a low-resolution and time-consuming technique for designing 3D tissue. Therefore, compared to the visible light-based photoinitiation, the bioprinting UV light-based process consumes more time during exposure to light and possesses a low cell survival rate (Ozbolat et al. 2016).

15.7 Applications of 3D Bioprinting

It is an exciting platform for developing medical and engineering technologies and biomimicking living animal cells, tissues, and organs. After modifying conventional technology, the contemporary 3D bioprinting technique forms a base for the evaluation of numerous biomedical applications such as scaffolds, tissue fabrication, artificial organs, development of drugs, and medical devices. Apart from the above-cited applications, it can also be used to create prosthetics, implants, anatomical models, and drug delivery.

15.7.1 Tissues and Organs Printing

3D printing technology provides benefits in the fabrication of cells, cell-laden biomaterials, and tissues to create 3D organs (Ozbolat and Yu 2013). 3D printers have been employed to develop a spinal disk, knee meniscus, heart valve, cartilage and bone, and an artificial ear (Gross et al. 2014).

15.7.2 Customized Implants and Prostheses

Due to advancements in 3D technology, many techniques such as X-ray, MRI, or CT scans are used for making similar geometry of organs that can transfer images into digital 3D files. The strategy has been practiced in spinal, dental, and hip implants (Šljivić et al. 2019).

15.7.3 Anatomical Models

Anatomical models are customized for specific patients and pathology. On the basis of customized models, it helps in educating patients on their exact condition. Neurosurgeons widely use 3D-printed neuroanatomical models to understand the complexity of the human body (Klein et al. 2013).

15.7.4 3D-Printed Dosage Forms and Drug Delivery Devices

Bioprinting has earned worldwide popularity in the last decades, making a significant impact on the pharmaceutical industry. It has shown high reproducibility and capability in manufacturing dosage forms with complex drug release profiles. This technique can also create complex drug manufacturing methods. For the control release rate of the drug, internal structure and shape structure can be adjusted by choosing suitable models, parameters, and materials. The surface area is modified to create a customized shape of the pill by 3D printing (Goyanes et al. 2015). It works on the principle of the concentration gradient to regulate and control the strength and time of the drug release. ZipDose Technology enables the administration of a high drug load, up to 1000 mg in a unit dose. SPRITAM is one example through which the largest quantity of levetiracetam is administered with only a single sip, thereby enhancing patient compliance (Jose and Christoper 2018).

15.7.5 Unique Dosage Forms

3D printing offers the opportunity to produce abundant dosage forms that overcome the limitations of conventional drug fabrication. It has currently been used to manufacture various novel drug formulations, for instance, microcapsules, antibiotic printed micropatterns, nanosuspensions, mesoporous bioactive glass scaffolds, and multilayered drug delivery devices (Ursan et al. 2013). Sustained-release formulation can be developed as single or multilayer printed tablets. Such type of dosage forms are known to reduce the frequency and number of dosage form units taken by the patient on a regular basis. One of the major potentials of 3D printing is to manufacture an individualized dosage form known as polypill. Any polypill is a combination of two or more doses, which includes sustained-release (SR) layer and immediate-release (IR) layer. For example, aspirin and hydrochlorothiazide are placed in the IR layer, while the SR layer helps in the separation of the three drugs, namely, atenolol, pravastatin, and ramipril, to ensure the peculiar release of the individual drug. The polypill helps in treating patients with comorbidities, such as hypertension, chronic renal failure, and diabetes (Khaled et al. 2015).

15.7.6 Drug Testing

3D printed biomimetic model has been employed in drug testing, where it is accelerating the new drug development process and ultimately reduces the usage of animals for preclinical studies. For this, personalized cell-laden pattern and fabricate models such as in vitro liver for drug screening are fabricated. The 3D microenvironment is useful in investigating the toxicological and pharmacological properties of microtissues (Satpathy et al. 2018; Peng et al. 2017).

15.7.7 Other Applications with Some Challenges

Repairing of skin has gained more attention due to its simple structure, but the application of 3D bioprinting on burned skin would be challenging to regenerate scar-free skin. Vascular tissue engineering is complicated as vessels exist throughout the body. Myocardial repair has also attracted massive attention. Currently, there are many cardiac patches used to treat myocardial infarction. Simple tissues (e.g., skin, bone, and cartilage) are fabricated easily, while complex tissues, such as vascular or connective tissues, are hard to fabricate (Aljohani et al. 2018).

15.8 Tissue Engineering and Regenerative Medicine

Tissue engineering is an interdisciplinary field that applies engineering and life sciences principles in developing biological tissue that focuses on creating and fabricating natural substitutes to reproduce functioning tissues or organs. The objective of tissue engineering is to achieve a physiological structure that maintains, restores, or improves damaged tissues or whole organs and to promote health. It constructs alternative structures that mimic the body's normal tissue for substituting injured or diseased tissue (Wardhana and Valeria 2020).

Regenerative medicine is a multidisciplinary area that involves the body utilizing its systems (directly) or sometimes with the help of foreign biological material (indirectly releasing factors that can induce endogenous tissue healing) to reform damaged cells, tissues, or organs.

The words "tissue engineering" and "regenerative medicine" are often used as interchangeable terms, as the ultimate target is to concentrate on cures rather than treatments for multiple diseases (Tonelli et al. 2016). For example, artificial skin and cartilage have been approved by the FDA as common engineered tissues; however, they have limited use in human patients due to certain limitations.

15.8.1 Components of Tissue Engineering

The aim of constructing tissues or regenerating tissues is achieved by tissue engineering. The following three components are required to fabricate artificial tissues.

15.8.1.1 Scaffold

The scaffold is an artificial three-dimensional structure that carries cells and acts as their microenvironment. It mainly contains proteoglycans and glycoproteins that serve as a simulation of the extracellular matrix in a human cell. Scaffold structures should be made of biodegradable materials like collagen and some polyesters. Generally, polylactic acid is used as a scaffold material. To produce an ideal scaffold, it should possess precise regulation of macrostructural (e.g., mechanical strength, spatial form, density, porosity) and microstructural (e.g., pore size, pore interconnectivity, pore distribution) features (Leong et al. 2003). Micropores are

necessary for the growth of cells, diffusion of nutrients, and waste. It has been seen that space for blood vessels is imperative and exhibits the limiting factor in scaffold (Chen et al. 2006).

15.8.1.2 Cells

Cells are essential components that fill the empty scaffold and convert it into the target tissue. Currently, the cells available can be classified in several ways. The simplest way is to classify as follows: (1) allogenic cells (cells from the same person), (2) autologous cells (cells from the same individuals), and (3) xenogenic cells (cells from another species) (Hasirci and Hasirci 2018). The other method has classified cells into five types, which are available for tissue engineering (Lanza et al. 2020).

(a) Adult stem cells: These cells are isolated from the same person. It is a good method of production because the donor is the recipient, and rare chances of infection occur. Moreover, immune suppressive therapy is not needed during transplantation.
(b) Mesenchymal stem cells: MSCs exist in the bone marrow and are responsible for the homeostasis of musculoskeletal tissue. They have the potential to differentiate into a variety of cells that are lacking for reparation of tissue by modifying themselves. They are also the right way of replacing adult stem cells.
(c) Embryonic stem cell: ES is capable of differentiating into all somatic cells and proliferating indefinitely. However, they are less reliable due to their tumorigenic properties.
(d) Induced pluripotent stem cells (iPSC): This method is widely accepted. However, these cells are integrated into the host genome and exhibit oncogenic behavior because this type of stem cells rely on the retroviral vector.
(e) Other types of cells: Some of them are from placental tissue, umbilical cord, amniotic fluid, and umbilical cord. Unlike embryonic stem cells, it does not have tumorigenic properties.

15.8.1.3 Growth Factors

It is essential to add bioactive compounds (growth factors) that guide their attachment to the scaffold in tissue regeneration. Growth factors (GFs) are signaling molecules that provide direction for cell growth, helping in stem cell proliferation, migration, and differentiation. Nevertheless, the main limitation of growth factor selection is dosage-related adverse effects (Subbiah and Guldberg 2019).

15.8.2 Limitations

Adequate pore volume and permeability are necessary for the penetration of the cells into the scaffold. Otherwise, it would be confined within the scaffold peripheries leading to cell necrosis. Due to the lack of nutrients, the degradation of scaffold starts, resulting in long-term failure of new tissue grafts (O'Connor et al. 2020). The

thickness of artificial tissue is a significant parameter for supplying nutrients to central parts of the tissue by diffusion. This is one of the vital and large areas for developing angiogenesis in tissue engineering (TE) (Shafiee and Atala 2017). The drawback of TE is a deficiency of donor organs for people who are waiting for transplantation. Another drawback is that regular immunosuppressive therapy can harm the health of the recipients. In addition to the cells and scaffolds, temperature (37 °C) and growth and differentiation factors (hormones, chemical, and metabolic factors) are essential for tissue development (Castro et al. 2020).

15.8.3 3D Bioprinting and Tissue Engineering

3D bioprinting is becoming the future of tissue or organ construction. It is difficult to mimic the natural process to establish viable organs due to the slow growth and development of tissues in an individual (Kawasaki et al. 2017; Van Linthout et al. 2014). For these reasons, organ regeneration and complex tissue formation seem to be incompatible through a natural process. Therefore, the biomanufacturing of complex tissue/organ models that imitate specific tissues/organs' biological functions could help overcome organ failure or rejection (Homan et al. 2016; Pourchet et al. 2017; Neiman et al. 2015; Liu and Wang 2015). The traditional engineering systems are not useful because of their complexity. Each organ has its specific anatomical, histological, and morphological structures that perform various physiological activities (Wang et al. 2010). Thus, the 3D bioprinting technology plays an imperative role in creating external and internal tissue structures. In conventional methods, including cell transplantation, tissue repairing, the anatomy of tissue models, and regulation of vital physiological processes, relies on a self-healing attribute of the recipient cells. Today, 3D bioprinting methods are capable of fabricating tissues in vitro but still face challenges in controlling the process of a complex organ after implantation. The extracorporeal systems can help to regenerate organ function permanently. Thus, bioadditive manufacturing is focusing on engineering extracorporeal organ systems to print, fabricate, and maturation before implantation (Hartupee and Mann 2016).

15.8.4 Tissue Engineering of Different Organs

15.8.4.1 Cardiac

It becomes crucial to design functional myocardium for repairment. Alginate scaffolds can cultivate myocardium models with human cardiomyocyte progenitor cells (hCMPCs) by using 3D bioprinting (Gaetani et al. 2012). Indeed, a cardiac patch of polyesterurethane urea (PEUU) co-cultured with human umbilical vein endothelial cells (HUVECs) and human mesenchymal stem cells (hMSCs) through laser-induced forward transfer (LIFT) cell printing method has been developed. Transplantation of these patches in the infarcted region of the rat heart has shown vascularization and improved physiology of cardiac muscles. Moreover, in 2016, a

study has declared that the possibility of 3D bioprinting to recapitulate the whole heart through CAD software (Oklu et al. 2016).

15.8.4.2 Liver

Recently, liver-like microstructures have been fabricated by employing printing technology. For instance, one of the first bioprinting companies, Organovo™ constructed high cell viability and 3D vascularized liver by using bioprinting of high-density hepatocyte cells, hepatoma cells, and endothelial cells, a scaffold which resembles native hepatic lobules. On the other hand, single hepatic cells were replaced by liver spheroids, which can prevent the exerted shear stress on cells throughout the printing process. Consequently, such spheroids demonstrated constant hepatic biomarker secretion of alpha-1 antitrypsin, ceruloplasmin, transferrin, and albumin that helps in the evaluation of hepatotoxic drugs (Nguyen et al. 2015).

15.8.4.3 Cartilage Bone

PCL/nHA/PPF and PCL/nHA scaffolds were set in in the rabbit's femurs, with or without seeding of rabbit bone marrow mesenchymal stem cells (BMSCs). After 4 and 8 weeks, the evaluation was done by micro-CT, histological test, and mechanical test (Buyuksungur et al. 2017). As evaluated by micro-CT and bone mineral density, scaffolds seeded by BMSC revealed progress in bone tissue regeneration. The test results were significantly better than healthy rabbit femur and showed improvement in regeneration of bone. At present, many researchers are focusing on hybrid biomaterials for the construction of bone scaffolds and their applications. Recently, Oladapo et al. (2019) have fabricated a hybrid bone implant material with the composition of carbohydrate particles (cHA) and polylactic acid (PLA) by using the 3D bioprinting technique (Oladapo et al. 2019). As a result, there is a surge in the multiplicity of components and usage of biomaterials by a combination (Beheshtizadeh et al. 2020).

15.9 Organ-on-Chip

These are the micro-engineered biomimetic systems that combine the ideas of microfluidics, tissue engineering, and diagnosis that can simulate living tissues more closely. It generally consists of transparent 3D polymeric microchannels bound by mammalian cells and replicates three essential aspects of printed organs, which can be employed as remarkable in vitro models for biological activity. Various steps are involved in the printing of organ-on-chip platform (Esch et al. 2015) (Fig. 15.2).

Modification of 3D bioprinting technology with specific cell pattern enables function, tissue regeneration, and high rate of production (organs-on-a-chip) which opens the wide range of research on the organ-on-chip (Park et al. 2018a).

In one such study, living soft tissue was fabricated by using a bioink which combines the shear thinning properties of nanofibrillated cellulose (NFC) and alginate (cross-linker). Moreover, MRI and CT images were used as blueprints to

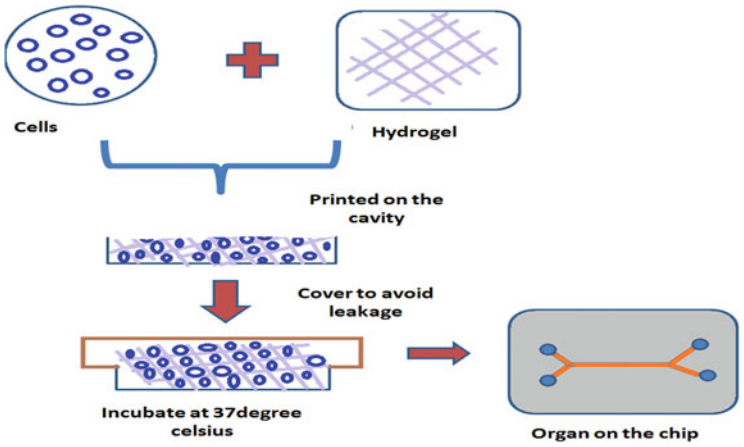

Fig. 15.2 Schematic representation of the organ-on-chip

design an atomically shaped human ear and sheep meniscus (cartilage structures) (Markstedt et al. 2015). In another study, Choi et al. (2016) concluded that extracellular muscle matrix (mdECM)-based bioink and bioprinting technology mimicked the functional and structural characteristics of native muscle. This provides new hope for the treatment of muscular injuries. The printed structure is known to enhance myogenic differentiation with high cell viability, contractility, and maturation (Choi et al. 2016). These in vitro tissue models serve as an alternative source in multiple fields, for example, toxicological, drug discovery, and micro-level physiological studies.

15.9.1 Applications of Organ-on-Chip

The main objective of an organ-on a chip is to fabricate a functional unit of the required organ instead of the entire organ that mimics human physiology. This technology can be used for the engineering of tissues that can be used on a large scale for human cell testing and organoids in the integrated fluidic environment (Fetah et al. 2019). It can also be used for the assessment of toxicology parameters in the drug development process where the two-dimensional (2D) cell culture fails to precisely imitate the physiological environment of intra-organ interactions (Wu et al. 2020). So, we can evaluate the impact of drugs upon disease condition in real-time.

15.9.1.1 Liver-on-the-Chip

The liver is the central core of xenobiotic metabolism and is more prone to drug and cosmetic toxicity. During the hepatic injury, liver cirrhosis is encountered in later stages due to the aggregation of collagen-like extracellular matrix proteins from the hepatic stellate cells (HSCs), which are intermediated by numerous pathways within the liver parenchyma. By this technology, human co-culture models are developed to

test hepatotoxicity (Leite et al. 2020). The sinusoid is the functional unit of the liver which contains different cells of the liver. It has bile duct cells, hepatic artery, and vein, which perform respective functions such as bile removal, oxygen transport, and toxin removal. Ultimately, several strategies have been printed to mimic the functions of an organ on an organ chip (Radhakrishnan et al. 2020).

15.9.1.2 Tumor-on-the-Chip

Cancers are characterized by cell angiogenesis, proliferation migration, and intravasation of tumor cells. In vitro cell culture fails to replicate the complex nature of the 3D tumor microenvironment. A microfluidic platform has been established to overcome this problem, which mimics the biological tumor microenvironment for appropriate conditions to generate chemical gradients. This makes easier to study the cellular responses of biomolecules and chemicals with changing concentration during cancer metastasis (Yu and Choudhury 2019). Before loading tumor cells or tumor organoids of patients, quiescent perfused 3D microvascular structure (provide nutrients or drugs to the tumor) is printed in an adjacent compartment (Shirure et al. 2018). An easy method to design 3D tumor tissue via employing a microfluidic platform through direct cell writing has been developed. In the first step, an automatic direct cell writing (DCW) technique designs the 3D tissue; secondly, it is placed in a polydimethylsiloxane (PDMS) device engineered with soft lithography (Chang et al. 2008).

15.9.1.3 Lung-on-the-Chip

Horváth et al. (2015) reported the first bioprinted in vitro lung model. This air-blood tissue barrier model is separated by Matrigel membrane, which is made of an epithelial cell layer and endothelial cell layer, printed on porous membrane substrate with a bottom-up approach (Horváth et al. 2015). Decellularized extracellular matrix bioink was obtained from porcine tracheal mucosa (tmdECM), which enclosed fibroblasts and endothelial cells into the polycaprolactone (PCL) frame for printing. From the physiological point of view, this model presents respiratory symptoms such as allergen-induced asthma exacerbation and asthmatic airway inflammation. As 3D bioprinting possesses adaptable nature, it is expected that this will offer a wide platform for preclinical research studies (Park et al. 2018b).

15.10 3D Bioprinting of Medical Devices

3D bioprinting offers many advantages in the area of healthcare and medical devices. The surgeon can use the 3D model of the desired patient anatomy to help MRI or CT scan images for the surgical approach (Aimar et al. 2019). Further enhancement in additive manufacturing technology enables the construction of patient-specific implants from 3D imaging data (Ahangar et al. 2019). The FDA-approved 3D printed titanium device (*FastForward Bone Tether Plate*) represents a novel approach in treating hallux valgus deformities (FDA 2016).

15.11 3D Bioprinting for Improved Drug Delivery

3D bioprinting has proven to have a disruptive effect on drug delivery systems as this technology has potentially promoted the creativity in fabricating the solid oral dosage form with complex structures, various geometries, torture channels, porosity gradients, and multi-compartment systems, for instance, polypills that consist of multiple active pharmaceutical ingredient in unit dosage form for control release of drug (Samiei 2020). Goyanes et al. (2015) evaluated the drug release property of 3D printed tablets on the basis of geometry and found that printed geometry of acetaminophen tablets resulted in different rate of drug release pattern with high degree of personalization. In this study, acetaminophen-loaded filaments of PVA (polyvinyl alcohol) were produced by taking an aqueous solution of paracetamol (2% w/w) in small pieces of PVA with Varicut (plasticizer). A single-screw filament extruder was printed in various shapes such as a pyramid, cube, cylinder, and sphere. The pyramid-shaped tablet of acetaminophen has the fastest release rate as compared to the cylindrical shaped tablet because the former had the largest surface area-to-volume ratio (Goyanes et al. 2015). Moreover, Spirtam® (levapiracetam) is an immediate-release pyramid-shaped tablet manufactured by powder bed binding method that decreases the lag time for the onset of action because a high amount of the drug is present for absorption through the oral mucosa (Jamróz et al. 2018).

FDM technology was used to enhance the dissolution rate and bioavailability of poorly soluble drug "domperidone." The conclusion of the study was that the formulation of the 3D tablet could disintegrate slowly and offer a sustained release rate with a floating ability up to 10 h both in vivo and in vitro (Chai et al. 2017). Apart from this, inkjet-based printing systems and nozzle-based deposition systems that rely on natural products have been used for drug delivery systems (Aguilar-de-Leyva et al. 2020). 3D printed implant materials are used for gynecologic and obstetric applications to elute progesterone or estrogen that are entrapped within polycaprolactone (PCL) biodegradable polymer. These implants show extended hormonal release for a period of 1 week (Tappa et al. 2017).

15.12 Disadvantages of 3D Bioprinting

Limitations of raw material: currently, 3D printers can employ approximately 100 different raw materials, which is significantly less than conventional systems. Specifically, the designing of a complex native structure depends on 3D bioprinting, which is still lagging due to the lack of appropriate bioinks with high printability, biocompatibility, and mechanical properties (Noor et al. 2019). Low resolution is caused by inkjet and extrusion-based bioprinting, which is imposed by the physical confinement of the nozzles. Thus, appropriate nozzle size variation is imperative to enhance printing speed, resolution, and compatibility for biomaterials (Xie et al. 2020). Limitations of size: The 3D printing technique is restricted by size limitations. 3D printers are still not capable of printing very large objects. The development of microvasculature plays a pivotal role in restoring the high cell viability of printed

organs for long-term survival. However, using 3D bioprinting to fabricate a similar native vascular network is still inadequate because the size of the bioprinted tissues is larger than tens of micrometers (Yu et al. 2020). Cost of printers: There is less probability of purchasing a 3D printer by the average householder. Moreover, printing different types of objects require different types of 3D printers. Even printing of colored objects is costlier than the printing of monochrome objects. Hence, the average population would not be able to afford such expensive treatment. Limited manufacturing jobs: It is an undeniable fact that advancement in technologies leads to a decrease in manufacturing jobs. This drawback can have a heavy impact on the economies of many countries (Ghadage et al. 2019). Unchecked production of dangerous items: One of the major concerns is that the ease of understanding of 3D bioprinting could lead to the undisciplined fabrication of tissues without ethics. Another disadvantage is that it may potentially be used to construct a bioweapon for bioterrorism that endangers people's lives (Gokhare et al. 2017).

15.13 Conclusion

The chapter encompasses the concept of 3D bioprinting, history, bioinks, type of bioprinting methods, and its varied applications in different research areas. Currently, 3D bioprinting is in the initial stage of development and has generated some notable results by creating a variety of functional tissues. Bioprinting enables the equal distribution of cells throughout a scaffold, but it is still under progress and has to cross multiple obstacles before entering the clinical world, especially for in situ direct use. Various challenges have to be tackled out, which include suitable cell and material selection, tissue maturation, and appropriate vascularization. Major hurdles are biomechanics, sterilized environment, selection of stent material, and scaffolds. 3D bioprinting has shown significant advancement and has garnered the immense interest of the research community. Bioprinting will continue to evolve and develop and will transform the area of organ transplantation.

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Virtual Planning, Rapid Prototyping and 3D Printing in Orthopedic Surgery

16

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Abstract

Any orthopaedic surgical procedure requires meticulous planning and execution for better outcomes; investigative tools like radiographs and computed topography have been in vogue for understanding anatomy, injuries and deformities. With the advent of 3D printing into the medical field, a new dimension for pre-operative planning, prototyping, designing, and manufacturing related to orthopaedic surgeries have developed. The present chapter explores and highlights these aspects of utilisation of 3D printing in the field of orthopaedics, with specific focus on arthroplasty, trauma, musculoskeletal oncology and spine.

Keywords

3D printing · Rapid prototyping · Orthopaedics · Arthroplasty · Pre-operative planning · Additive manufacturing

16.1 Introduction

'Failing to plan is planning to fail'.

This famous quote by Benjamin Franklin sums up the essence and necessity of planning. Nowhere is this adage inherently more suited than in orthopedic surgical procedures. Orthopedic surgical procedures are complex and involve restoration of anatomy after trauma, correction of anatomy in case of deformities and augmentation or replacement of bony and soft tissue structures after infection, arthritis, tumours and even trauma.

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Meticulous planning is key to any surgical procedure and has been the cornerstone of orthopedic surgical procedures. Until the mid-1970s, plain radiographs were perhaps the only tools available to orthopedic surgeons for planning. The 'disruptive innovation' in medical imaging came with the invention of computed tomography (CT), by a British engineer, Gregory Hounsfield (Rubin 2014). Whole body CT scans became available in the early 1980s and resulted in a paradigm shift in the way human anatomy could be assessed. Orthopedic surgeons could now have a three-dimensional understanding of even the most complex of injuries and deformities. It was around the same time, in early 1980s, that two outstanding scientists started laying down the foundations of rapid prototyping. Dr. Hideo Kodama from Japan created the first ever 3D printed model, whereas Dr. Charles Hull from the United States described stereolithography (Lengua 2017). Whereas rapid prototyping was initially geared towards industrial design, it has slowly but steadily found its way into the medical field. In a significant evolution from planning on radiographs, orthopedic surgeons have moved to virtual planning, rapid prototyping and 3D printing.

16.2 Key Concepts

(a) *Virtual surgical planning* refers to the use of two- or three-dimensional imaging techniques to evaluate complex anatomy. In the orthopedic scenario, this typically involves planning on digital radiographs, CT scans or MRI scans, although other imaging modalities such as ultrasonography and nuclear medicine scans may also be used (Fig. 16.1).

(b) *Rapid prototyping:* In engineering terms, a *prototype* is a model or a replica of a final product or design. These are used to analyse complex designs and test key concepts before a final finished version is created.

Prototypes can be classified as 'low fidelity', which can be produced very quickly and are used to test a broad concept. Examples include sketches or crude models. On the other hand, a 'high fidelity' prototype is very close to the finished product, both in terms of structure and function.

'Rapid prototyping', as the name suggests, is a process by which prototypes, both low and high fidelity, can be created rapidly. This can be achieved by a number of methods and can include virtual (computer based) as well as physical prototypes.

(c) *Computer-aided design (CAD)* refers to the use of computer software to create designs or virtual models, which can be two- or three-dimensional.

(d) *3D printing/additive manufacturing: 3D (three-dimensional) printing*, also known as *additive manufacturing*, is a process by which a three-dimensional object can be created or 'printed' by adding material in layers. There many methods by which additive manufacturing can be performed, but the most commonly used method is 'fused deposition modelling' (FDM). Here, a thermoplastic material filament such as ABS (acrylonitrile butadiene styrene) is passed through a heated nozzle. The melted plastic is deposited on to a platform

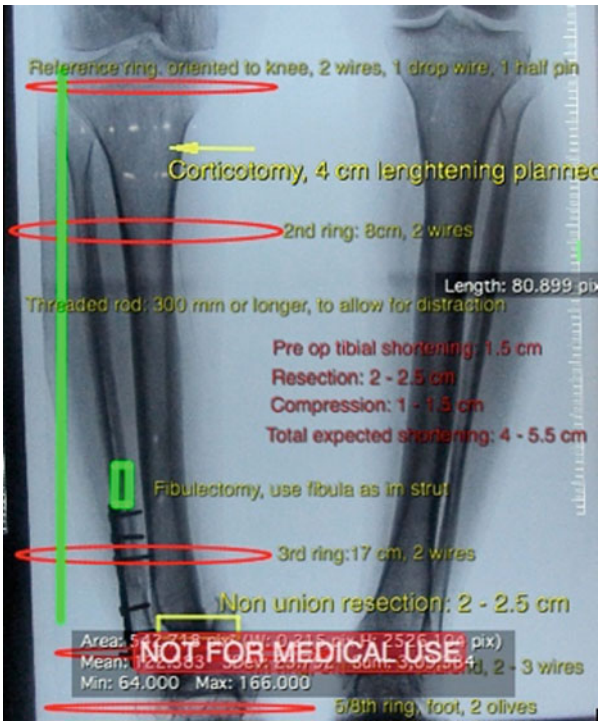

Fig. 16.1 An illustrative example of 2D virtual surgical planning on plain radiographs for reconstruction in patient with non-union of the tibia. The OsiriX Lite software package was used

layer by layer to create the 3D object. Although the terms *rapid prototyping* and *3D printing* are used interchangeably, it must be remembered that 3D printing can be used to create both rapid prototypes and final finished products.

16.3 Virtual Surgical Planning in Orthopedics

Virtual surgical planning is the norm in most orthopedic centres in developed nations. With the decreasing costs of computer-based technologies, it has also started to find its way into the tertiary care centres of many developing nations. Many free-to-use as well as paid software are also available that can help orthopedic surgeons perform virtual planning from their personal computers. A few examples of how virtual surgical planning is used in orthopedics are outlined below:

(a) *Virtual templating in joint replacement*: Joint replacement surgery (arthroplasty) involves cutting bones, termed as 'osteotomy', and implant placement. Templating is a planning process which includes, but is not limited to, planning the site of osteotomy and deciding how much bone to cut and remove and which size and type of implants to use. Templating is routinely performed for joint replacement surgeries (arthroplasty) (Si et al. 2015), but can be also used for practically any orthopedic surgical procedure. Of late, 3D templating on CT

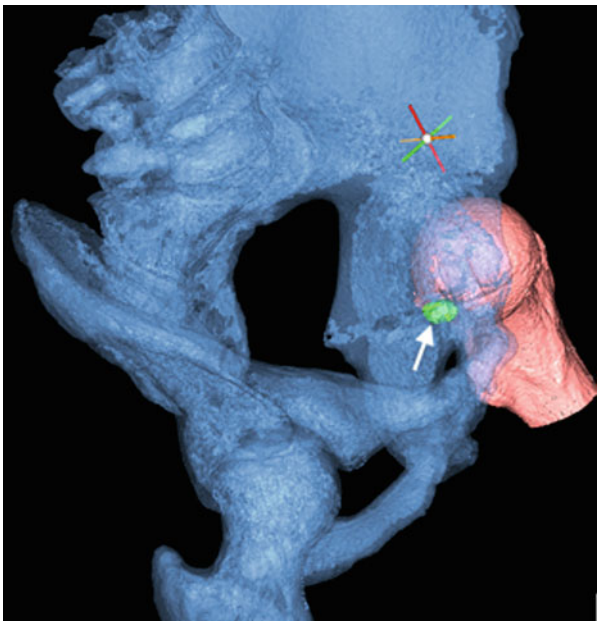

Fig. 16.2 The importance of 3D visualization in surgical planning. This 30-year-old male has a bullet (white arrow, green colour) lodged in his hip joint. Accurate localization of the bullet, which was made possible by advanced 3D visualization techniques as demonstrated here, is essential before the surgeon can attempt to remove it

scans has generated significant interest, though there is no evidence to suggest that 3D templating improves clinical outcomes, over 2D templating; however, it may have benefit in difficult cases (Colombi et al. 2019). The question then arises, how accurate is such planning? Holzer et al. (2019) in a study on virtual templating in uncemented total hip replacement showed that the exact implant size was predicted in 47% of the cases for the femoral stem and 37% of the cases for the acetabular cup. Furthermore, 87% of the femoral stem and 78% of the acetabular cups were within one size predicted by virtual templating. A fact to note is that templating was more accurate when performed by senior surgeons and more inaccurate in patients with obesity.

(b) *3D visualization*: Some regions of the human body, e.g. the spine, pelvis, joint surfaces and the small bones of the hands and feet, have complex three-dimensional anatomy (Wang et al. 2016; Mishra et al. 2019). It is often difficult for the average orthopedic surgeon to understand the three-dimensional pathoanatomy of fractures and deformity of such regions, which is essential in planning surgery in these situations (Fig. 16.2).
There are many PACS (picture archiving and communication system) software as well as CAD (computer-aided design) software that allow virtual 3D models to be created from axial slices of CT scans. Hence, such models have become an important tool for planning (Fig. 16.3). However, it must be remembered that 3D models may not be accurate for measurements, and therefore it is better to perform measurements on CT sections rather than 3D rendered models.

(c) *Long bone deformity correction*: Planning for correction of long bone deformities involves measurement of a number of parameters, which include

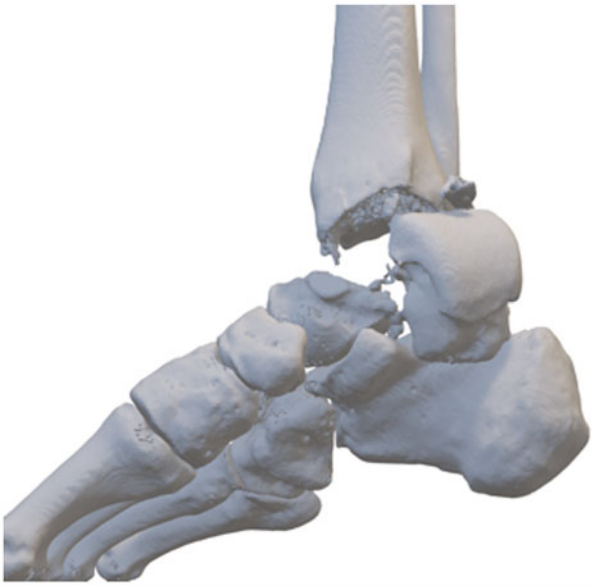

Fig. 16.3 A virtual 3D model of a patient's foot with talus fracture, which was created from CT scan sections. Such models help the surgeon to visualize and plan the fracture surgery from a 3D perspective

the overall alignment of limb (mechanical axis deviation), estimation of joint orientation angles at the proximal and distal ends of the bones as well as identifying and quantifying the apex, or the centre of rotation of angulation (CORA) of the deformity. Although this can be done on conventional radiographs, there are a few commercially available software that allow the surgeon to rapidly calculate these parameters (He et al. 2020; Hung et al. 2019; Whitaker et al. 2016). Some of them also have the option to perform the bone cut (osteotomy) 'virtually' and visualize the correction. This allows the surgeon to plan the osteotomy accurately and also enables the patients to visualize how the limb would appear after correction.

(d) *Virtual and augmented reality*: Virtual reality and augmented reality are two technologies that allow a person to visualize objects in the virtual environment. Whereas in *virtual reality (VR)*, the entire visualization is in a virtual medium, *augmented reality (AR)* allows an image to be superimposed on to a real-life image from a camera. Although these technologies have been around for a while, their routine use in orthopedic surgeries is in a nascent stage. Typical applications of AR could include guidance for placement of implants in the spine, pelvis, joint replacement or tumour resection (Jud et al. 2020).

16.4 Surgical Applications of 3D Printing

3D printing as a marvel of biomedical engineering and computer programming has been utilized in different orthopedic sub-disciplines like arthroplasty, pelvic acetabular/periarticular fractures and spine and musculoskeletal oncology. It focusses on

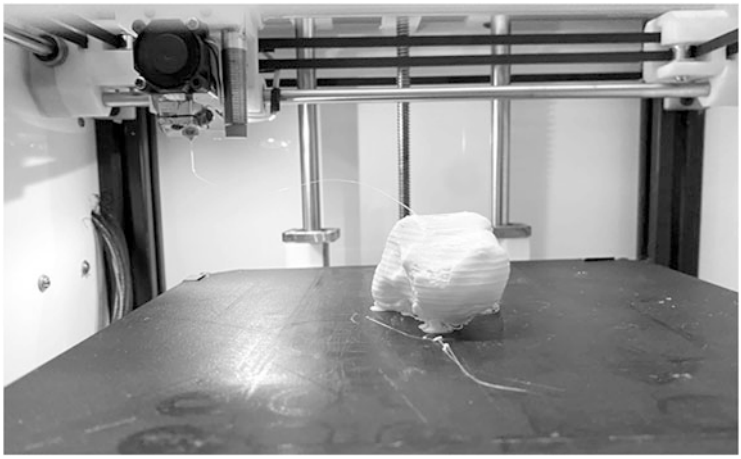

Fig. 16.4 A life-size 3D printed talus sits on the build plate of the authors' desktop FDM 3D printer. Such printers are being increasingly used for surgical planning

specific nuances of the musculoskeletal system and generates models which closely represent the anatomy specific to a patient. With the advent of technology, 'desktop' 3D printers are also readily available; these are being increasingly used for generating 3D models for surgical planning (Fig. 16.4).

3DP has demonstrated success in different phases of surgical training and patient care in the abovementioned sub-specialities and has been an evolution from preoperative planning to patient-specific instruments (PSI) and customized implants.

16.4.1 3D Printing and Arthroplasty

The hip and knee are the commonest regions where arthroplasties are done due to the high success rates in these joints. Primary total hip arthroplasties/replacements (THA/THR) in specific cases with distorted acetabulum or femoral head anatomy resulting from previous trauma, infection or dysplasia require a challenging recreation of native biomechanics in terms of offsets to provide optimal stability and limb lengths (Xu et al. 2015). Revision surgeries on the other hand could present with inadequate bone stock due to the primary surgery and osteolysis or infection creating complex anatomical defects (Zerr et al. 2016).

In knee arthroplasty certain factors increase the life of implants, implant design and material, cementing techniques and post-operative knee alignments (Gemalmaz et al. 2019). Post-operative knee malalignment has been established as a definite cause of shorter lifespan of the prosthesis.

There has been a huge spike in the recent literature on 3D printing in these areas of hip and knee arthroplasties, with specific focus being on patient-specific instruments and individualized implants.

3D printed customized cages in revision THAs with severe bony defects have been shown to provide stability and improved functional scores at 5.5 years of mean follow-up (Li et al. 2016). Similar results were shown by Mao et al. and Kieser et al. in a small series of cases (Mao et al. 2015; Kieser et al. 2018). Comparative trials have shown that 3D printed acetabular components provide earlier weight bearing and better functional outcomes as compared to conventional surgeries (Wang et al. 2017a).

Studies have shown that acetabular ante version achieved is closer to the safe zone with 3D printed patient-specific instruments and 3DP provides better accuracy of component positioning in THR (Small et al. 2014). For femoral neck cuts as well, 3DP has been shown to provide more accuracy (within 3 mm of the planned level) (Schneider et al. 2018).

Ogura et al. (2019) documented 92% survival rates of 3D printed customized knee prostheses in 59 cases after 5 years, while Sultan et al. showed 99% survival of a cement less design after 3 years of surgery with excellent pain relief and functional scores (Sultan et al. 2020).

However, in total knee replacements, the utility of PSI is controversial with contradictory results described in the literature. Sun et al. in their randomized study found no difference in post-operative knee alignments, range of motion and knee functional scores between 3D printed PSI and conventional surgeries (Sun et al. 2020). On the other hand, improved post-operative alignments with precise cuts, providing better functional scores, have been demonstrated in patients operated with 3D printed instrumentations (Qiu et al. 2017). For addressing periprosthetic joint infections, 3D printed liners have been described which are made of polylactic acid and are stronger and more ductile than the standard polymethylmethacrylate (PMMA) used conventionally. They have been shown to elude antibiotics in a sustained and controlled manner (Kim et al. 2017).

Overall 3DP has made massive strides in the field of complex primary and revision arthroplasty; however these new technologies need a more comprehensive testing over a longer run before assessing their cost-effectiveness and wider application.

16.4.2 3D Printing and Trauma

Trauma surgeries have witnessed an increased complexity of the fracture patterns, owing to the increasing high energy modes involved; more traffic and congested roads have increased the number of road traffic accidents in the urban areas. Specific trauma subsets like pelvic acetabular and periarticular fractures have been studied for assessing the utility of 3DP.

A recent meta-analysis assessed the use of 3DP in preoperative planning of fractures which included hip, pelvi-acetabular, ankle and intra-articular fractures at the tibial plateau, proximal humerus and elbow (Morgan et al. 2020). Across 17 included studies, it was concluded that 3DP usage reduced the overall surgical time, intra-operative blood loss and utilization of intra-operative fluoroscopy.

Pelvi-acetabular fractures are a result of high-energy trauma, and certain level of skills and expertise are required to fix these. The most commonly reported method is printing of two models; the fractured and the normal side. They provide the surgeon a tactile impression of the fracture fragments, and a mirror image of the intact side helps in determining the amount of pre-contouring needed for fixation. Hung et al. (2018) reported a 70-min reduction in average surgical duration and better radiological outcomes with this method. Similar results were shown by Zhang et al. (2018) with significant reduction in the mal-reduction of the fragments with this 3DP-based planning, when compared to conventional planning.

This approach is specifically useful in minimally invasive surgeries wherein percutaneous screws are inserted for unstable pelvic fractures. Cai et al. (2018) in their retrospective study compared 65 patients in whom preoperative 3D models were used for surgical simulation, with 72 patients with conventional X-ray and CT-based planning. They showed decrease in duration of surgery as well as number of fluoroscopy shots. The reduction and functional outcomes were comparable in both the groups, highlighting the potential of 3DP in such cases.

The use of patient-specific anterior plates to treat acetabular fractures has shown decreased surgical duration and blood loss when conventionally fixed implants were compared to the 3DP group. There was however no difference in reduction quality and function between the groups (Wu et al. 2020).

Periarticular fractures are other trauma subsets where 3DP has been utilized effectively. Bizzoto et al. (2015) described 3DP replica of articular fractures; areas like the distal radius, radial head, tibial plateau, calcaneus, ankle, etc. can be aided immensely by using these innovations in the evaluation of joint fragmentation and test suitable implants and screw length/orientation (Fig. 16.5).

In pilon fractures, the utility of preoperative planning and surgical simulation using 3D printed models has been evaluated and showed lesser surgical duration and blood loss, with higher rates of anatomical reduction with superior function, when compared to conventional surgeries (Zheng et al. 2018).

Similar results of decreased operative time and blood loss with preoperative planning and simulation has also been described in distal radius fractures.

To conclude, in trauma scenarios, 3DP has a potential role mainly in preoperative planning and surgical simulation to facilitate faster surgeries and adequate function. This becomes important in centres with high patient footfall so as to utilize the OR schedule more effectively with more turnover of cases.

16.4.3 3D Printing and Musculoskeletal Oncology

Surgical resection of bone tumours require precise cuts and osteotomies for tumour-free margins; this has been shown to be difficult especially in regions like the pelvis and spine despite conventional preoperative planning and experience of the surgeons (Thadani et al. 2018). 3DP allows creation of accurate geometrical replicas of a patient's tumour, and studies have shown that such models aid immensely in subsequent surgeries. The preoperative planning improves, and 3D printing could

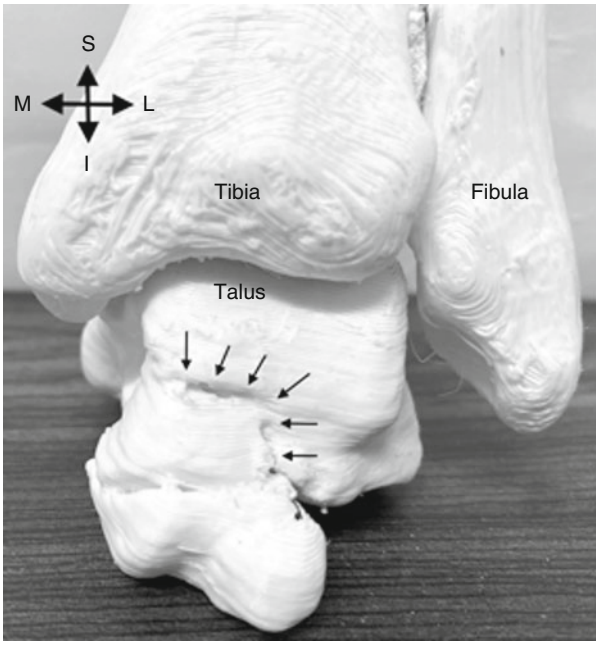

Fig. 16.5 A 3D printed model of the ankle joint in a patient with fracture of the posterior process of talus. The arrows demonstrate the fracture line (*S* superior, *I* inferior, *M* medial, *L* lateral)

also be used to manufacture resection guiding templates to facilitate intra-operative precise cuts for tumour-free margins. Utilization of 3D printing in osteosarcoma resection decreases blood loss, intra-operative fluoroscopy and surgical duration (Ma et al. 2016).

Utilizing 3D printed patient-specific resection jigs have been shown to give cuts that differed less than 1 mm from the preoperative plan (Wong et al. 2012). This difference in conventional surgeries have been shown to be up to 9 mm in cadaveric studies (Thadani et al. 2018). Comparative studies utilizing PSI in tumour resections and comparing them with conventional surgeries have shown that although the surgical duration did not differ, intra-operative blood loss, complication rates (infection, delayed healing, loosening, fracture, etc.) and the functional score of the knee joint statistically favoured the 3DP group (Thadani et al. 2018; Wang et al. 2017b).

When patients' anatomy is not suitable due to distortion and destruction by the tumour, for standard implants, customisation becomes the need of the hour; this is where 3DP has a potential role to play. Luo et al. (2017) demonstrated this technology in four cases of giant cell tumours of the proximal tibia. They designed a customized block which fitted well in the defect after resection of the tumour, and in combination with standard knee prosthesis, the authors achieved good soft tissue balance and knee stability with adequate range of motion, after average follow-up of 7 months. 3DP helps simplify the design and manufacture process of customized prosthesis for tumour surgeries, in complex situations like the pelvis, and it allows precise cuts and better implant position (Thadani et al. 2018).

This was demonstrated by Wang et al. (2018) in pelvi-acetabular bone tumours using 3DP hemipelvic prostheses, and at mean follow-up of 15 months, the functional scores were good with no abnormal radiological signs of loosening and resorption.

Metal bioscaffolds can be manufactured by 3DP, and they have excellent resistance to fatigue; these can be used to fill cortical weight bearing defects. Iron-based composite scaffold with $CaSiO_3$ have also been created, which have been shown to be a promising modality for tumour phototherapy via production of reactive oxygen species, causing lipid oxidation and DNA damage inside tumour cells and regeneration of bone defects by stimulating vascularisation and bone formation (Ma et al. 2018).

3DP also aids in altering the prosthetic material properties and porosity to match the bone that could allow better incorporation and longevity.

Microwave ablation is an accepted modality for bone tumours; however the irradiation tends to create bone defects and increased susceptibility to fractures. Ma et al. (2017) invented a 3D printed titanium plate customized to the patients' anatomy, which they implanted to assist the reconstruction in cases treated with microwave-induced tumour resection. They showed that there were no cases of fractures in the early post-operative stage in their patients and suggested that customized plates as per the region affected by the tumour provided a more rigid fixation as compared to conventional plates.

Overall, although 3DP has shown promising results, evidence is mainly available in forms of case reports and small series; further larger studies are warranted to ascertain the benefits of 3DP in comparison to conventional tumour surgeries.

16.4.4 3D Printing and the Spine

3DP has been described as a potential tool that could transform spine surgery in the near future (Cho et al. 2018). Its ability to accurately manufacture and represent anatomical nuances of spine has been validated. In the field of spine surgery, rapid prototyping has been utilized for spinal tumours, lumbar discectomies in failed back syndrome, spinal deformity correction as well as craniovertebral surgeries. It facilitates studying the pedicle sizes and facet joint morphology as well as preoperative planning in terms of calculation of screw and plate sizes and angles. Studies have shown 3DP to be advantageous in these scenarios, with decreased surgical duration, lesser blood loss and better reduction of vertebral dislocation (Cho et al. 2018). 3DP has also been utilized in minimally invasive spine surgeries, but this continues to be in a nascent stage (Garg and Mehta 2018).

Cervical spine tumours like chondrosarcoma and chordomas have been successfully resected with no local recurrence or instrumentation failure, by surgical planning on 3D printed models which allow better understanding of the anatomy of both the tumour and the cervical spine (Xiao et al. 2016).

Scoliosis surgeries utilizing 3DP have been shown to be shorter with lesser blood loss; however there seems to be limited advantage in terms of complication rates and

outcomes as compared to conventional methods. This does raise doubts in application of 3DP extensively in deformity correction, and more high-quality studies are needed to ascertain its overall efficacy in preoperative planning (Cho et al. 2018; Garg and Mehta 2018).

3DP has been used to manufacture drill guides for insertion of pedicle screws. It specifically becomes advantageous in cervical spine region where the pedicle size and narrow clearance present increased risk of iatrogenic damage in conventional surgeries. 3DP allows better precision of screw insertion and reduced iatrogenic complications with studies showing reduction in surgical time and radiation (Garg and Mehta 2018). The latter advantages hold true even at thoracic and lumbar levels, where screw placement is not as big an issue with conventional methods (Chen et al. 2015).

Customized implants in spine surgeries have been utilized in spinal tumours for reconstruction as well as for creation of individualized cages, screws and plates for fusion surgeries (Cho et al. 2018). Such designs tend to provide better stabilization and load bearing with decreased complications like implant subsidence and neuromuscular damage, along with better deformity correction.

3D printed vertebral bodies have been developed and reportedly used after anterior corpectomy in 6 cases of cervical spondylosis-induced myelopathy (Amelot et al. 2018). 3D printed intervertebral discs have also been described to mimic the viscoelastic properties of the natural disc, and they could see the light of the day in the near future. Various drug delivery devices have been described in forms of capsules, suspensions and scaffolds which could use fruitful in pyogenic or tubercular infections (Garg and Mehta 2018).

Overall, 3DP in spine surgeries have a potential role which needs to be further explored as the evidence is scarce and restricted to case reports and small series with low quality of evidence. Larger trials are needed to weigh in the production cost and required time against probable improvements in surgical precision, duration and patient-related outcomes.

16.5 Conclusion

Advances in science and innovations in technology have helped the medical arena tremendously; visualization of injuries and proposed solutions using virtual planning, leading to rapid prototyping, has revolutionized many surgical fields. 3D printing in orthopedic surgery has evolved both in its indications and use and in the cost reduction, and it is increasingly being used in a variety of fields in modern orthopedics. There is no doubt that future innovations and cost reductions will lead to better implementation of these technologies.

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Tissue Chips: Contemporary Applications and Advancements

17

Taranjot Kaur, Jigyasa Sharma, and Seemha Rai

Abstract

The tissue chip is an efficient and highly applicable novel product developed by the amalgamation of cell biology, engineering and biomaterial technology. It is a functional organ biomimic model built on a dynamic microfluidic chip that replicates the architecture, physiology and functionality of an organ as observed in vivo. This tissue chip technology has the potential to be utilized for drug screening and safety testing during preclinical trials. The current preclinical studies use the 2-dimensional cell culture and animal models, which do not always translate into successful clinical trials. The tissue chip or micro physiological system is a far more efficient preclinical model as it is designed to mimic the human organ system in its true sense, thus increasing the success rate during human clinical trials. It also holds the potential to completely eliminate the utilization of animals as drug testing models. These systems can also be used to model diseased state, thus providing a stronger apprehension of the mechanisms behind disease pathologies and development of new therapeutic agents. This technology is the future of personalized medicine as diseased cells from the patient can directly be used to understand the disease mechanism and to develop a novel treatment for the same. This tissue chip technology has surfaced in the past two decades and has recently been selected by the global economic forum as one of the top ten developing technologies. The article will discuss the various contemporary applications of the tissue chip technology, including its use in the testing for suitable drugs against the current novel coronavirus. Although, there are still a few limitations to the organ-on-a-chip technology, it is continuously evolving as a potential model for translational research and precision medicine.

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Keywords

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17.1 Introduction

17.1.1 What Is a Tissue Chip?

Tissue chips or more commonly known as organ-on-a-chip (OOAC) are the devices that reiterate the structure, role and physiology of the organs of our body. These micro physiological systems (MPS) are designed in such a manner that allows them to position the cells (mostly stem cells) in a structurally accurate 3-dimentional (3D) formation that recapitulates the function and physiology of the various organs of the body and also elicits a response to various stimuli such as interaction with drugs, hormones, cell signalling molecules and biomechanical stimuli (Low and Tagle 2017). The major goal of an OOAC, as a biomimic apparatus, is to imitate the comprehensive environment of the human organ (Bhatia and Ingber 2014a) and simulate a biologically accurate response to external stimuli, hence bridging the gap between in vivo and in vitro data (Fig. 17.1) (Wu et al. 2020; Ramadan and Zourob 2020). These tissue chips are based on the microfluidic technology. Microfluidics is a branch of science that exactly controls and processes (10^{-9} to 10^{-18} L) fluids in the micro range (Wu et al. 2020).

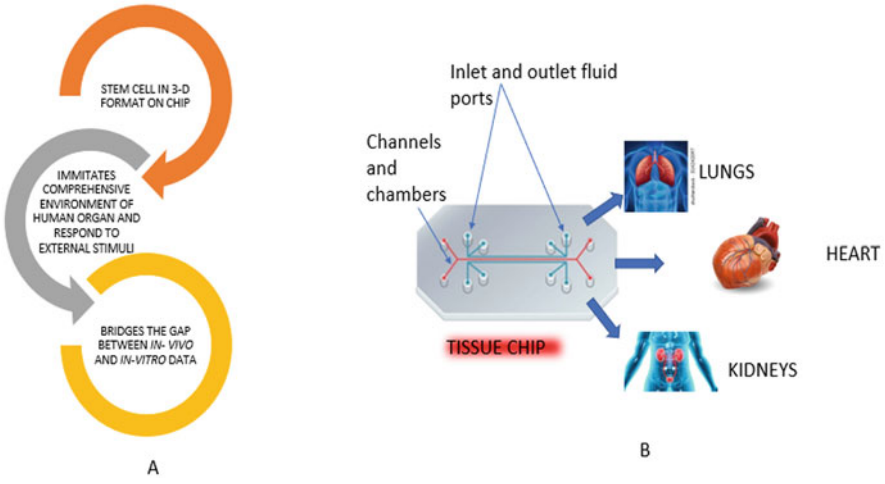

Fig. 17.1 (**a**) Major goal of an OOAC technology. (**b**) OOAC apparatus imitating the comprehensive environment of the human organ

17.1.2 Why Do We Need Tissue Chips?

It has been clearly observed that most of the newly developed drugs do not progress beyond phase 2 and phase 3 clinical trials. The average cost involved in the research and development of a new medicine is estimated to be around $2.6 billion by PhRMA (Pharmaceutical Research and Manufacturers of America). It has also been reported that for a medicine to gain approval, it takes at least 10 years starting from the initial discovery stage. The clinical success is achieved by those drugs that are able to enter the clinical trials stage, and this amounts to 12% (Scannell et al. 2012; Tufts 2014). Once a lead compound for a particular biological target has been discovered, it has to be analysed on the basis of its pharmacological properties to be able to be tested on preclinical models (Low and Tagle 2017; Mittal et al. 2019).

Currently, 2D cell cultures and animal models are being utilized as drug screening models at preclinical level. Although both these methods have some level of credibility, neither of these models adequately mimic the human physiological responses against the drug being tested (Fig. 17.2a). This leads to a higher rate of failure of drugs at a clinical trial level. Therefore, there is a need for a model system that accurately depicts the structural and functional heterogeneous environment of multiple cell types and their interactions in a human organ in 3D (Fig. 17.2b). Hence, OOAC is the required predictive tool for risk evaluation in the process of developing a drug and is a unique platform to help potent treatments succeed with more assurance (Fig. 17.2c) (Coller and Califf 2009).

Organs-on-chips are being used as tools for choosing the drug and checking the toxicity. The creation of neural microsystems has highlighted examples of tissue chip's potential use for checking the toxicity (Schwartz et al. 2015), which will be discussed in the later sections of this article. Tissue chips can also be used as an excellent platform for disease modelling. This technology is highly helpful for the patients suffering from rare diseases. It provides new ways for understanding pathologies and to discover potent therapeutics for various disorders. Diseases could be modelled on the chip using stem cells of the patient donors to induce a diseased phenotypic condition that can be observed in vitro (Azizipour et al. 2020; Low and Tagle 2016; Chen et al. 2020; Marx et al. 2016).

17.1.3 Tissue Chips: Preparation and Design

The entire process of preparation of OOAC is called soft lithography. Soft lithography is the technology used to fabricate substances composed of elastomeric materials. The first step of the process consists of photolithography of a silicon wafer which then produces a negative copy of the desired mould (Huh et al. 2013). The photoresist material is then put on the top of the silicon wafer, and uncovered region is etched by ultraviolet light. Subsequently, polydimethylsiloxane (PDMS) is dispensed into this mould, which thus creates a positive copy of the desired design. Then, this PDMS copy is sealed to a glass slide, forming closed-circuit channels (Fig. 17.3) (Bhatia and Ingber 2014b). The chip is now ready to be connected to

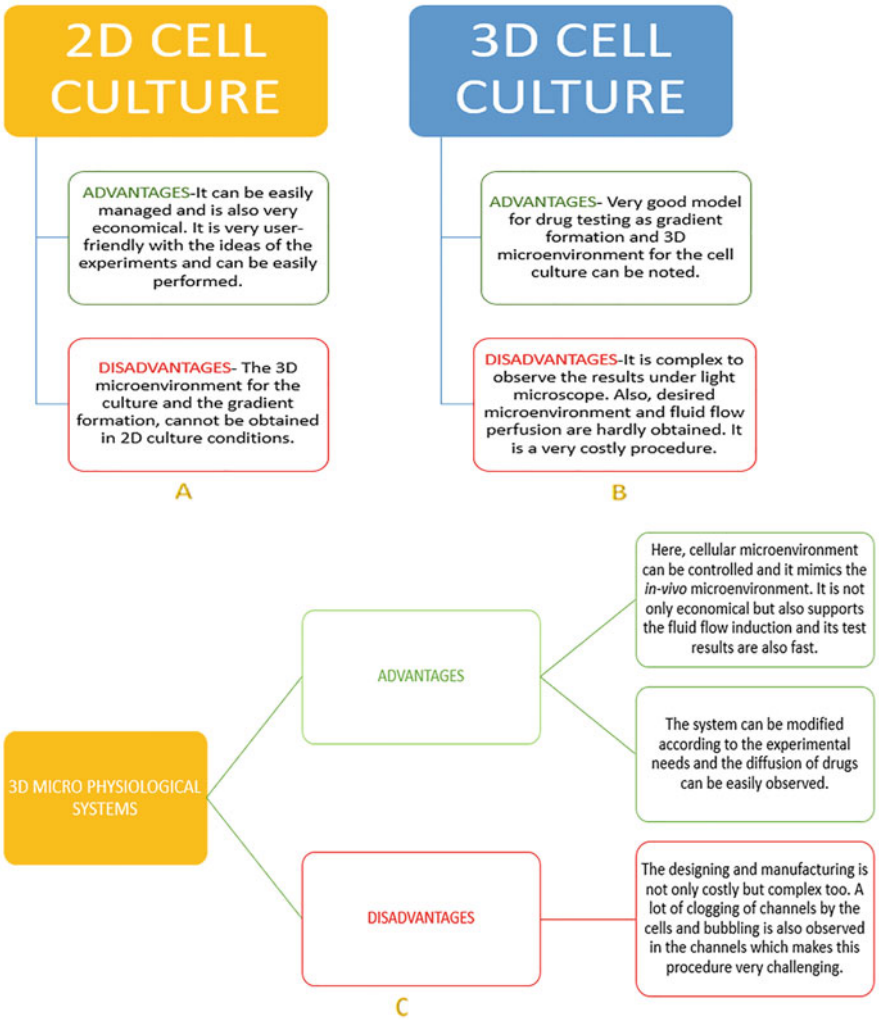

Fig. 17.2 (**a**, **b**, **c**) Comparison between the advantages and disadvantages of macroscopic 2D and 3D cell culture models and micro physiological cell culture platforms

microfluidic reservoirs and pumps using microfluidic tubing. PDMS is biocompatible, easy to mould and is transparent, therefore allowing for easy representation with photography. This makes PDMS optimum to be used in organ miniaturization (Huh et al. 2013; Bhatia and Ingber 2014b).

Further designing and mechanism of the tissue chips are dependent on the following key components:

- *Fluid shear force*: The cells cultured in a dynamic nature through micropump perfusion is enabled through microfluidics. It aids the management of nutrients

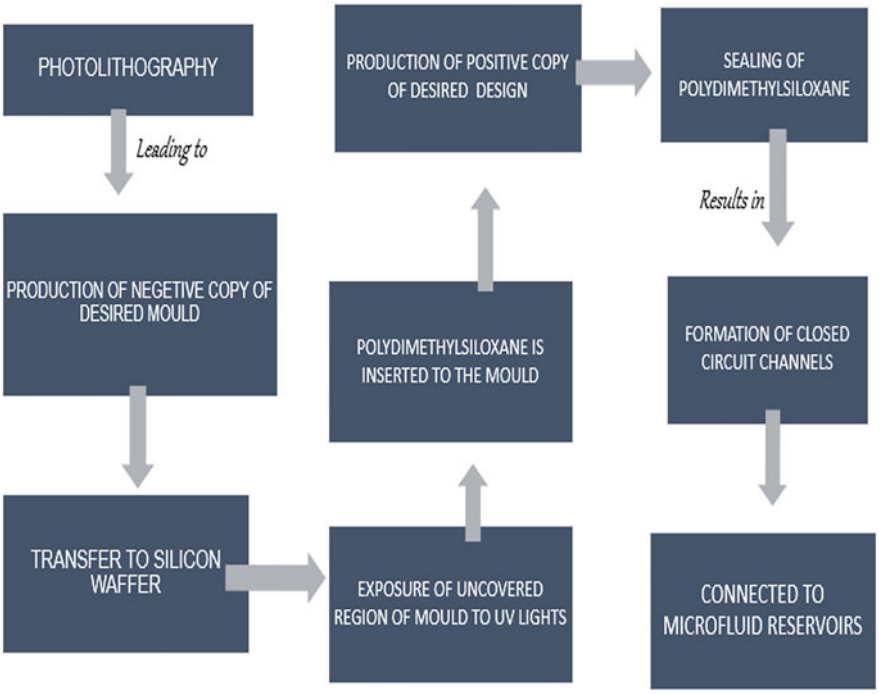

Fig. 17.3 Flow chart representing the process of *Soft Lithography*

and timely waste release on the chip. This microfluidic potent environment of the chip resembles the in vivo conditions. Also, the organ polarity is caused by fluid shear stress (Theobald et al. 2018). The flow to the organ chip system is ensured through a simple "rocker" on a chip fluid motion. The flow can also be ensured with the help of a "pulsatile" set-up (Wu et al. 2020; Ronaldson-Bouchard and Vunjak-Novakovic 2018).

- *Concentration gradient*: The fluid in the organ chips acts as a laminar flow. It leads to the flow of biological molecules in the form of a stable gradient and controls it both with regard to space and time at a microscale level. Various concentration gradient-driven biochemical signals exist in biological phenomenon such as angiogenesis, invasion and migration (Wu et al. 2020; Yang et al. 2014; Nguyen et al. 2013; Song et al. 2012).
- *Dynamic mechanical stress*: Organ pressures inside the body such as lung pressure, blood pressure and bone pressure are responsible for the maintenance of the mechanically stressed tissues (skeletal muscle, bone, cartilage, etc.) in the body (Sato and Clevers 2013; Sellgren et al. 2015). Using microfluidics, elastic porous membranes are used on the MPS to create periodic mechanical stresses. These mechanical stimulations are a major factor in determination of differentiation of cells during physiological processes on the chip (Yang et al. 2017; Kshitiz et al. 2012).

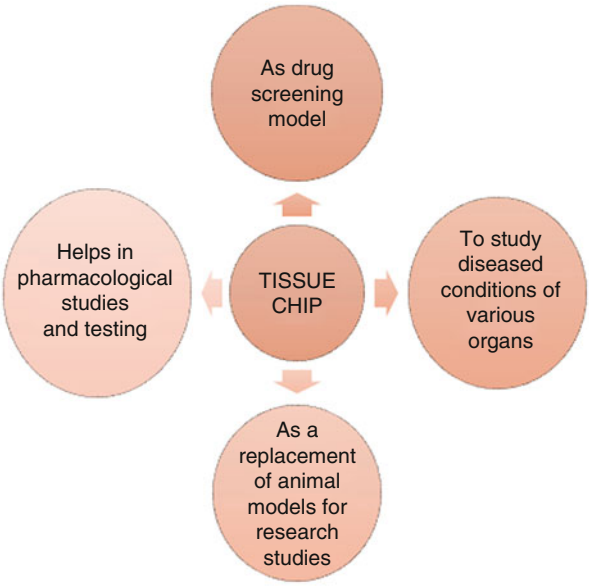

Fig. 17.4 Uses of tissue chip/Organ on a chip technology

- *Cell patterning*: In vitro micro physiological models have complex geometries. The cell patterning for these micro physiological models is controlled by microfluidics. Cell patterning on the chip is contributed by surface modifications, templates and 3D printing (Zhou et al. 2012; Tibbe et al. 2018; Xue et al. 2018). The use of 3D printing allows and enables the development of user-defined customized moulds to enhance adaptability and utility in cell patterns which are essential to recapitulate the cellular microenvironment on the tissue chip.

Tissue chips have extensive usage in drug screening and disease modelling areas (Fig. 17.4). Some of the recent applications are discussed in the following sections.

17.2 Tissue Chips as Drug Screening Model for Coronavirus and Other Viruses of the Respiratory Tract

In the light of the recent events, it has been proven that a respiratory tract virus such as SARS-CoV-2 can create havoc in the entire world and lead to a global pandemic. Respiratory tract viruses such as influenza and coronavirus are a major threat to public health and world economy. The pandemic Spanish flu in 1918, which developed due to H1N1 influenza A virus infection, is estimated to have been responsible for the deaths of 50–100 million people and lasted for 2 years. These rapidly spreading virus pandemics require rapid action, and therefore development of therapeutic treatments against the viruses of the respiratory tract is the need of the hour. Since the efficient diseased model for preclinical studies has not been developed so far, hence this is the biggest challenge in producing effective drugs against

these viruses. Currently, 2D in vitro cell cultures and animal models are being used as preclinical models that often result in failed association to human clinical trial responses. These methods are usually low throughput and do not mimic the function and physiology of the human body in its true sense. To fight the current COVID-19 pandemic, a team of researchers from Harvard University quickly leveraged the lung airway chip platform to test and repurpose the existing FDA-approved drugs as potent therapeutic molecules against SARS-CoV-2 (Tang et al. 2020).

The human airway chip is a microfluidic instrument consisting of two parallel microchannels, which are separated by a porous membrane coated with the extracellular matrix (Benam et al. 2016a, b). The air-liquid interface (ALI) of the chip is surrounded by primary human lung airway basal stem cells in the airway channel and primary human lung endothelium in the parallel vascular channel. This device leads to the differentiation of the lung airway basal stem cells. The stem cells differentiate into a mucociliary, pseudostratified epithelium along with other airway-specific cell types such as mucus-producing goblet cells, club cells, ciliated cells and basal cells. The organ chip also leads to the establishment of mucus production levels and permeability barrier properties comparable to the levels perceived in human airway in vivo (Yaghi and Dolovich 2016). The underlying human pulmonary microvascular endothelium also leads to a continuous planar cell monolayer where cells are interconnected by VE-cadherin containing adherens junctions as observed in vivo.

The differentiated epithelial cells in the airway chip are shown to express increased levels of various serine proteases such as TMPRSS2, TMPRSS4, TMPRSS11D and TMPRSS11E (DESC1) in comparison to MDCK cells that are generally tried to investigate influenza infection in vitro. These serine proteases are found to play an important role in the stimulation and spread of influenza and SARS-CoV-2 viruses in the human body. Moreover, differentiation of the airway basal stem cells at an ALI on-chip leads to overexpression of ACE-2 protein and mRNA of ACE-2 (angiotensin converting enzyme-2), which acts as a receptor for SARS-CoV-2 (Hoffmann et al. 2020).

Upon the infection of GFP-labelled H1N1 influenza virus into the air channel of the human airway chip to recapitulate the airborne influenza in vivo, it was observed through real-time fluorescence microscopic analysis that the virus has infected the airway epithelial cells. This resulted in damage to the epithelium, including absence of apical cilia, reduced barrier function as well as disruption of tight junctions. On the other hand, significantly less infection was observed in undifferentiated airway basal epithelium prior to being cultured on the airway chip. The lung endothelium also got damaged on the airway chip and was characterized by absence of VE-cadherin. The disruption was found to be consonant with the blood vessel leakage that is observed during an influenza infection in human lung in vivo (Benam et al. 2016a; Si et al. 2019, 2021; Armstrong et al. 2013). It was observed that the H3N2 virus strains were 10 times more replication efficient than the H1N1 virus strains and caused increased cilia loss and high barrier function damage in the microfluidic chip, which supports the finding that H3N2 causes more severe clinical symptoms and is more virulent and highly infectious (Cheung et al. 2002). When the

primary human neutrophils were perfused through the vascular channel of airway chips infected with H1N1 or H3N2, it was found that circulating immune cells were recruited to the apical surface of the activated lung endothelium within minutes. The neutrophils further transmigrated through the endothelium followed by the ECM-coated membrane, and finally reached into the airway epithelium in few hours (Si et al. 2021). The influenza nucleoprotein (NP)-expressing infected airway cells were targeted by neutrophils. Upon being targeted by neutrophils, the infected airway cells converged into clusters, and their size decreased over time, which resulted in clearance of the virus from the chip. More neutrophil recruitment was observed by H3N2 on the airway chip, which is concurrent with the observation that H3N2 virus leads to stronger and more dangerous inflammation as compared to H1N1 in vivo (Cheung et al. 2002). Oseltamivir (Tamiflu), the anti-influenza molecule used clinically, was tested to inspect if the airway chip could be used to analyse its efficacy. It was observed that Oseltamivir (1 μM) strongly repressed the replication of the influenza virus. It prevented disruption of epithelial tight junctions and defects of barrier function and diminished the synthesis of many cytokines and chemokines induced by the influenza virus on the airway chip. This implies that the airway chip truly repeats the effects of oseltamivir as observed in humans, indicating clearly that it can cater as an efficient 3D in vitro model to assess and understand the efficacy of potent anti-influenza molecules/drugs for virus-infected human lung disease as a part of preclinical studies (Si et al. 2021).

As microfluidic chip was faithfully able to recapitulate the human lung response to influenza, it was then studied as a model for SARS-CoV-2.

SARS-CoV-2 pseudo particles (SARS-CoV-2pp) were created to imitate human airway infection by airborne SARS-CoV-2. These pseudo particles carrying the SARS-CoV-2 spike protein (one of the main factors) were added into the air channel of the chip and were thus present in close proximity to the human lung epithelial cells expressing increased levels of TMPRSS2 and ACE2 (Tang et al. 2020; Armstrong et al. 2013). To explore the potential of using existing FDA-approved antiviral drugs, as COVID-19 preventive therapies, the CoV-2pp-infected human airway chips were treated by introducing drugs like *chloroquine*, *arbidol*, *toremifene*, *clomiphene*, *amodiaquine*, *verapamil*, or *amiodarone* into their vascular channel. Through these studies it was observed that only two drugs, i.e. *amodiaquine* and *toremiphen*, statistically repressed the viral infection (Si et al. 2021).

Another team of researchers from Massachusetts built a high-throughput human primary cell-based airway model to analyse and study respiratory viruses in vitro. The platform is known as PREDICT96- ALI and is based on the PREDICT96 organ-on-a-chip model, which consists of a microfluidic culture plate with 96 individual devices and a perfusion system driven by 192 microfluidic pumps integrated into the plate lid. The PREDICT96-ALI platform is observed to be effective as it is high throughput and summarizes healthy human airway structure and function. The model expresses ACE-2 and TMPRSS2 and also supports infection with human coronavirus. Thus, it can be considered as a potential cell-based airway model to study respiratory viruses in vitro (Gard et al. 2020).

In another study, a micro-engineered airway lung chip was modelled to study the viral induced exacerbation of asthma. A micro-engineered model of terminally differentiated human mucociliary airway epithelium was induced with IL-13 which stimulated a Th2-type asthmatic characteristic, and it was then infected with live human rhinovirus 16 (HRV16) to recapitulate the phenotypic features of asthma caused by virus (Nawroth et al. 2020).

17.3 Tissue Chips in Space

It has been well established that micro physiological systems or tissue chips serve as excellent models to study the diseased condition of the human organs and also for drug screening processes. It is observed that spaceflight leads to various changes in the body of the astronauts. Microgravity environment exerts numerous stresses and pathological and physiological disturbances on the human body leading to reduced cardiopulmonary function, osteoporosis, suppressed immune response and muscle wasting (Demontis et al. 2017; Tanaka et al. 2017; Fitzgerald 2017; Watenpaugh and Hargens 1996; Nicogossian et al. 2016). These changes directly correlate to aging and diseased pathology on Earth. The cellular and molecular mechanisms behind these changes remain unknown due to the limited availability of biosamples of the astronauts. Research works in mice as surrogate have been carried out that included transcriptomic, biochemical and proteomic analysis following a mission on the International Space Station by the mice (Hammond et al. 2018; Mao et al. 2018). However, whether these studies show that same human health effects and diseased conditions are produced as are produced in mice is still unknown. When a recent study was conducted on the jugular vein flow and morphology, it was first time found that obstructive thrombosis occurred in an astronaut in space (Aunon-Chancellor et al. 2020; Marshall-Goebel et al. 2019). Treatment was carried out using the available anticoagulants on board the International Space Station (ISS); although spontaneous flow was not found even after 3 months of treatment with apixaban on the ISS, it was surprisingly observed on landing (Aunon-Chancellor et al. 2020). The challenge is that the current knowledge of drug pharmacological kinetics and dynamics during spaceflight and return to Earth is uncertain (Eyal and Derendorf 2019), due to lack of research in the microgravity environment.

To overcome this challenge, a robust and accurate model system is required to mimic the physiology and functions of the human organs. Therefore, micro physiological systems or organ chips are being employed on board the ISS to identify the effects of microgravity on human bodies and to understand the mechanisms at the level of molecules, involved behind the changes that occur in a microgravity environment. The tissue chips research on board the ISS might give us an insight on two broad aspects; firstly, the physiological changes associated with microgravity are also associated with aging and diseased conditions on Earth. Therefore, tissue chips on board the ISS exhibit disease pathologies in expedited time frame which would take years on Earth. This research will help scientists to develop therapeutics against age-related diseases. Secondly, tissue chips serve as a model system for

changes in physiology and function of a human body during a spaceflight. This research will enable scientists to plan explorations deep into the space like future missions to the Moon and Mars at a reduced risk to health of the astronaut (Eyal and Derendorf 2020).

According to the "Tissue Chips in Space" move, by collaboration of the NCATS (National Center for Advancing Translational Sciences) and ISS, the following nine projects are being carried out at ISS. The goal of the Tissue Chips in Space initiative is to use tissue chip platforms and the exclusive microgravity environment of the ISS to develop models of human disease, with the ultimate goal of expediting the discovery of therapeutics for people on Earth (Low and Giulianotti 2019; Yeung et al. 2020).

A team from University of California, San Francisco (UCSF), is working on a project that investigates microgravity-induced aging of the immune system (generated through simulated microgravity and spaceflight) and its role in tissue-specific healing and regeneration using micro physiological systems (Low and Giulianotti 2019). It is known that spaceflight induces dysregulation of the immune system and inflammatory responses and thus can be considered as a surrogate model for Earth-based immunosenescence.

A team of researchers from MIT (Massachusetts Institute of Technology) is utilizing tissue chips to find therapeutics to treat post-traumatic osteoarthritis (PTOA). The tissue chip system comprised of human cartilage, bone and synovium that is challenged with inflammatory cytokines and an acute impact injury which is believed to be an appropriate model for PTOA. The model will be used to test therapeutic options and monitor their effectiveness through the use of intracellular and extracellular biomarkers (Low and Giulianotti 2019).

A study of proximal tubule proteinuria and distal tubule kidney stone formation is being conducted by a group from University of Washington (UW), leveraging the ISS environment. As disease progression of proteinuria, osteoporosis and kidney stone formation is often slow and difficult to model in vivo in terrestrial studies, the group is utilizing microgravity to accelerate the onset of diseased state using a human cell-derived kidney tissue chip system (Low and Giulianotti 2019; Yeung et al. 2020).

Another team from the UW, is using an engineered heart tissue chip to study aspects of cardiomyopathy related to human health on Earth and in space. The study is based on automated real-time continuous functional readouts of the engineered heart tissues using a novel magnetometer-based motion sensor. The ultimate goal is to test pharmaceuticals and mechanical stimulations as potential therapeutics (Low and Giulianotti 2019).

The first line of defence of a human body is the innate immune response which recruits innate immune cells to the infected organ or the site of action. A human airway tissue chip connected to a bone marrow tissue chip is being developed by the Children's hospital of Philadelphia (CHOP). By utilization of this interconnected tissue chip system, the team can infect the airway chip and keep track of the recruitment of neutrophils from the bone marrow as a model of innate response. The project is focused on the finding that spaceflight induces changes that involve

dysregulation of the immune system. This ISS project is expected to serve as a model for a compromised immune system (Low and Giulianotti 2019).

A team from the University of Florida (UF) aims to use the accelerated muscle wasting property of the microgravity by using a human muscle tissue chip as a model for sarcopenia in terrestrial settings. Sarcopenia is a disorder that usually affects the older adults and can be characterized as loss of skeletal muscle mass, similar to muscle wasting during spaceflight. The study will employ cells from different types of patients (human muscle cells isolated from young, healthy and older, sedentary volunteers) and will thus monitor the progression of the sarcopenia phenotype on a time scale not possible in terrestrial settings (Low and Giulianotti 2019).

Human induced pluripotent stem cells (iPSC) from healthy patients are being utilized by a team of researchers from Stanford University to fabricate engineered heart tissue platforms for use on the ISS and on the ground. By manipulating the microgravity-induced weakening of the heart muscle to their advantage, the team will validate the ISS platform as a tool to model ischemic cardiomyopathy in humans on Earth and later to screen for potential drug candidates to treat patients (Low and Giulianotti 2019).

A team of researchers from Emulate is developing an automated BBB (blood-brain barrier) tissue chip platform derived from human cells for use both on the ground and on the ISS. This model will allow the monitoring of the dysregulation of the BBB which could provide applications for studying neurological disorders as well as the transport of drugs and toxins to the central nervous system. Another team from Emulate will use plug-and-play technologies to modify their automated platform developed for the BBB ISS experiment to study dysregulation in the gut. This system will be infected to study the innate and probiotic-induced response (Low and Giulianotti 2019).

17.4 Micro Physiological Human Brain or Neural System on a Chip

Neurological diseases and disorders lead to majority of deaths worldwide (Feigin et al. 2020). Neurodegenerative diseases (NDDs) consist of hundreds of different types of neural system disorders in human beings in the globe. According to the World Health Organization (WHO), there is expected to be nearly 50% increase in the number of people suffering from NDDs by 2030 (Mofazzal Jahromi et al. 2019; Menken et al. 2000). Neurological disorders, diseases and injuries (NDDIs) are more common in the elderly and cause substantial degradation in the well-being of the patient.

Therapeutic treatments for various NDDIs have been developed over the years using 2D cell culture followed by animal models and subsequently clinical trials. But these are "reductionist approaches" that many a times fail to recapitulate the dynamic features, functionalities and trajectories of the in vivo nervous system (Pampaloni et al. 2007; Breslin and O'Driscoll 2013). Due to the many limitations of these preclinical model systems, "micro physiological nervous systems" (MPNS) come

into picture leading to advancement of the knowledge of the highly ordered structural features, role and disease of the human nervous system, which therefore would lead to the development of innovative medicines for NDDIs (Haring et al. 2017).

Chip-based MPNS are 3D culture systems based on a scaffold that replicate highly ordered structural aspects as well as roles of the nervous system. Design and engineering of a neural tissue chip is very challenging as it requires precise modelling of human neural circuitry, anatomy and microenvironmental conditions to achieve higher-order and dynamic pathophysiology or neurophysiology of the human body. Brain-on-a-chip or neural tissue chip is designed using a distinctive "structure-function-disease" logical perspective. Structural and functional components essential for handling system framework to attain higher performance levels are catered to (Haring et al. 2017). Hence, microfluidic brain-on-a-chip in conjugation with stem cells has great potential to be used as an effective research tool to study novel therapeutics for the treatment of various nervous system disorders.

Parkinson's disease (PD) is a complex neurodegenerative disorder observed by reduced number of neurons in the brain's basal ganglia. It affects 70% of the dopaminergic neurons of the *substantia nigra* of the midbrain, associated with onset of motor dysfunction (Davie 2008; Obeso et al. 2008). The in vitro models and animal models for the PD are not able to mimic the human pathology of PD and hence results in the failure of development of efficacious therapeutic molecules for PD in the clinical trials (Poewe et al. 2017; Meissner et al. 2011).

A group of researchers from the University of Luxembourg worked with 3D cultures for studying the high content phenotype and drug analysis of dopaminergic neurons involved in the development of pathology of PD (Bolognin et al. 2018). The same group has also shown that neuroepithelial stem cells derived from iPSCs can be differentiated into midbrain-specific dopaminergic neurons using 3D microfluidics instrument (Moreno et al. 2015). G2019S mutation in LRRK-2 (leucine-rich repeat kinase 2) commonly leads to PD. Mutated LRRK2 has been found to hinder various cellular pathways like cell proliferation, protein trafficking and cytoskeletal integrity (Healy et al. 2008; Wallings et al. 2015). 3D tissue chip culture showed that LRRK2-G2019S mutation leads to dopaminergic neuronal cell death. It was also observed that LRRK2-G2019S caused mitochondrial abnormalities and cell death in young neurons. When LRRK2 inhibitor (Inh2) was administered, it was found that it salvaged only few phenotypes. This indicates that apart from LRRK2-G2019S mutation, other genetic factors also contribute to phenotypes associated with PD. For this purpose, identification of these LRRK2-G2019S-dependent and independent phenotypes was done using high-content image analysis platform. It can be used to check the efficacy of potent therapeutic compounds in a high-throughput manner. Using iPSCs-derived human neuroepithelial stem cells on 3D tissue chips, they stated that in place of the LRRK2-G2019S mutation, it was the genotype of the patients that was found to be a major discerning factor among the lines (Bolognin et al. 2018). Matrigel as a scaffold was chosen as it contains high levels of extracellular matrix proteins such as laminin, collagen and heparin sulphate proteoglycans which also have been reported to be present in high levels in the brain extracellular matrix (Hughes et al. 2010). The 3D scaffold given by Matrigel and also other

hydrogels such as collagen and alginate gels has shown to expedite neuronal network formation on the chip (Choi et al. 2014; Ortinau et al. 2010).

According to another study using organ chips, it was shown that the vascular system leads to neuronal maturation of the spinal cord neural tissue. The signalling pathways have been found to be common in brain microvascular endothelial cells (BMECs) and neurons early in development, but their role in human neuronal maturation is mostly unknown. Scientists were able to develop both spinal motor neurons and BMECs from human induced pluripotent stem cells. The organ chips showed an elevated calcium transient function and gene expression in comparison to 96 well plates. Addition of BMECs in the organ chip led to specific gene expression and vascular-neural interaction that, in addition, augmented the neuronal function and in vivo-like physiology (Sances et al. 2018). It clearly shows that use of organ chips can direct stem cells cultured within them to move closer to an in vivo structure and functionality.

A recent study also stated the use of 3D brain-on-chip platform with human iPSC-derived brain cells as a model to evaluate the neurotoxicity of organophosphates (OP) exposure and to evaluate beneficial compounds for cure (Liu et al. 2020). OPs are nerve agents that inhibit acetylcholinesterase (AChE) and thus cause severe neurotoxicity (Eddleston et al. 2008). OPs are also observed to cause necrosis, apoptosis and alteration of oxidative stress-mediated pathway (Kashyap et al. 2011). Butyrylcholinesterase (BuChE) has been used as a cure for OP toxicity as BuChE and AChE have similar active sites and both efficiently catalyse the break-down of acetylcholine (Ach). Inactivation of BuChE activity does not cause toxicity, as BuChE is not known to play an important role in vivo (Lee and Harpst 1971*;* Reiner et al. 2000*;* Masson and Lockridge 2010*;* Vijayaraghavan et al. 2013*;* Li et al. 2006*;* Broomfield et al. 1991*;* Lenz et al. 2005, 2010*;* Mumford et al. 2013*)*. BuChE is a bioscavenger of OPs and does not allow them to reach their physiological targets (Nachon et al. 2013). A 3D brain-on-chip platform with human induced pluripotent stem cell (iPSC)-derived neurons and astrocytes to simulate human brain behaviour was developed. The chip consisted of two compartments: the first compartment contained a hydrogel embedded with human iPSC-derived GABAergic neurons and astrocytes, and the second compartment consisted of a perfusion channel with dynamic medium flow. These brain tissue constructs were treated with various concentrations of malathion (MT), an organophosphate insecticide which acts as an AChE inhibitor and subsequently exposed to BuChE after 20 min of MT treatment. Results depict that the iPSC-derived neurons and astrocytes came in close proximity of each other and developed synapses in the 3D matrix. Also the exposure to BuChE enhanced the viability after MT treatment (Liu et al. 2020). It was also observed that neurons require co-culturing with astrocytes for their growth, enhanced neuronal synaptic activity and heightened synaptic transmission (Johnson et al. 2007). A previous research conducted by the same team also showed the effects of astrocytes on viability using human iPSC-derived 3D brain-on-chip model (Liu et al. 2019). It was found that a higher number of astrocytes in comparison to neurons enhanced the viability following severe MT exposure which is also consistent with the previous studies indicating that astrocytes have been found to protect

against diazinon- and diazonxon-induced inhibition of neurite outgrowth (Pizzurro et al. 2014).

Opioid overdose leads to 69,000 deaths each year around the world, and 15 million people suffer from opioid addiction (Parthvi et al. 2019). Opioid abuse is a significant crisis to public health worldwide; therefore there is a desperate need to develop therapeutics for treatment of opioid use disorder (OUD) and also to develop pain treatments that are non-addictive. The addictive quality of opioid drugs involves variations in the activity of brain neurons that utilize dopamine as a neurotransmitter. The search for drugs that can reverse such changes and potentially treat addiction to opioid drugs, as well as the search for analgesics that are devoid of addictive qualities, will be aided by model systems based on human cells. A team of researchers from University of California, Los Angeles, is currently working to develop a multi-organ, micro physiological systems (MPSs) based on the use of human induced pluripotent stem cell (iPSC)-derived midbrain-fated dopamine (DA)/GABA neurons on a three-dimensional platform that incorporates microglia, blood-brain barrier (BBB) and liver metabolism components. The model chip will focus on a key component of addictive circuitry—the dopaminergic and gamma-aminobutyric acid (GABA)ergic neurons of the midbrain, which have been recognized as responsible for mediating the reinforcing properties of many classes of abused drugs, including opioids (Ashammakhi et al. 2019).

The blood-brain barrier (BBB) is a highly selective semipermeable border of endothelial cells. It prevents the non-selective crossing solutes in the circulating blood into extracellular fluid of the central nervous system. The blood-brain barrier plays an important role in protecting and controlling entry of drugs to the brain tissue. The tight and stable BBB on the organ chip models has been difficult to maintain. According to a study by researchers from Harvard University, a tight blood-brain barrier can be maintained for a long period of time by regulating the amount of oxygen in an organ chip with stem cell-derived blood vessel cells (Park et al. 2019; van der Meer 2019). In a study carried out by researchers at Seoul National University in South Korea, the interactions were observed between glial cells and vascular cells, using the 3D micro vessel chip. It was observed that these interactions are important in tuning the geometry and function of brain microvascular networks. The extracellular matrix in which the cells are present has been found to control these interactions in part (van der Meer 2019; Lee et al. 2020).

Neural tissue chip platforms are also being used to observe the higher-order pathophysiology of Alzheimer's disease (AD). In a study at Korea University, it was found that when interstitial flow was applied to 3D neurospheroids on a microfluidic neural tissue chip, then Aβ was found to be significantly more damaging as compared to static conditions (Park et al. 2015).

17.5 A Few Other Recent Interesting Applications of the Organ-On-A-Chip Technology

17.5.1 Blood Vessels on a Chip

Graaf et al. used human induced pluripotent stem cells as a source of endothelial cells (hiPSC-ECs), along with a technique called viscous finger patterning (VFP) to develop in vitro cultures of human vascular cells inside microfluidic chips. In this finding, by providing the mechanical stimuli such as shear stress, they can emulate characteristics of the in vivo microenvironment. This is a robust model as it shows good results as far as the repeatability of the diameter between and within channels is concerned. All these features make hiPSC-EC-based MPS a cost-effective and efficient system for future studies in individualized disease modelling (de Graaf et al. 2019).

Duinen et al. studied a robust, high-throughput method to culture endothelial cells as 96 3D and perfusable micro vessels. They also fabricated a quantitative, real-time permeability assay to evaluate their barrier potential. As this assay has high-throughput value, high efficiency and robustness, hence it will be helpful for the researchers to make the transition from 2D to 3D culture models to observe and learn vasculature (van Duinen et al. 2017).

Poussin et al. have created a 3D model of endothelial micro vessels on a chip using primary human coronary arterial endothelial cells. This model will be used to study the attachment of monocyte to endothelium under flow and its applications in system toxicity (Poussin et al. 2020).

Duinen et al. developed a culture of perfusable 3D angiogenic sprouts in a membrane-free platform. A cocktail of angiogenic factors was added to the other side of the ECM gel, against which endothelial cells were grown on the chip. The resulting gradient of angiogenic factors induced the formation of endothelial sprouts through the ECM gel within 4 days. This perfused 3D angiogenesis model is amenable to high-throughput screening (van Duinen et al. 2019).

17.5.2 Tissue Chips as Disease Model for Oncological Studies

Lanz et al. analysed the use of a high-throughput organ-on-a-chip platform to select potent therapeutics for breast cancer. Triple negative breast cancer cell lines were seeded in the microfluidic platform. Cisplatin dose responses were generated in the simultaneous culture of 96 perfused micro tissues. The results have been promising and have created a possibility to the use of the organ-on-a-chip technology in individualized drug to ensure selection of efficient drugs and to determine the expected response to a particular treatment in real time (Lanz et al. 2017).

Aleman et al. described a metastasis-on-a-chip device that consisted of multiple bioengineered 3D organoids, made by a 3D photopatterning technique using extracellular matrix-derived hydrogel biomaterials. The cancer originated in colorectal cells chip and subsequently could be detected in other chips such as lung and liver

which are connected to the origin chip. This study is beneficial to gain knowledge of the mechanisms leading to metastasis, further in identification of anti-tumour drug targets (Aleman and Skardal 2019).

17.5.3 Organ-On-A-Chip Technology to Mimic the Fetomaternal Membrane Interface

Richardson et al. in 2019 created the first OOAC to mimic the components of foetal membranes to know the cell-cell interactions and paracrine crosstalk between maternal and foetal cells during pregnancy and parturition. The FM-OOC (fetomaternal organ-on-chip) platform used the vertical co-culture OOC design and was formed by two orthogonal vertically stacked cell culture chambers containing equal surface areas. The FM-OOC was used to analyse membrane permeability, oxidative stress and toxin-induced senescence, as well as cytokine production (Richardson et al. 2019a).

Richardson et al. in 2019 also created an amnion membrane OOC (AM-OOC), which was the first of its kind. This model successfully showed the interactive and transitional characteristics of amnion cells (epithelial-to-mesenchymal transition and mesenchymal-to-epithelial transition), under normal and oxidative stress conditions, similar to how they function and respond in utero (Richardson et al. 2019b).

Tissue chip has also been commercialized, and many companies such as Tissue, Emulate, Tara Biosystems, etc. have emerged from this technology. These companies develop specific tissue chips to meet the needs of their clients.

17.6 Discussion and Future Directions

The article has established that there are many exciting and innovative applications of the OOAC technology to study drug development and disease models. The tissue chip is a flexible model that can be utilized to study any diseased condition and pathology in the future. On the basis of the design and utility, they can be used to ask and answer the questions for which answers are not available by conventional means. Despite numerous advantages, there are still many tasks that need to be conquered for this technology to be considered as an ultimate model for drug screening.

Although OOAC has evolved and established quickly, the human on a chip theory remains far-off. There are disadvantages associated with the most commonly used material for production of tissue chips (i.e. PDMS). As the PDMS film is thicker than the in vivo morphology, hence this causes a decreased absorbance of small hydrophobic molecules which influence solvent efficacy and toxicity. Therefore, it is necessary to find suitable substitute materials. Another challenge in the use of this technology is that the cost of manufacturing is high and experimental use of organ chips is expensive and hence not suitable to the extensive usage of organ

chips. To overcome this challenge, the components of the organ chips must be cost-effective and easy to dispose.

But there is no doubt that tissue chip technology holds great future as a model to mimic disease pathology, hence proving to be beneficial for development of therapeutics for the rarest of the rare diseases.

In conclusion, microfluidic 3D models are very potent tools that will help the progressions in the field of biomedical research, leading to the development of novel therapeutics for the treatment of various human diseases.

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Flow Cytometry for Measuring Neutrophil Function

18

Paul Hutchinson

Abstract

Neutrophils are crucial cells for host defence. In particular, their phagocytic function is a major mechanism through which the immune system eliminates extracellular pathogenic microorganisms. The production of reactive oxygen species (ROS) by neutrophils, referred to as the 'oxidative burst', is a critical step in the destruction of these phagocytosed bacteria. Flow cytometry is an ideal technology to use for the measurement of these functions. In this paper I describe methods for doing this that can be applied to the study of bacteria-neutrophil interactions and used in the clinical diagnosis of neutrophil defects and as a measure of an individual's immune system status.

Keywords

Flow cytometry · Oxidative burst · Host defence · Neutrophil defects

18.1 Introduction

Neutrophils are the primary cell involved in the initial host defence to pathogenic microorganisms (Nathan 2006). They have evolved a number of mechanisms to kill phagocytosed pathogens, of which the most important is the respiratory oxidative burst, where the nicotinamide dinucleotide phosphate (NADPH) oxidase complex produces reactive oxygen species (ROS) to kill the phagocytosed microorganisms (Condliffe et al. 2006). Deficiency of the neutrophil respiratory oxidative burst is

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associated with impaired neutrophil killing (Quie et al. 1967) and recurrent infection (Lekstrom-Himes and Gallin 2000). The most well-recognised deficiency of neutrophil function is the condition chronic granulomatous disease (CGD) which occurs due to an abnormality of one of the four NADPH sub-units resulting in an absent (or more rarely reduced) oxidative burst (Lekstrom-Himes and Gallin 2000).

Given its single cell measurement capabilities, flow cytometry is an ideal technology to measure neutrophil ROS production and phagocytic function. To measure ROS, neutrophils can be loaded with the non-fluorescent dihydrorhodamine 123 (DHR 123). Any stimulus that causes these cells to produce reactive oxygen species can then be measured by the oxidation of the DHR123 to the fluorescent Rhodamine 123 (Emmendörffer et al. 1990). Neutrophil phagocytosis can be measured using fluorescently labelled particles or organisms such as propidium iodide (PI)-labelled *Staphylococcus aureus* (Pansorbin), with the amount of neutrophil associated fluorescence proportional to their phagocytic activity (Bohmer et al. 1992). In this paper methods to do this are described in detail. Using these techniques it is possible to analyse bacteria-neutrophil interactions, diagnose defects such as chronic granulomatous disease (Jirapongsananuruk et al. 2003), and measure immune system status (Hutchinson et al. 2003).

18.2 Using Flow Cytometry to Measure Neutrophil Function

Three assays are presented that measure human peripheral blood neutrophil ROS production and phagocytosis function, two using whole blood and the other using purified or isolated neutrophils.

18.2.1 Whole Blood Phagocytosis and Reactive Oxygen Species Production Assay

This assay was adapted from Perticarari et al. (1994) and Bohmer et al. (1992). We have published on using it to assess neutrophil function in bronchiectasis (King et al. 2009) and kidney transplant patients (Hutchinson et al. 2003).

18.2.1.1 Specimen Requirements

The whole blood assay to measure ROS production and phagocytosis function of neutrophils requires at least 4 mL of Li heparin blood.

18.2.1.2 Reagents

Stock Solutions

Dihydrorhodamine 123 5 mM (1.73 mg/mL) in DMSO [THERMOFISHER Cat # D23806]. Aliquot and store at −20 °C.

Propidium iodide 1.0 mg/mL solution in water [THERMOFISHER Cat# P3566]. Keep covered from light and store at 4 °C.

Pansorbin cells—10%W/V [Merck Millipore 507,858]. Keep covered from light and store at 4 °C.

18.2.1.3 Method

- To make enough Pansorbin-PI solution for 1 sample, add 200 μL of Pansorbin (10% W/V suspension, 0.1% Az in buffer) to 200 μL of 100 μg/mL propidium iodide in PBS. Incubate at room temperature for 30 min, covered from light.
- Add 1 mL of PBS. Spin at 500 *g* for 4 min. Repeat wash. Resuspend in 400 μL PBS (final concentration of PI-Pansorbin 5% W/V).
- Setup 4 aliquots of blood (Li Hep), 450 μL each. To two of them, add 50 μL of PI-Pansorbin. Incubate all tubes in 37 °C water bath. Important: Always check water bath temperature is exactly 37 °C before use.
- After 20 min add 1 μL DHR123 stock (final concentration—10 μM) to all of the + PI-Pansorbin and no Pansorbin-PI aliquots; mix thoroughly and then incubate in 37 °C water bath for a further 10 min.
- After the 30 min of total incubation, remove 100 μL of blood from each sample. Add to 2 mL of FACS lysing solution (Becton Dickinson, New Jersey). Incubate for 10 min at 25 °C to lyse the red blood cells. Run on flow cytometer.

The type of controls used for the experiment depends on the application, but as a minimum use the no PI-Pansorbin sample to serve as the negative control for the fluorescence measurements.

NOTE: The time of addition of DHR123 and the lysing of samples is crucial to this assay; do not vary the times by more than 5%.

18.2.1.4 Data Analysis

Exclude doublets by forward and side scatter height vs. pulse histograms. Identify the neutrophils by forward and side scatter, and then gate to measure and quantify (using mean fluorescence intensity) the level of fluorescence of the probes for ROS and phagocytosis function (Fig. 18.1).

18.2.2 Separated Neutrophil Phagocytosis and Reactive Oxygen Species Production Assays

Use density gradient separation medium Polymorphprep (Progen, Germany) to isolate neutrophils from whole blood.

18.2.2.1 Specimen Requirements

This assay requires at least 6 mL of Li heparin or EDTA blood.

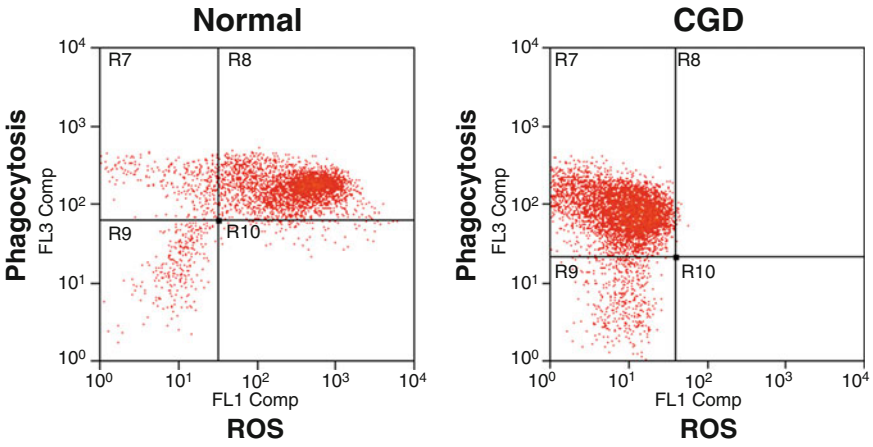

Fig. 18.1 Neutrophil ROS and phagocytosis function. Example histograms of neutrophil ROS and phagocytosis staining for a normal (top) and chronic granulomatous disease (bottom) donors after using the whole blood phagocytosis and ROS production assay. Neutrophils were gated by forward and side scatter

18.2.2.2 Method

Isolating Neutrophils

- Up to 5.5 mL of blood is layered on top of 3.5 mL of Polymorphprep in a 12 mL round-bottomed Falcon tube. It is then centrifuged for 35 min at 450 *g* at room temperature.
- Remove the neutrophils from the second layer of cells. Add an equal amount of 0.45% NaCl solution to the recovered neutrophils, and spin down for 10 min at 450 *g*.
- To lyse the residual red cells, remove supernatant and resuspend the cell pellet in 1 mL distilled water. Leave for 30 s only, and then add 0.9% saline solution (NaCl), and fill up to 10 mL. Centrifuge for 5 min at 450 *g*.
- Remove supernatant, and resuspend pellet in 10 mL of 0.9% NaCl. Then determine cell concentration by doing cell count.
- Centrifuge cells for 5 min at 450 *g*. Remove supernatant, and resuspend cells in PBS/5% normal human serum at a concentration of 0.5×10^6 cells/mL.

Reactive Oxygen Species Production

- Make up a Phorbol Myristate Acetate (PMA) solution of 20 μg/mL by diluting 20 μL of PMA stock (2 mg/mL in DMSO, stored at −20 °C) in 2 mL PBS/5% FCS. This must be done fresh on the day of the assay.
- Setup two tubes with 1 mL of the isolated neutrophils. To one tube add 5 μL of the 20 μg/mL PMA solution (final concentration 100 ng/mL PMA), and to the other add nothing. Incubate both at 37 °C for 10 min.

- Add 1 μL of DHR123 (final concentration—5 μM) to both and incubate again at 37 °C for 10 min. After this place samples on ice, and run all samples on flow cytometer within 10 min.

Phagocytosis

1. Add 100 μL of Pansorbin from Calbiochem (10% W/V suspension, 0.1% Az in buffer) to 100 μL of 100 μg/mL propidium iodide in PBS. Incubate at room temperature for 30 min, covered from the light. This must be done fresh on the day of the assay.
2. Add 1 mL of PBS. Spin at 500 *g* for 4 min. Repeat wash. Resuspend in 200 μL PBS (final concentration of Pansorbin-PI 5% W/V).
3. Set up 3 tubes with 0.9 mL of the isolated neutrophils. To two of the tubes, add 100 μL of the Pansorbin-PI solution, and add nothing to third tube. Incubate one PI-Pansorbin tube and the no PI-Pansorbin tube at 37 °C; the other PI-Pansorbin tube incubate on ice. After 30 min place 37 °C tubes incubated on ice. Run on flow cytometer within 10 min.

18.2.2.3 Data Analysis

Exclude doublets by forward and side scatter height vs. pulse histograms. Identify the neutrophils by forward and side scatter, and then gate to measure and quantify (using mean fluorescence intensity) the level of fluorescence of the probes for ROS and phagocytosis function.

The type of controls used for the experiment depends on the application, but as a minimum use the PMA sample as the negative for the DHR123 measurement and the 4 °C sample and no PI-Pansorbin samples as the negative for the phagocytosis measurement.

18.2.3 No Lyse No Wash Whole Blood Phagocytosis and Reactive Oxygen Species Production Assay

In this assay pHrodo BioParticles from ThermoFisher are used as the targets for phagocytosis by the neutrophils. These particles are inactivated, unopsonized *E. coli* reagents that are conjugated to the pHrodo dye. This dye becomes much more fluorescent as the surrounding pH becomes more acidic. Therefore, we can measure the ingestion of this particle based on the increased fluorescence due to the acidic environment of the phagolysosome. At the same time, we can measure the induced oxidative burst with the DHR123 dye. By adding the DyeCycle Ruby stain at the end of the phagocytosis incubation, which binds to the DNA of the leukocytes, we can distinguish the white blood cells from the red blood cells by triggering on the red fluorescence signal from the red laser and thus eliminate the need to lyse the RBCs before flow cytometric analysis.

Materials recommended but not provided with the pHrodoBioParticle kit:

- Whole blood sample collected in sodium or lithium heparin collection tube.
- Water bath or incubator set to 37 °C.
- Ice bath/bucket.
- Analysis tubes for your flow cytometer.
- Water bath sonicator.
- Flow cytometer with 488 nm, 561 nm and 640 nm excitation wavelengths.

18.2.3.1 Specimen Requirements

The whole blood assay to measure ROS production and phagocytosis function of neutrophils requires at least 4 mL of Li heparin blood.

18.2.3.2 Method

Preparing the BioParticles® Solution

1. Bring the Lysis Buffer A (Component A) to room temperature before use.
2. Add 2.2 mL Buffer B (Component B) to the vial containing the lyophilized product to resuspend the pHrodo™ BioParticles® conjugate. This provides sufficient pHrodo™ BioParticles® conjugate in a 20 µL aliquot for a 20:1 particle-to-phagocyte ratio.
3. Vortex for 1 min. Sonicate for 5 min until all the fluorescent particles are homogenously dispersed.
4. Store the pHrodo™ BioParticles® solution on ice for ~10 min prior to use.

Please note: It has been found that excess unused pHrodo dye can be frozen at −20 °C in aliquots, but avoid repeat freeze thawing (Fine et al. 2017).

Phagocytosis and Staining Protocol

1. Transfer 50 µL aliquots of whole blood (Li heparin) to each of two flat bottom 96-well plates; the number of wells will depend on the number of assay conditions. One plate will be kept at 37 °C and 5% CO_2 and the other plate at 4 °C as a negative control.
2. Add the concentration indicated in the product insert of the pHrodo *E. coli* BioParticles conjugate to the wells (10 µL). Set up samples as detailed in Table 18.1.
3. Bring volumes up to 100 µL per well with RPMI-1640 Medium; incubate one plate at 37 °C and 5% CO_2 and the other plate at 4 °C for 10–20 min.
4. Add DHR123 (final concentration—10 µM). Pipette up and down to mix the well, and incubate one plate at 37 °C and 5% CO_2 and the other plate at 4 °C for 5–10 min.
5. Prepare flow cytometry tubes with 500 µL of RPMI 1640 Medium and 1 µL of Vybrant DyeCycle dye.
6. Transfer 5 µL blood from each well of the 96-well plate into the tubes prepared in step 4; incubate all tubes for 15 min at 37 °C and 5% CO_2.

Table 18.1 Sample setup

37 °C, 5% CO_2			
Time: 15 min − pHrodo − DHR123[a]	Time: 15 min − pHrodo + DHR123[a]	Time: 15 min + pHrodo − DHR123[a]	Time: 15 min + pHrodo + DHR123[a]
Time: 30 min − pHrodo − DHR123[b]	Time: 30 min − pHrodo + DHR123[b]	Time: 30 min + pHrodo − DHR123[b]	Time: 30 min + pHrodo + DHR123[b]
4 °C			
Time: 15 min − pHrodo − DHR123[a]	Time: 15 min − pHrodo + DHR123[a]	Time: 15 min + pHrodo − DHR123[a]	Time: 15 min + pHrodo + DHR123[a]
Time: 30 min − pHrodo − DHR123[b]	Time: 30 min − pHrodo + DHR123[b]	Time: 30 min + pHrodo − DHR123[b]	Time: 30 min + pHrodo + DHR123[b]

[a] Add DHR123 at $T = 10'$
[b] Add DHR123 at $T = 20'$

7. Dilute the samples to 4 mL with RPMI 1640 Medium in each flow cytometry tube.
8. Acquire the samples on flow cytometer.

Flow Cytometer Setup

Trigger off Red Laser APC/Alexa 647 detector signal (DyeCycle Ruby).
DyeCycle Ruby—Red Laser 640 nm APC/Alexa 647 detector.
pHrodo Red—Yellow Green Laser 561 nm PE detector.
DHR123—Blue 488 nm Laser FITC/GFP detector.

18.2.3.3 Data Analysis

For the no lyse no wash whole blood method, make sure the trigger or threshold signal is set to the DyeCycle Ruby channel and the level set so that positive cells are visible, but the negative cells are not. Exclude doublets by forward and side scatter height vs. pulse histograms. Next use forward and side light scatter to identify the neutrophils. Then gate to measure and quantify (using mean fluorescence intensity) the level of fluorescence of the probes for ROS and phagocytosis function.

The correct controls to use does depend on the purpose of the experiment, but using the listed controls in Table 18.1 will enable you to determine the negative background staining of the pHrodo and DHR123 probes.

18.3 Clinical Applications of these Assays

Patients have been identified with defects in the intracellular killing of phagocytosed microorganisms, usually due to failure of production of superoxide anion, singlet oxygen and hydrogen peroxide (ROS). This has been called chronic granulomatous disease (CGD) and is characterised by recurrent life-threatening opportunistic

infections and uncontrolled inflammation, often with granuloma formation (Mauch et al. 2007). The flow cytometry assays described here can be used to determine this defect in neutrophils and help diagnose patients with CGD.

18.4 Summary

Neutrophils are a vitally important component of the immune system, and defects in their function can have serious consequences for an individual. This paper details methods for the measurement of neutrophil ROS and phagocytosis function using flow cytometry. The methods offer relatively simple and cost-effective ways to measure neutrophil function in both the clinical and research setting.

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Flow Cytometer as Tool to Understand the Variation in Genome Size Among Medicinal Herbs and Nonedible Oil Yielding Trees of Northeast India

19

Supriyo Basak and Latha Rangan

Abstract

The principal evolutionary processes of plant (speciation, new trait generation, invasion success of plant, domestication of crops, and community assembly) are governed by polyploidy. Hampering of population continuity is one of the major concerns in biodiversity conservation. However, the potential role of polyploidy in conservation of Zingiberaceae and nonedible oil yielding species of Northeast (NE) India is largely ignored. Here, we reviewed the potential role of polyploidy for understanding the conservation of Zingiberaceae and nonedible oil yielding species of NE India. Our work showed that the herbs are characterized by the higher level of ploidy whereas the trees were characterized by lower ploidy level.

Keywords

Biodiesel yielding plants · Cell cycle analysis · Fluorescence inhibitors · Polyploidy · Medicinal plant

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19.1 Introduction

The recent breakthrough in biological science has been governed by the ability of our technological invention to differentiate and enumerate multiple cell populations through flow cytometry. Flow cytometry-based analysis has permitted characterization of nuclear DNA content and cell sorting feasible. A blue argon ion laser or diode laser serves as one of the light sources. The forward scatter placed at the flow of light and the side scatter placed at the right angle of the flow of light give the dimension of particle size granularity when estimating the sample for size determination. A set of dichroic mirrors that capture the fluorescence signal from the nuclei gives the data of the relative fluorescence intensity (Hamelik and Krishan 2009). Because of this and other crucial features, flow cytometry is serving as an inseparable tool for plant ploidy analysis that has extensive application in the field of ecology, plant systematic, and epigenetics.

Polyploidy is a characteristic feature of plant evolution associated with ancient and recent whole genome duplication events (Jiao et al. 2011; Vanneste et al. 2014). Polyploidy can be of two types: autopolyploidy and allopolyploidy. Autopolyploidy involves the presence of multiple sets of chromosomes of the same origin, whereas alloploidy involves the presence of multiple sets of chromosomes of different origins (Stebbins 1971). Polyploidy is present in one third of the species of angiosperms (Wood et al. 2009) with higher frequency of polyploids observed in plants predominant in the poles (50%) compared to the plants growing in the equator (30%) of the earth (Rice et al. 2019). Though the possible mechanism for polyploid abundance was discussed (Levin 1983; Ramsey and Ramsey 2014; Soltis et al. 2016; Van de Peer et al. 2017), the hypothesis of advantage of polyploid distribution in ecological concept is recently being evaluated (Ramsey 2011; Chao et al. 2013).

Our research work focuses using flow cytometry as a tool to estimate genome size of medicinally important herbs (Zingiberaceae) and biofuel yielding tree and shrubs (*Pongamia pinnata*, *Mesua ferrea*) from Northeast (NE) India. The advent of a suitable buffer is a daunting task which can be used to estimate genome size of different kinds of plant species. In this book chapter, we focused on composition analysis of different isolation buffers, biological standards, the key hurdles in optimization for nuclei isolation, and our recent progress in the field of genome size research involving a flow cytometer (Ramesh et al. 2014).

19.2 Historical Development of Nuclear DNA Content Analysis of Plant

The pivotal role of DNA as heredity material was carried out after preliminary effort to investigate the nuclear DNA amounts of cell nucleus (Caspersson and Schultz 1938). Within few years, Swift (1950) established the constancy of nuclear DNA amount and termed it as "C value." C value was defined as the nuclear DNA content of an unreplicated haploid chromosome complement (n). The term 2C DNA content was represented as the total nuclear DNA amount in a G1 phase of cell cycle

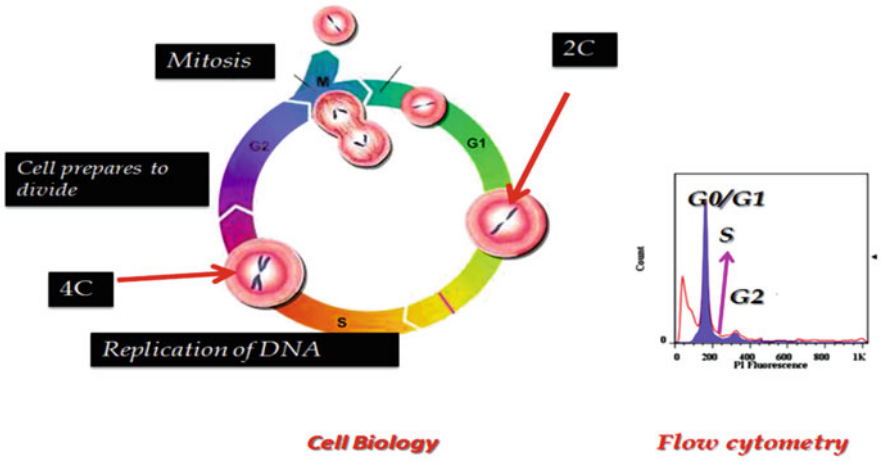

Fig. 19.1 Nuclear DNA content: a cell biology flow cytometric interface

(Fig. 19.1). Consequently, no correlation between nuclear DNA content and organism complexity was shown (Mirsky and Ris 1951). Thomas (1971) coined the term "C value paradox" to answer this question. Though the clue to this problem was solved by the discovery of non-coding DNA [C value enigma (Gregory 2001)], the significance, origin, and function of this variation of nuclear DNA content remained to be elucidated. Flow cytometer was used to estimate nuclear DNA content of flowering plants in 1980, and nowadays the number of laboratories attached with a flow facility has increased since then. Scientific fields which can be advanced through the knowledge of nuclear DNA content are systematics, ecology, molecular biology, and DNA sequencing programs (Bennett et al. 2000). In spite of the growing interest, genome size of only 1.4% angiosperms were known (Leitch et al. 2005).

19.3 Nuclear DNA Content: Various Definitions

The following paragraphs demonstrate the various terms associated with nuclear DNA content estimation of flowering plants.

Haploid number (n): It is the number of chromosomes present in the gamete (n). The number of chromosome present in the somatic cell ($2n$) is double that of the gamete.

Monoploid number(x): The number of chromosomes in a single non-homologous set is called the monoploid number (x) and is different from the haploid number (n). The term C_x is used for the monoploid genome.

Holoploid genome size: This is abbreviated as *C* value, and this genome size is not taking care of the ploidy level of the species.

Table 19.1 Terminologies to represent nuclear DNA content

Genome status	Monoploid	Holoploid
Chromosome number designation	x	n
Covering term for genomic DNA content	Genome size	Genome size
Kinds of genome size	Monoploid genome size	Holoploid genome size
Short terms	Cx value	C value
Short terms quantified	1Cx, 2Cx, etc.	1C, 2C, etc.

A summary of the key terminologies are presented in Table 19.1.

19.4 Units for Presenting DNA Amounts and Their Conversion Factors

Nuclear DNA content can be presented relative to the DNA content of biological standard nuclei (% ratio), as mass units (usually picograms, pg) or as number of base pairs (bp, Mbp, Gbp). The amount of nuclear DNA of the unknown sample is calculated as follows:

Sample 2*C* value (DNA pg or Mbb) = Sample 2*C* mean peak position/Reference 2*C* mean peak position × Reference 2*C* value.

A derivation of the factors has been published (Dolezel et al. 2003), which is as follows:

DNA content (bp) = $(0.978 \times 10^9) \times$ DNA content (pg)

DNA content (pg) = DNA content (bp)/(0.978×10^9)

19.5 Major Techniques

19.5.1 Available Techniques for Determination of Nuclear DNA Content

Initial attempt to nuclear DNA content estimation was obstructed by the lack of suitable approaches and instruments and used the biochemical methods which produced the average values of many cells (cytochemistry) through Feulgen absorbance cytophotometry. Due to the occurrence of the high intraspecific variation and time-consuming methodology, this technique became obsolete, and flow cytometer has occupied its position (Dolezel and Bartos 2005; Greilhuber 2008). The progress of flow cytometry was expanded when Galbraith et al. (1983) developed a protocol for estimating nuclear DNA content from the plant cell. This method has generally become convenient and was well taken.

19.5.2 Biological Standards

In the flow cytometer, nuclear DNA content has been estimated based on the relative fluorescent intensities. To represent the data, a universally accepted reference standard is required. The standardization process is either internal or external. The external standardization means acquiring the fluorescence intensity of the standard and unknown in the same voltage settings of the instrument, whereas the internal standardization means the co-processing of the standard and unknown species. The recommended standard for estimating nuclear DNA content in absolute unit requires internal standardization, and animal standards are not advised to estimate nuclear DNA content of plants (Greilhuber et al. 2007). The literature review suggested the higher percentage of scientific manuscript published following internal standardization (91.8%) compared to external standardization (6.1%). In some manuscript (7.1%) of all literatures analyzed, both methods of nuclear DNA content estimation were given priority (Greilhuber et al. 2007). Also, cytological stability, wide availability, near absence of secondary metabolites, and easy germination success are the primary requirements of the ideal calibration standard used for nuclear DNA content estimation (Suda and Leitch 2010). Apart from the above mentioned features, resolution and reproducibility of the histogram were the characteristic features of the ideal references standards. The secondary requirement of the standards is the less than fourfold variation between the standard and unknown species to avoid the problems of linearity in photomultiplier tubes (Suda and Leitch 2010). The most popular standards used by different investigators to estimate nuclear DNA content of angiosperms were *Zea mays* (Poaceae; 8%), *Petunia hybrid* (Solanaceae; 11%), *Hordeum vulgare* (Poaceae; 12%), and *Pisum sativum* (Fabaceae; 14%) (Fig. 19.2). The usage of the four reference standards with the bright field and fluorescence nuclear image and the clear flow cytometric peak for G0/G1 nuclei used by our group to estimate nuclear DNA content of the plants of NE India was shown (Fig. 19.3). The reference standards used for flow cytometric estimation of nuclear DNA content is shown in Table 19.2.

19.5.3 Optimization of Protocol for Nuclear DNA Content Estimation

19.5.3.1 Selection of Target Tissue

Investigation of the nuclear DA content of an unknown species requires the optimum tissue. Most of the literatures showed the prevalence of fully expanded leaves as the optimum tissue type for flow cytometric analysis (Greilhuber et al. 2007). Caution is also shown in some literature to use the colorless plant tissue type to reduce the interference of fluorescence inhibitors. A large number of literatures have investigated seeds as the target material for flow cytometric investigation (Sliwinska et al. 2005).

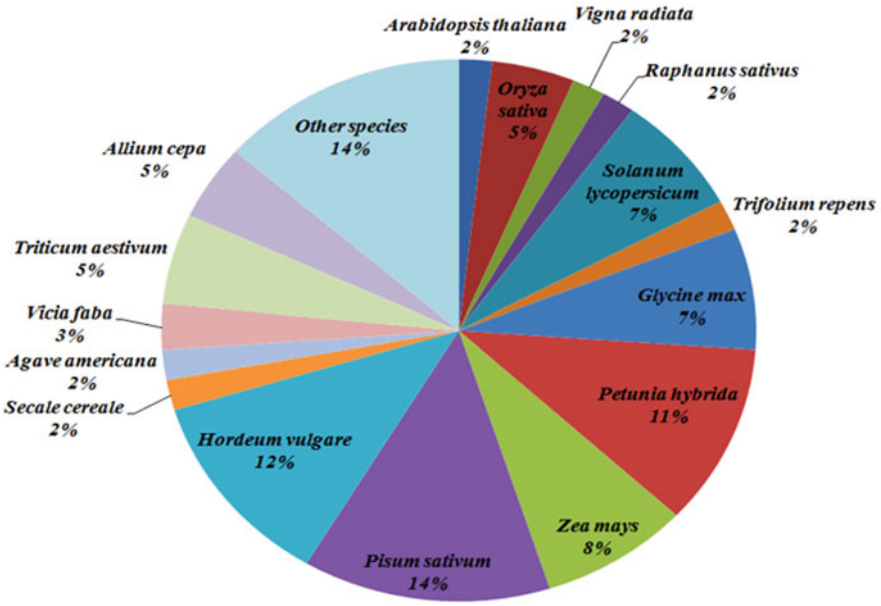

Fig. 19.2 Plant reference standards used for flow cytometric estimation of nuclear DNA content

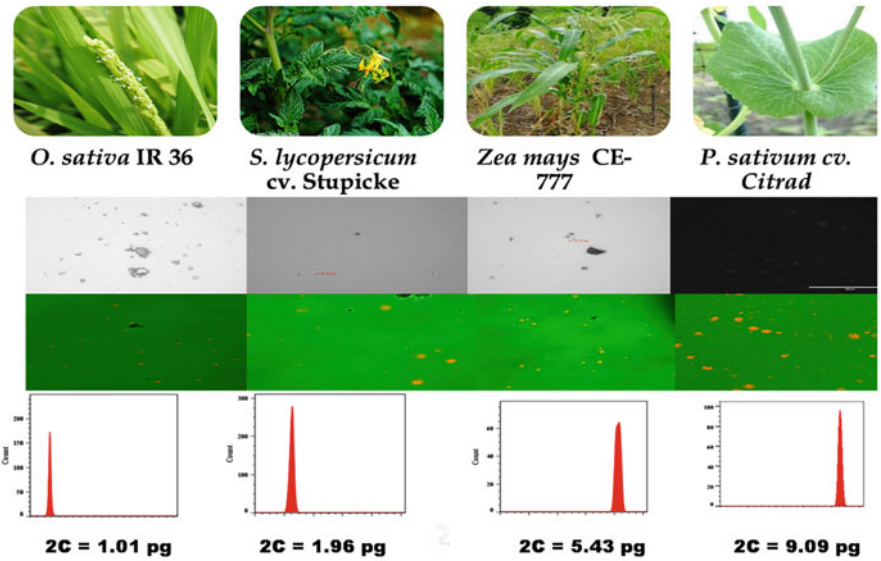

Fig. 19.3 Reference standards grown in IIT Guwahati. Top panel, bright field; middle panel: confocal microscopic image; bottom panel, histogram for flow cytometric study

Table 19.2 Some DNA reference standards recommended for estimation of nuclear DNA amounts in absolute units

Sl no	Plant species and cultivars	2C DNA content (pg DNA)	1C Genome size (Mbp)	References
1	*Oryza sativa* "IR36"	1.01	493	Price and Johnston (1996)
2	*Solanum lycopersicum* L. "Stupicke polni rane"	1.96	958	Dolezel et al. (1992)
3	*Zea mays* L. "CE-777"	5.43	2655	Lysak and Doležel (1998)
4	*Pisum sativum* L. "Ctirad"	9.09	4445	Doležel et al. (1998)

19.5.3.2 Nuclear Isolation Buffers

An ideal buffer should help in the isolation of large amount of nuclei which is free from the cellular debris and which should give stability to the isolated nuclei (Loureiro et al. 2007). The buffer should provide an appropriate micro environment for the stoichiometric binding of fluorescence dye to the nucleus (Loureiro et al. 2007). The most commonly used nuclear isolation buffers (composition and usage in different literatures) for estimating nuclear DNA content of different species were shown (Table 19.3 and Fig. 19.4). Galbraith's buffer and the commercial buffer were having the maximum usage for estimating nuclear DNA content of different species (Fig. 19.4). Literature also suggested that Galbraith and LB01 were the buffer used for estimating nuclear DNA content of woody species, whereas the Arumuganathan and Earle buffer and Tris-$MgCl_2$ were used for herbs. Hypotonic citrate buffer (Krishan 1975) was used once for estimating nuclear DNA content of angiosperms (Bharathan et al. 1994). Inspired by the lack of usage of the hypotonic citrate buffer, we were interested in finding out nuclear DNA content of large number of angiosperms of NE India. The method of obtaining the suspension of nuclei is as follows: chopping of plant materials in the hypotonic citrate buffer (Krishan 1975) with a scissor followed by the sieving through 30 μm nylon filter and acquisition in flow cytometer. The protocol was shown in Fig. 19.5.

19.5.3.3 Inhibition of Fluorescence Intensity by Intercalation of Secondary Metabolites

Plant contains a variety of secondary metabolites. Inhibition of propidium iodide (PI) fluorescence involves the binding of secondary metabolites to the DNA and/or direct reaction with the dye molecule. Adjusting the buffer composition to the specific requirement of a plant is a challenging task (Price et al. 2000). Although several studies were published regarding the mode of action of staining inhibitors, no complete method could avoid interfering substances and its effect on nuclear DNA content estimation (Greilhuber et al. 2007). The addition of antioxidants such as polyvinylpyrrolidone, β-mercaptoethanol, or dithiothreitol to the nuclear isolation

Table 19.3 Composition of buffer for flow cytometric estimation of nuclear DNA content

Buffer	Composition	Reference
Galbraith's buffer	45 mM $MgCl_2$; 30 mM sodium citrate; 20 mM MOPS; 0.1%(w/v) Triton X-100; pH 7.0	Galbraith et al. (1983)
LB01	15 mM TRIS; 2 mM Na_2EDTA; 0.5 mM spermine.4HCl; 80 mM KCl; 20 mM; NaCl; 15 mM β-mercaptoethanol; 0.1% (v/v) Triton X-100; pH 7.5	Doležel et al. (1989)
Arumuganathan and Earle	9.53 mM $MgSO_4.7H_2O$; 47.67 mM KCl; 4.77 mM HEPES; 6.48 mM DTT; 0.25% (w/v) Triton X-100; pH 8.0	Arumuganathan and Earle (1991)
Marie's nuclear isolation buffer	50 mM glucose; 15 mM KCl; 15 mM NaCl; 5 mM Na_2EDTA; 50 mM; sodium citrate; 0.5% (v/v) Tween 20; 50 mM HEPES; 0.5% (v/v) β-mercaptoethanol; pH 7.2	Marie and Brown (1993)
Otto buffers	Otto I buffer: 100 mM citric acid; 0.5% (v/v) Tween 20 (pH approx. 2.3); Otto II buffer: 400 mM $Na_2HPO_4.12H_2O$ (pH approx. 8.9)	Otto (1990) and Doležel and Göhde (1995)
Tris-$MgCl_2$	200 mM TRIS; 4 mM $MgCl_2.6H_2O$; 0.5% (v/v) Triton X-100; pH 7.5	Pfosser et al. (1995)
Baranyi's buffer	Baranyi solution I: 100 mM citric acid monohydrate 0.5% Triton X-100 Baranyi solution II: 400 mM $Na_2PO_4.12H_2O$; 10 mM sodium citrate; 25 mM sodium sulfite	Baranyi and Greilhuber (1995)
Bergounioux's buffer	Tissue culture salts supplemented with 700 mM sorbitol; 1.0% (v/v) Triton X-100; pH 6.6	Bergounioux et al. (1986)
Rayburn's buffer	1 mM hexylene glycol; 10 mM $MgCl_2$; 0.5% (v/v) Triton X-100; pH 8.0	Rayburn et al. (1989)
Bino's buffer	200 mM mannitol; 10 mM MOPS; 0.05% (v/v) Triton X-100; 10 mM KCl; 10 mM NaCl; 2.5 mM DTT; 10 mM spermine.4HCl; 2.5 mM $Na_2EDTA.2H_2O$; 0.05% (w/v) sodium azide; pH 5.8	Bino et al. (1993)
De Laat's buffer	15 mM HEPES; 1 mM $EDTANa_2.2H_2O$; 0.2% (v/v) Triton X-100; 80 mM KCl; 20 mM NaCl; 15 mM DTT; 0.5 mM spermine.4HCl; 300 mM sucrose; pH 7.0	De Laat and Blass (1984)

buffer is helpful in reducing the interference, but it is not always the case (Ramesh et al. 2014) (Fig. 19.6).

19.5.3.4 Development of Protocol for Estimation of Nuclear DNA Content Through Microscopy and Flow Cytometry

In parallel to the development of flow cytometric protocols for plant cells, a discovery was observed for the mammalian cells for flow cytometric determination of lymphocytes (Krishan 1975). Incubation of cells in a hypotonic solution containing sodium citrate and propidium iodide resulted in the disruption of cell

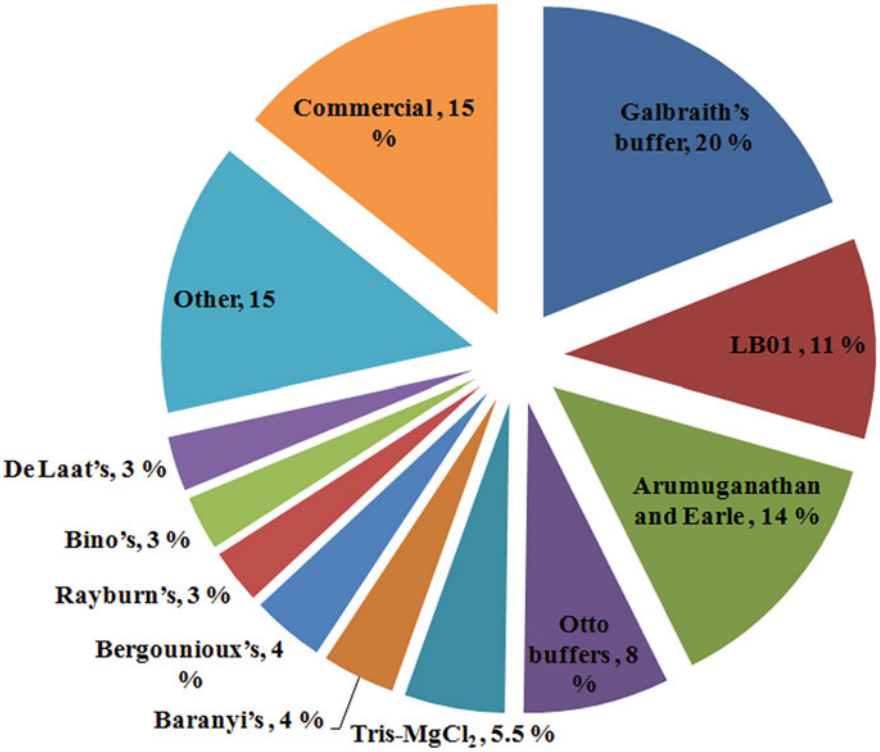

Fig. 19.4 Pie chart representing the nuclear isolation buffer used for flow cytometric estimation of nuclear DNA content. Data obtained from 1982 to 2013

membrane followed by rapid staining of the nuclear chromatin. The quality of the histograms generated by this technique was equivalent to the method of producing cell prepared by fixation and RNase digestion. The additional benefit of this technique was the rapid processing (5 min), requiring minimum amount of starting material and formation of negligible cell debris (Krishan 1975). This protocol worked for the most of the herbs and tree species of NE India with minor modifications.

19.5.3.5 Effect of Filter Mesh

Initially we were interested in the optimizing of the filter mesh dimension required for our work. The usage of unfiltered, 100 μm and 30 μm filter mesh had no significant effect on the nuclei released from the standard plant material (*P. sativum*) (Fig. 19.7a, d, h). However, the side scatterplot was very intact for the filtered plant material through the 30 μm nylon mesh (Fig. 19.7b, e, i). Hence, 30 μm filter mesh was optimized for all the standard plant materials for subsequent flow cytometric analysis. Nevertheless, the bright field microscopic view of the

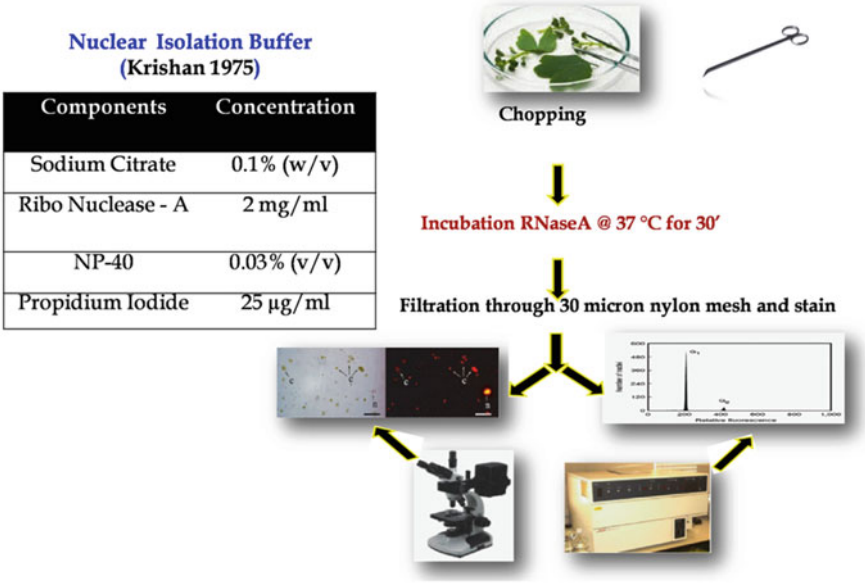

Fig. 19.5 General flow cytometric procedure for estimation of plant nuclear DNA content

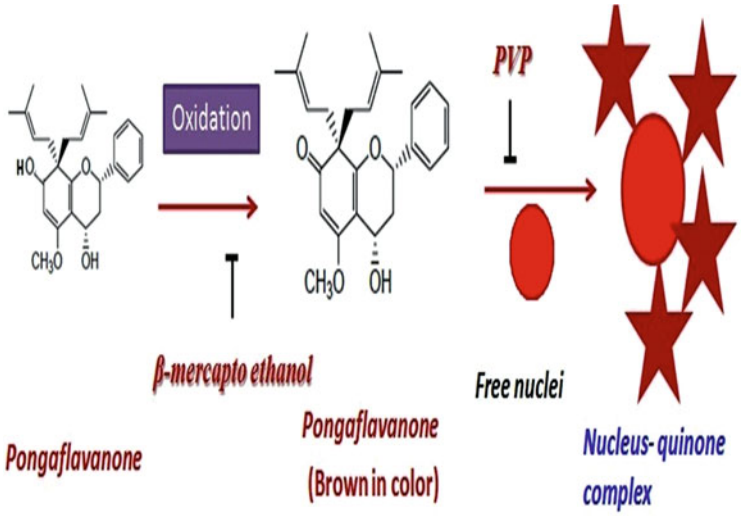

Fig. 19.6 Mode of action of the stripping of inhibitors from *P. pinnata* by β-mercaptoethanol and polyvinylpyrrolidones (Ramesh et al. 2014)

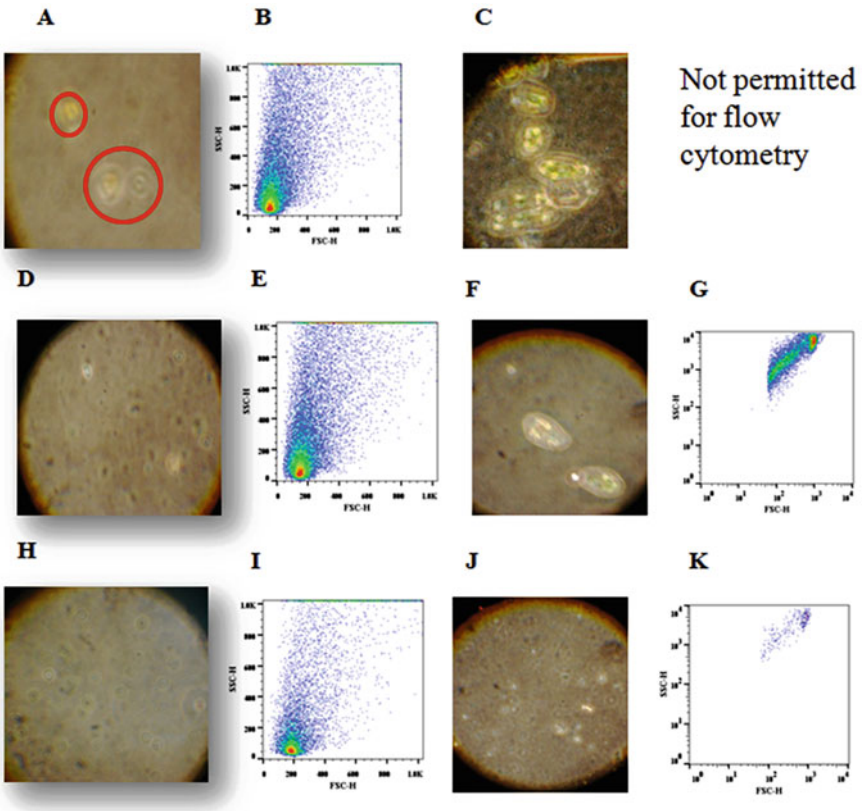

Fig. 19.7 Bright field microscopy and flow cytometric comparison. Light microscopic image and scatterplot of standard (*P. sativum*) and test (*C. zanthorrhiza*) of the plant samples chopped in nuclear isolation buffer (Krishan 1975) before filtration through nylon mesh [(**a**) bright field (40×) and (**b**) scatterplot of *P. sativum* and (**c**) bright field (40×) of *C. zanthorrhiza*] after filtration through 100 μm nylon mesh [(**d**) bright field (40×) and (**e**) scatterplot of *P. sativum*, (**f**) bright field (40×) of *C. zanthorrhiza*, (**g**) scatterplot of *C. zanthorrhiza*] and 30 μm nylon mesh [(**h**) bright field (40×) and (**i**) scatterplot of *P. sativum* (**j**) bright field (40×) of *C. zanthorrhiza* (**k**) scatterplot of *C. zanthorrhiza*]

unfiltered suspension of a sample (Zingiberaceae species) showed the appearance of cells but no free nuclei. The light microscopy confirmed that unfiltered suspension was unsuitable for flow cytometric study as it may block the fluidics. To add the trouble, the usage of 100 μm and 30 μm filter mesh resulted in decrease of the analysis particles and also nuclei (Fig. 19.7c, f, j). The decreasing trend of the cell cluster was also observed in the scatterplot produced by the usage of 100 μm and 30 μm filters (Fig. 19.7g, k).

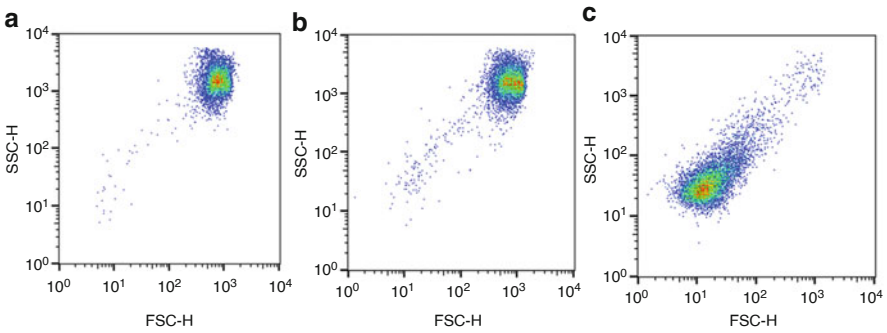

Fig. 19.8 Effect of detergent concentration on the lysis of cells of *C. zanthorrhiza*. (**a**) Nuclear isolation buffer (Krishan 1975) containing 0.03% (v/v) NP 40. (**b**) Nuclear isolation buffer with 0.10% (v/v) NP 40. (**c**) Nuclear isolation buffer with 0.3% (v/v) NP 40

19.5.3.6 Effect of Detergent Concentration

Although the hypotonic PI [sodium citrate 0.1% (w/v), 0.03% (v/v) NP 40, 25 μg mL^{-1} and 0.5 mg mL^{-1}] was able to release the nuclei from all the standard species, the buffer at the current form was not able to release nuclei from the tested Zingiberaceae species. The prolonged incubation at higher temperature (37 °C and 65 °C) even was unsuccessful in releasing the nuclei from the cells. Ultimately, the nuclear release was optimized by increasing the detergent concentration (NP40: 0.03% to 0.10% to 0.30%) for Zingiberaceae species (Fig. 19.8). The same protocol was found to be successful in releasing nuclei from three different genera of Zingiberaceae species (*Curcuma*, *Hedychium*, and *Kaempferia*) (Fig. 19.9).

19.5.3.7 Microscopic and Flow Cytometric Interface: External Standardization

Microscopy and flow cytometry should be going simultaneously to optimize the nuclear DNA content of a species. Our result showed the difference between the size and fluorescence intensity of the *Z. mays* CE777 (2C = 5.43 pg) with the *C. zanthorrhiza* nuclei. For *Z. mays*, free nuclei were observed, but in the case of *C. zanthorrhiza*, some free nuclei and some attached to the cell debris were observed (Fig. 19.10).

19.6 Concluding Remarks

Our aim was to develop a suitable flow cytometric protocol which can be successfully used for a large number of plant species for estimating the genome size. We were successful in isolating the nuclei from a broad range of species from herbs to trees. The findings of this research show that the tree species and the medicinal herbs are characterized by huge difference in genome size. The difference in genome size is attributed to the life form of these two kinds of species. This research provide the

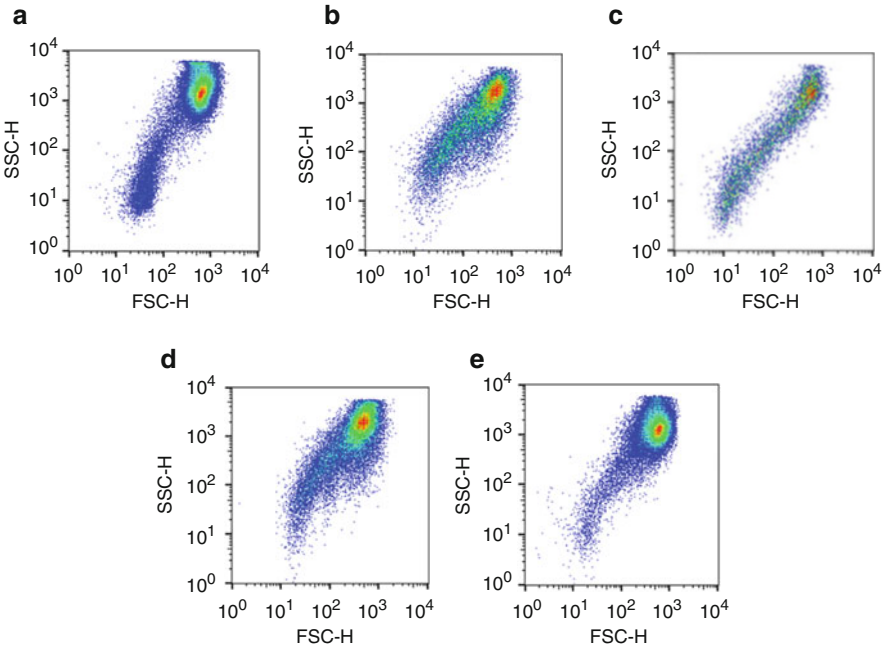

Fig. 19.9 Differential release of nuclei in 0.3% of detergent NP 40. (**a**) *C. longa* (**b**) *C. amada.* (**c**) *H. coronarium* (**d**) *K. galanga* (**e**) *K. angustifolia.* The figure demonstrates that nuclei release was optimized for a broad range of Zingiberaceae species

key material for carrying out genome size mechanism research for the life history traits in the plant kingdom which has not yet been evaluated.

19.6.1 Genome Size Estimation of Biodiesel Yielding Trees of NE India

Our group is interested in the genome size variation of biodiesel yielding tree species, for example, *P. pinnata* and *M. ferrea*. The popular name of *P. pinnata* (Fabaceae) in India is "Karanj" which has versatile application in nitrogen fixation, eutrophication, and biodiesel production, among many others. The genome size of this plant was unknown for a long time. Our group has invested quality time to identify the candidate plus tree from the rich genetic diversity of Assam followed by the in vitro propagation of the mother plant and studying the genome size stability of the mother plant and the tissue culture-raised plant. The development of appropriate protocol for estimating the genome size of *P. pinnata* was quite challenging for us as different buffers tested were found to be insignificant in producing viable nuclei. Then, by reducing the detergent concentration in the modified propidium iodide/ hypotonic citrate buffer of Krishan, nuclei isolation was optimized. The reported

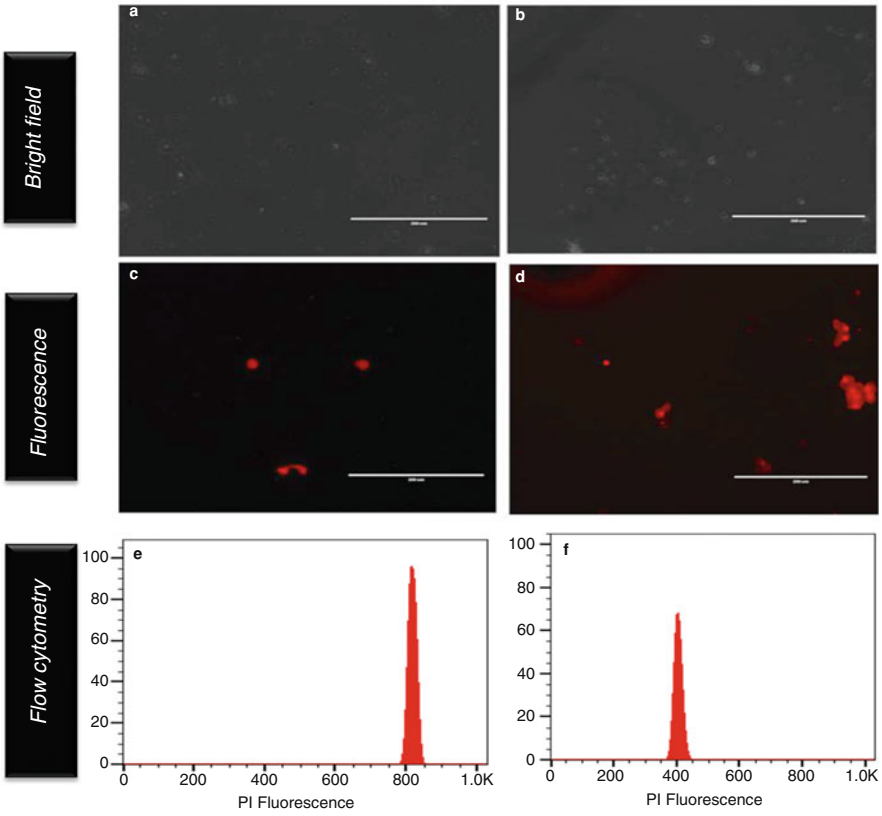

Fig. 19.10 Bright field and fluorescence microscopic photograph of *Z. mays* and *C. amada*. **a** and **d** are the bright field microscopy image of the nuclear suspension prepared in hypotonic PI of *Z. mays* and *C. amada*, respectively. **b** and **e** are the fluorescence microscopic image of the nuclear suspension prepared in hypotonic PI of *Z. mays* and *C. amada*, respectively. **c** and **f** histogram of median fluorescence intensity of the nuclear suspension prepared in hypotonic PI of *Z. mays* and *C. amada*, respectively

genome size of *P. pinnata* in our study was 2.66 pg with approximately predicted 1C value of 1300 Mb using *Z. mays* as internal standard (Table 19.4). The chromosome number was found to be $2n = 22$ (diploids). The ecological study of variation of genome size showed that genome size has a decreasing trend with altitude for this tree species (Ramesh et al. 2014; Choudhury et al. 2014).

M. ferrea is another abundantly growing tree species of NE India which can grow up to a medium-sized or fairly large evergreen tree up to 36 m tall. The tree is recently being investigated for different therapeutic applications (anti-inflammatory activity and anticancer activity). The genome size of this plant reported by our group is 1.40 pg (Table 19.4), and diploidy was observed as the characteristic feature of this plant (Das et al. 2018).

Table 19.4 List of plants for which genome size has been published by our group in the last 5 years

Serial number	Plant name	Genome size (2C, pg) (mean ± SE)	Genome size (1C, Mbp)	Literature cited
Medicinal herbs of Northeast India				
1	*Curcuma longa*	2.74 ± 0.09	1339	Basak et al. (2017)
2	*C. zanthorrhiza*	2.84 ± 0.01	1388	Basak and Rangan (2018)
3	*C. amada*	1.86 ± 0.01	909	Basak and Rangan (2018)
4	*C. angustifolia*	2.18 ± 0.09	1066	Basak and Rangan (2018)
5	*C. leucorrhiza*	2.73 ± 0.01	1334	Basak and Rangan (2018)
6	*C. caesia*	2.80 ± 0.01	1369	Basak and Rangan (2018)
7	*C. aromatic*	2.86 ± 0.00	1370	Basak and Rangan (2018)
8	*Hedychium coronarium*	1.96 ± 0.01	958	Basak and Rangan (2018)
9	*H. chrysoleucum*	2.15 ± 0.02	1051	Basak and Rangan (2018)
10	*H. spicatum*	3.51 ± 0.06	1716	Basak and Rangan (2018)
11	*H. gardnerianum*	4.30 ± 0.02	2102	Basak and Rangan (2018)
12	*H. ellipticum*	3.70 ± 0.06	1809	Basak and Rangan (2018)
13	*Kaempferia galanga*	8.15 ± 0.02	3985	Basak and Rangan (2018)
14	*K. angustifolia*	4.72 ± 0.06	2308	Basak and Rangan (2018)
15	*K. elegans*	3.48 ± 0.05	1701	Basak and Rangan (2018)
16	*K. rotunda*	3.54 ± 0.02	1731	Basak and Rangan (2018)
17	*Alpinia nigra*	4.58 ± 0.02	2239	Basak et al. (2018)
18	*Boesenbergia longiflora*	9.02 ± 0.05	4410	Basak et al. (2018)
19	*Globba bulbifera*	2.53 ± 0.01	1237	Basak et al. (2018)
20	*Zingiber officinale*	3.61 ± 0.09	1765	Basak et al. (2018)
21	*Zingiber* sp. (Moran)	3.68 ± 0.02	1799	Basak et al. (2018)
Bio-fuel yielding trees				
22	*Pongamia pinnata*	2.51 ± 0.01	1191	Choudhury et al. (2014) and Ramesh et al. (2014)
23	*Mesua ferrea*	1.40 ± 0.02	684	Das et al. (2018)

19.6.2 Intraspecific and Interspecific Variation in Genome Size of Medicinal Herbs of NE India

Alpinia nigra (Gaertn.) B.L. Burtt is one of the abundant wild Zingiberaceae germplasm of Guwahati city of Assam. This plant plays a dominant role in the conservation of Brahmaputra riverbank, roadside, and also forests of Guwahati city. Moreover, the plant is growing in the wild on mountains inside the Indian Institute of Guwahati (IITG) campus. The campus of IITG was established in 1996 creating a geographical isolation of the *A. nigra* growing inside and outside of the IITG campus. We speculate that 30 years of isolation might have caused the variation of genetic diversity between the *A. nigra* populations of IITG campus and outside area. This is because higher priority was given to conserve the wild species of IITG in the natural state, whereas the frequent anthropogenic activity was observed in the

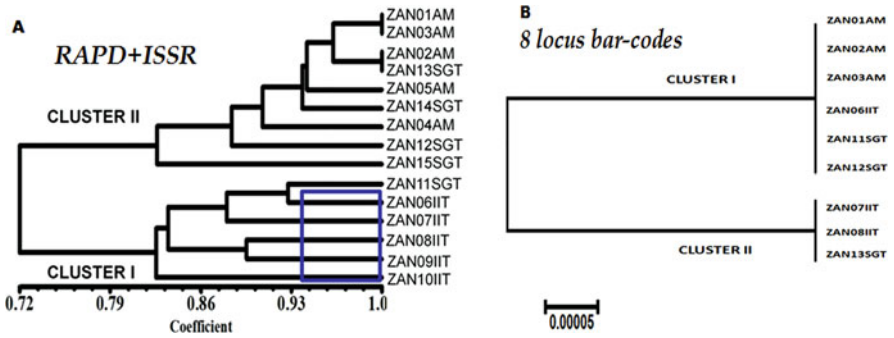

Fig. 19.11 (**a**) RAPD-ISSR marker data showed that *A. nigra* population of IIT Guwahati was clustering separately from the Guwahati city populations. (**b**) Eight locus (plastid and mitochondrial) confirmed habitat fragmentation of *A. nigra*

populations of *A. nigra* in the Guwahati city. To understand the nature of genetic variation, leaf samples were collected from five individual plants from IITG campus. Moreover, leaf samples from two populations which are undergoing different anthropogenic pressures were collected. The two areas were Amingaon region which is located in the riverbanks of the Brahmaputra and Saraighat region where different industrial development (expansion of tea estate and road construction) is taking place for the last 30 years. Markers such as RAPD and ISSR PCR analysis of the isolated genomic DNA were performed to assess the genetic diversity.

The genetic diversity assessment of three populations showed that genetic diversity of wild *A. nigra* populations of IITG was higher compared to Amingaon and Saraighat populations. The dendrogram and principal component analysis (PCA) showed that *A. nigra* populations of IITG were forming a different cluster (Fig. 19.11a). The study of eight loci barcode also confirmed that IITG populations were partially segregated from the Amingaon and Saraighat populations (Fig. 19.11b). We termed the observation as the habitat fragmentation and confirmed that extinction of demes is the reason for the process (Basak et al. 2019a, b). Yet, it is still unknown whether genome size variation is playing a big role in the habitat fragmentation process of wild *A. nigra*.

In *Curcuma*, we have done work on the turmeric landraces of NE India. We have collected turmeric landraces from the farmers of the region. We got inspiration for the work following the turmeric production statistics of different Indian states. The statistics showed us that the average production of turmeric from NE India is lower compared to the average production of other states. We wanted to assess the genetic diversity distribution of different NE states (Assam, Arunachal Pradesh, Manipur, and Meghalaya). Our research showed that among the NE states, turmeric landraces of Assam have the highest variation compared to three other states. Turmeric landraces from higher-altitude areas were clustered separately from turmeric landraces from lower-altitude areas. Genome size estimation of the turmeric landraces showed that higher-altitude turmeric landraces of NE India were characterized by statistically higher genome size compared to lower-altitude areas (Fig. 19.12; Basak et al. 2017).

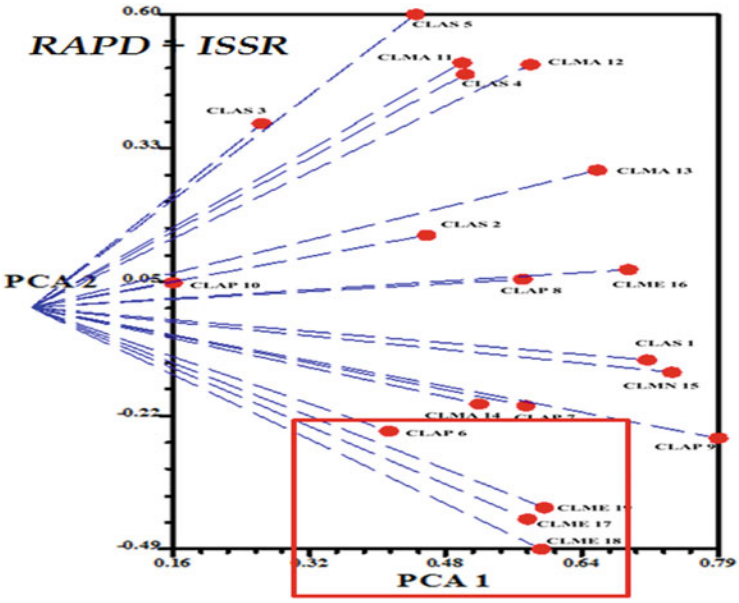

Fig. 19.12 Turmeric landraces of highlands were clustered in the terminal of the principal component analysis showed geographical isolation leading to evolutionary younger origin

Zingiberaceae is characterized by high level of polyploidy. Cytology is one of the taxonomic markers and in collaboration with genome size estimation by PI flow cytometry. The cytological data help in understanding the diversification and evolution of Zingiberaceae species. Using the technique of flow cytometry, we have characterized chromosome number of 9 species and genome size estimation of 21 species out of which four were new counts. The research showed that the intraspecies variation of genome size of wild Zingiberaceae species was lower, whereas the cultivated species were characterized by higher intraspecific genome size variation. The lowest genome size (2C) was found in *C. amada* (1.860 ± 0.006 pg). The highest genome size (2C) was observed for *K. galanga* (8.150 ± 0.020 pg). Among the 20 species, a 4.38 fold variation in genome size was observed (Basak and Rangan 2018; Basak et al. 2018). The research finding of flow cytometric investigation is tabulated (Table 19.4).

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Raman Spectroscopy Applications for Bacterial Detection: Potential and Challenges Towards Development of Field Deployable System

20

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"Everything is energy and that's all there is to it. Match the frequency of the reality you want and you cannot help but get that reality. It can be no other way. This is not philosophy. This is physics".

—Albert Einstein

Abstract

Detection of pathogens in air, water, soil, or food is essential due to their infectious nature. Raman microspectroscopy has emerged as a potential diagnostic technique for detection of bacteria. Vibrational spectroscopic technique such as Raman spectroscopy is nondestructive in nature and probes the bond molecular vibrations to yield structural information. Biological systems such as cells,

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tissues, and bacteria are composed of biomolecules like lipids, proteins, carbohydrates, and nucleic acids. Therefore, this spectroscopic technique can provide information pertaining to the molecular structure of the components. Various Raman-based techniques such as surface-enhanced Raman spectroscopy (SERS), resonance Raman spectroscopy (RR), etc. have been used for sensitive and selective detection of pathogens. Employing suitable substrates for accommodating biological entities that also provide high sensitivity and highly reproducible SERS can be a state-of-the-art method for pathogen detection. Use of required wavelength for excitation and proper sampling with advanced data analytics approach has led to pathogen detection by RRS. In addition, combination of chemometric tools aids in discrimination and classification of samples which is important in clinical and field environment. This chapter discusses the use of Raman spectroscopy-based approaches for detection of pathogens. Further, critical steps such as sampling, importance of pre-processing and post-processing, and instrumental considerations have also been discussed.

Keywords

Raman spectroscopy · Bacterial detection · Pre-processing · Univariate analysis · Multivariate analysis

20.1 Introduction

Spectroscopy is the study of interaction of light with matter. The interaction may result in changes in the energy level of the probed system. Depending upon the energy of light, the effects may be manifested in electronic, vibrational, rotational levels of molecules. Figure 20.1 depicts the typical wavelengths of different types of radiations. The left-hand side corresponds to high-energy rays, while the right-hand side shows low-energy rays. Vibrational spectroscopy, such as infrared (IR) and Raman spectroscopy, occupy the UV, visible, and IR region of the electromagnetic spectrum. Figure 20.2 shows the Jablonski diagram for Raman, IR, and resonance Raman processes.

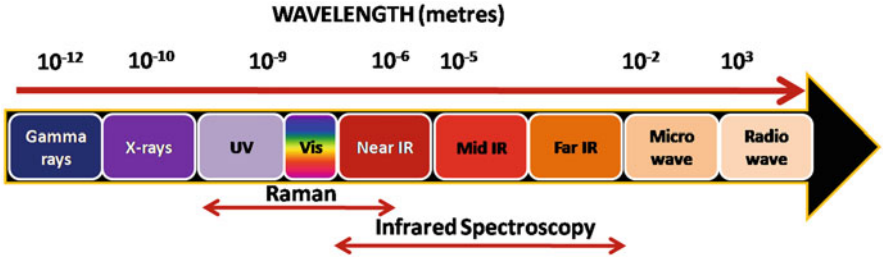

Fig. 20.1 Schematic representation of typical wavelengths of the electromagnetic spectrum. Conventional Raman techniques use visible near-infrared region for various applications

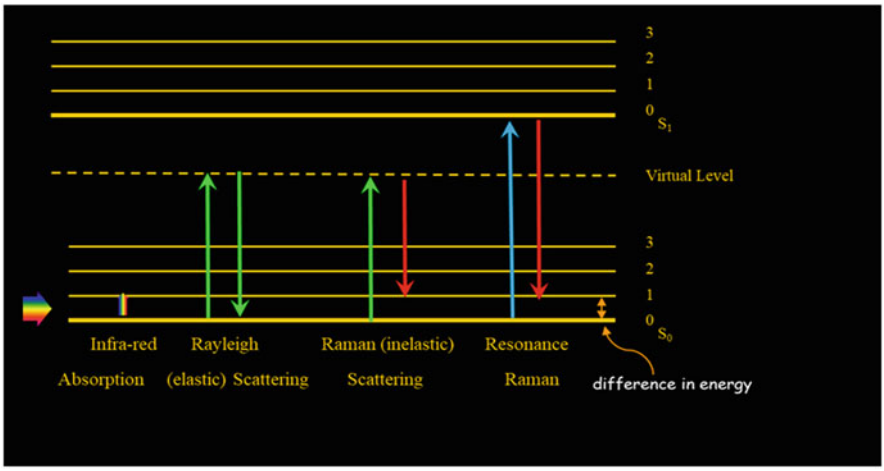

Fig. 20.2 Jablonski diagram for Raman, IR, and resonance Raman spectroscopic processes

Historically, the concept of spectroscopy originated in the process of dispersion of white light through a prism. Later, the concept was expanded to include any interaction with radiative energy as a function of its wavelength or frequency. "I propose this evening to speak to you on a new kind of radiation or light emission from atoms and molecules." With these farsighted words, Professor C. V. Raman from the Indian Association of Cultivation of Science, Kolkata, began his lecture in the South Indian Science Association in Bangalore on March 16, 1928. He described his work involving around 60 liquids whose wavelength changed upon interaction with light, later on known as "Raman effect" (Raman and Krishnan 1928). In 1930, he was awarded Nobel prize for this discovery. In the next 10 years, there were a number of publications mostly by the physicists to understand "Raman effect" (Long 2002; Smith and Dent 2005). When light impinges on a molecule, most photons are elastically scattered. This means that the scattered photons possess the same energy as the incident photon. This process is commonly known as Rayleigh scattering. Simultaneously, another process takes place where a small fraction of the photon, ~1 in 10^7, interacts with the molecule and an energy exchange takes place, wherein the optical frequencies of the scattered photons are different from the frequency of the incident photons. The process leading to this inelastic scattering is termed the Raman effect.

Raman scattering is a two-photon absorption-emission process as the incident photon is different from the scattered photon. The difference in this energy corresponds to the bond vibration of the molecule. Figure 20.2 describes the Raman process. The Raman shift, $\overline{\nu}$, in wavenumbers (cm^{-1}), can be numerically represented as follows:

$$\overline{v} = 1/\lambda_{\text{incident}} - 1/\lambda_{\text{scattered}}$$

where $\lambda_{\text{incident}}$ and $\lambda_{\text{scattered}}$ are the wavelengths (in cm) of the incident and Raman scattered photons, respectively.

A plot of intensity of scattered light versus the energy difference is a Raman spectrum.

Although powerful, the technique lay dormant for many years due to the inherent weak nature of the inelastic scattering process. The years after the invention of lasers prompted Raman spectroscopic studies in all areas of science and engineering. This was majorly attributed to the lasers as an intense light source coupled with the progress in the development of spectrometers, gratings, and detectors (Fig. 20.3). One key development in Raman spectroscopy took place in the 1970s with the integration of an optical microscope to a Raman spectrometer resulting in the development of Raman microspectroscopy. This opened up avenues for analysis of biomolecules such as cells, tissues, and cellular components with high spatial resolution.

Over the past few decades, different types of biological systems have been analyzed in different conditions with the help of Raman microspectroscopy. Relatively advanced techniques like resonance Raman spectroscopy (RRS) (Clark and Dines 1986), surface-enhanced Raman spectroscopy (SERS) (Schlücker 2014), coherent anti-Stokes Raman spectroscopy (CARS) (Evans and Xie 2008), tip-enhanced Raman spectroscopy (TERS) (Zhang et al. 2016), spatially offset Raman spectroscopy (SORS) (Matousek et al. 2005), and Raman optical activity (ROA) (Barron et al. 2004) have contributed in garnering sensitive selective and specific information. Many other novel Raman-based methods are also being studied such as ultrafast Raman loss spectroscopy (URLS) (Umapathy et al. 2009) and universal multiple angle Raman spectroscopy (UMARS) (Sil and Umapathy 2014) in the last decade to obtain molecular specific information in time and space domain. Use of chemometrics (multivariate analysis and statistical analysis) (Gautam et al. 2015) in the vibrational spectroscopic data has led to better understanding and interpretation of the experimental results.

20.2 Raman Spectroscopy and Bacterial Detection

Raman microspectroscopic techniques are widely applied in understanding various biochemical changes in different kinds of biological samples such as cells, tissues, bacteria, and biofluids. It has been applied to diverse applications such as identification of bacteria (Popp and Bauer 2015), understanding growth- and stress-related changes in bacteria (Hlaing et al. 2016; Mukherjee et al. 2020a, b), understanding fundamental processes of cells such as cell division and apoptosis (Panikkanvalappil et al. 2014), cell-drug interaction (Dutta et al. 2015), etc. The technique has been used in pathogen detection in a number of studies and has potential to be used as a field deployable system. The major biomolecules in the bacterial cell and their subunits along with an average percentage composition of the dry cell weight is as

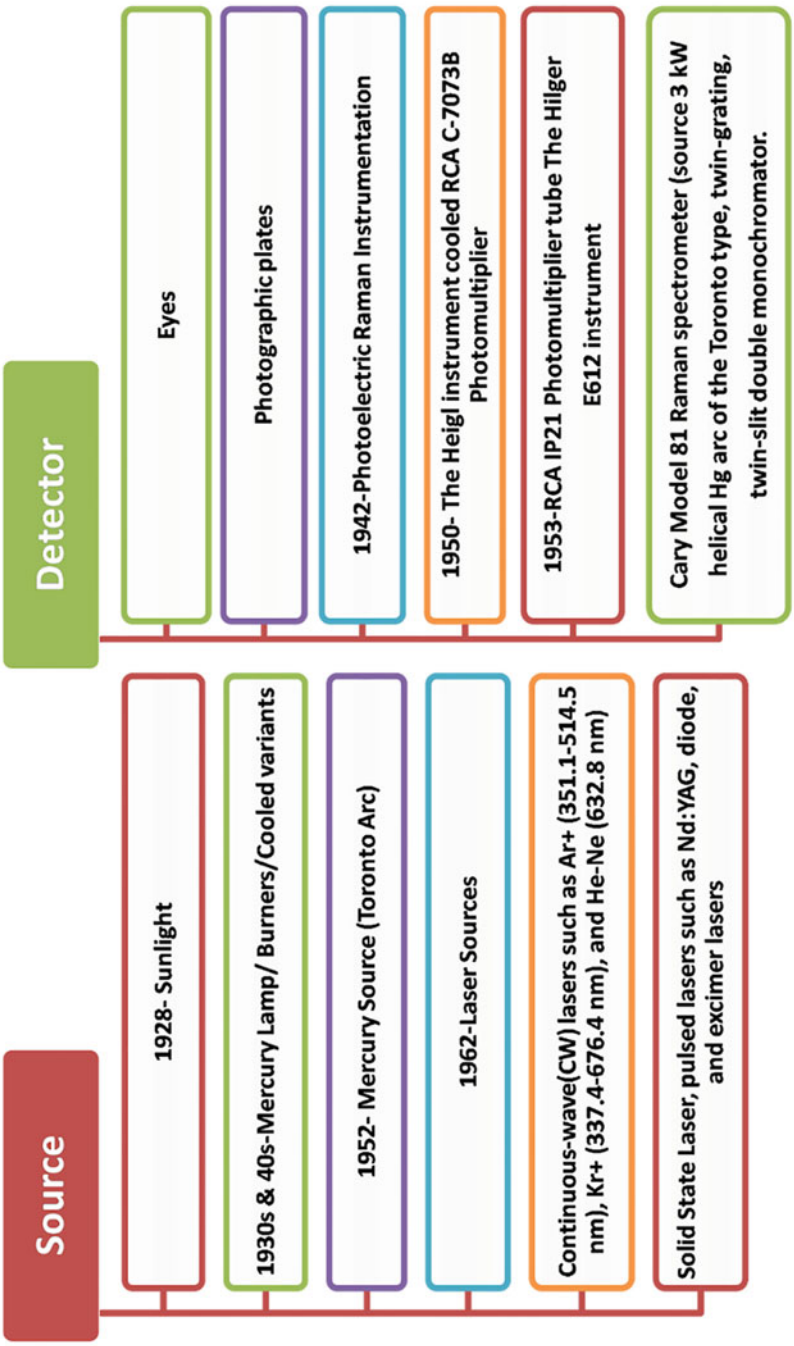

Fig. 20.3 The development of source and detectors for Raman spectroscopy

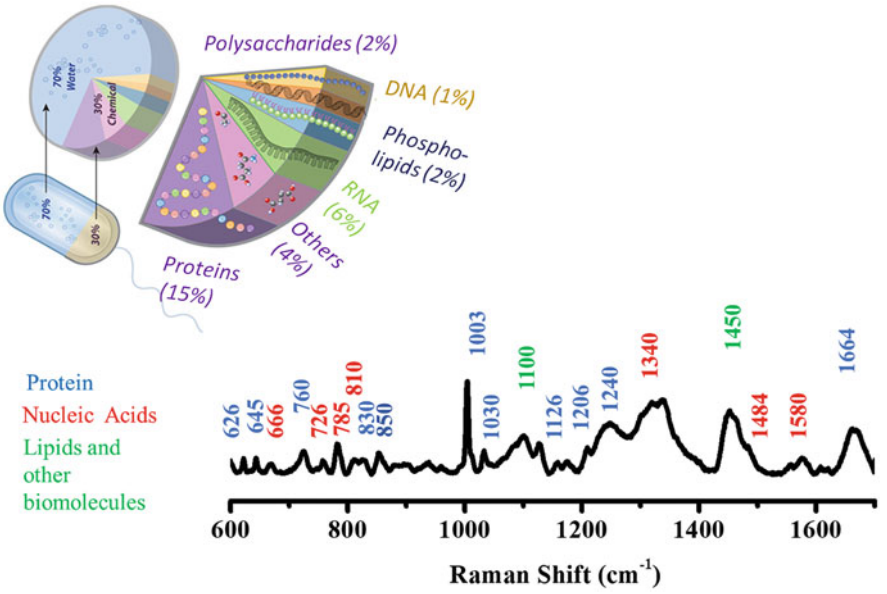

Fig. 20.4 Biochemical constituents of bacteria and Raman spectra of bacteria coming with contribution from different constituents

follows: proteins, which is an integral constituent of cell wall, cell membrane, pili, flagella, ribosomes, and enzymes in the cytoplasm take up ~55%. Ribonucleic acid (RNA) takes up ~20.5% of the dry weight. This is followed by lipids, polysaccharides, lipopolysaccharides, and deoxyribonucleic acid (DNA) which constitute ~9, 5, 3.4, and 3.1% of dry weight, respectively. A representative Raman spectrum of a bacteria is shown in Fig. 20.4.

These biomolecules are composed of numerous bonds, and Raman spectroscopy, in principle, can probe the structure of the bond vibrations in these molecules. Subsequent sections present different Raman-based techniques employed for bacterial detection. In addition, the challenges with respect to sampling, instrumentation, and data processing have also been explained for young spectroscopists.

20.2.1 Surface-Enhanced Raman Spectroscopy (SERS)

One of the greatest challenges of Raman spectroscopy is the low sensitivity. Only one in 10^7 photons are inelastically scattered resulting in the demand for increased concentration of the analyte to obtain a spectrum. However, this problem is addressed through the enhancement of Raman signals by using surface plasmon of nanoparticles. Precisely, surface-enhanced Raman spectroscopy (SERS) refers to Raman spectroscopy performed in the presence of metal nanoparticles in order that the surface plasmons of the nanoparticle can couple with the incident electric field

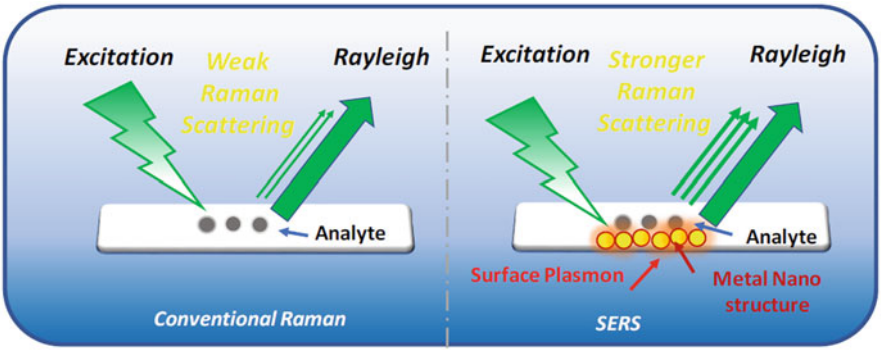

Fig. 20.5 Schematic depiction of conventional Raman and SERS technique

resulting in an increased resultant field to probe the molecular vibrations (Fig. 20.5). There are two mechanisms by which this enhancement can happen, chemical enhancement and electromagnetic enhancement. These mechanisms are discussed briefly in the subsequent paragraph. The enhancement factor due to this phenomenon can be as high as 10^6 to 10^{15}, making this technique an important one in detection of analytes in lower concentrations. The first SERS spectra were recorded on 1976 (Fleischmann et al. 1974), and since then the technique has been used in a wide variety of applications starting from explosive detection to pathogen detection (Prakash et al. 2019).

Plasmons, which are collective movements of interacting particles such as electrons, occur on the surface of the metals (Nayak et al. 2014; Ru and Etchegoin 2009). The collective oscillations of the plasmons are induced when the electron cloud of the metal interacts with the incoming electromagnetic (em) radiation leading to its excitation ("Introduction to nanoscience" 2008). The plasmon location (λ_{max}) and its strength depend upon the wavelength of the incident em radiation, the particle composition, its size, shape, orientation, etc. The following equation describes the plasmon frequency:

$$\omega_p^2 = N_e e^2 / \varepsilon_0 m \tag{20.1}$$

where,

ω_p = the plasmon excitation frequency
e = the electron charge (1.6×10^{-19} C),
N_e = the electron density in reciprocal cubic meters.
m = mass of the electron (9.1×10^{-31} kg),
ε_0 = vacuum permittivity of free space (8.8×10^{-12} Fm^{-1}).

It is evident from the above equation that metals with a higher electron density would have plasmons shifted to the blue part of the spectrum relative to metals with low electron densities. Another manifestation of the above equation is that as the

particle size of the nanoparticle diminishes, λ_{max} shifts to the blue region indicating an apparent increase in the quantum confinement of the electron. When the excitation is confined to the near surface of the metal, it is called a surface plasmon. Surface plasmons can either be propagating or localized, e.g., on the surface of a spherical particle. When the surface electrons are exposed to the electric field of the light, the free charges are displaced producing a polarization field. In case of particles smaller than the wavelength of incident radiation, the nanoparticle experiences a uniform or homogeneous field giving rise to one resonance. However, as the particle shapes and sizes vary, multipolar charge distributions can be induced. The displacement of the electron clouds become inhomogeneous even in the case of spherical particles of size >40 nm. Therefore, the strength of the plasmon oscillation would be determined by the shape as well as the orientation of the metal nanoparticle (Np). This implies that the more pointed a nanoparticle is, the stronger the opposing field becomes. It is therefore desirable to synthesize or fabricate Nps with a high aspect ratio or with many facets.

The amplification of the Raman signals in the SERS process has been attributed to two mechanisms, namely, the electromagnetic enhancement and the chemical enhancement (Campion and Kambhampati 1998). The electromagnetic field of the light at the surface can be greatly enhanced under conditions of surface plasmon excitation; the amplification of both the incident laser field and the scattered Raman field through their interaction with the surface constitutes the electromagnetic SERS mechanism. Chemical enhancement occurs when the adsorbed analyte molecules have inherent charge transfer capability. For instance, molecules with a lone pair of electrons show the strongest SERS signals, e.g., pyridine.

20.2.1.1 Electromagnetic Enhancement Mechanism

The electromagnetic enhancement occurs due to the induced dipolar and higher-order resonance of the surface plasmon on the metal Np. In a nutshell, the electromagnetic enhancement mechanism can be explained in the following manner:

Consider a spherical particle whose size (radius) is much smaller than the wavelength of the incident em radiation. This particle, therefore, experiences a uniform field around it which can be approximated within the Rayleigh regime. The field that will be induced at the surface of the particle shall be proportional to the incident electric field (e.g., the laser source) and can be represented as follows:

$$E_{\text{induced}} = \{[\varepsilon_1(\omega) - \varepsilon_2]/[\varepsilon_1(\omega) + 2\varepsilon_2]\}E_{\text{laser}} \tag{20.2}$$

where

$\varepsilon_1(\omega)$ = the complex, frequency-dependent dielectric function of the metal
ε_2 = the relative permittivity of the surrounding medium.

At resonant condition,

$$Re\ \varepsilon_1(\omega) = 2\varepsilon_2 \tag{20.3}$$

Therefore, at resonant condition, there is an enormous increase in the local field that is experienced by the molecule adsorbed/in the vicinity of the metal Np. The factor 2 from the equations is for a spherical particle. This equation can be generalized for other shapes of nanoparticles as well. Coinage metals (e.g., Au, Ag, Cu) are the most favored candidates for SERS substrates as these support plasmons in the visible region which is usually the laser wavelength employed in the Raman spectroscopic studies. It should be mentioned here that for the SERS condition to be satisfied, the real part of the frequency-dependent dielectric function ($Re\,\varepsilon_1(\omega)$) of the metal should match the resonance condition, while the imaginary part ($\mathrm{Im}\,\varepsilon_1(\omega)$) should be close to zero (Campion and Kambhampati 1998). In addition, the field enhancement diminishes as the distance between the molecule (analyte) increases from the surface of the metal Np. The relation between the SERS intensity and the distance is given by the following equation:

$$I_{\mathrm{SERS}} = (r/r+d)^{12} \tag{20.4}$$

d = the distance of the molecule from the surface of the sphere,
r = the radius of the sphere.

Therefore, the enhancement arises due to the fact that intensity of the Raman scattering is directly proportional to the square of the induced dipole moment, which is the product of the Raman polarizability and the incident electric field. Due to the excitation of the localized surface plasmon of the metal nanoparticles, both the incoming and the scattered electric field are enhanced by E^4.

20.2.1.2 Chemical Enhancement Mechanism

The existence of the chemical enhancement mechanism as one of the SERS enhancement contributors has been a source of controversy partly because it is difficult to experimentally validate this effect and partly due to the existence of multitude chemical effects that may contribute to enhancements (Ru and Etchegoin 2009). In chemical enhancement effect, the Raman polarizability tensor is modified upon the adsorption of the analyte molecule on the surface of the metal Np. The modification may occur due to the formation of a charge transfer metal-adsorbate complex, which is also the most studied and so far accepted mechanism for chemical enhancement. This mechanism is represented in Fig. 20.6.

In the first case, when the analyte does not adsorb covalently on the metal, the electronic structure of the analyte is only slightly perturbed, which leads to a subtle change in the electronic distribution. Any change occurring in the electronic distribution is manifested as a change in polarizability of the particular Raman mode. In the second case, a surface complex may form. This formation can be either via direct covalent bonding of the analyte with the metal or by indirect binding assisted by the

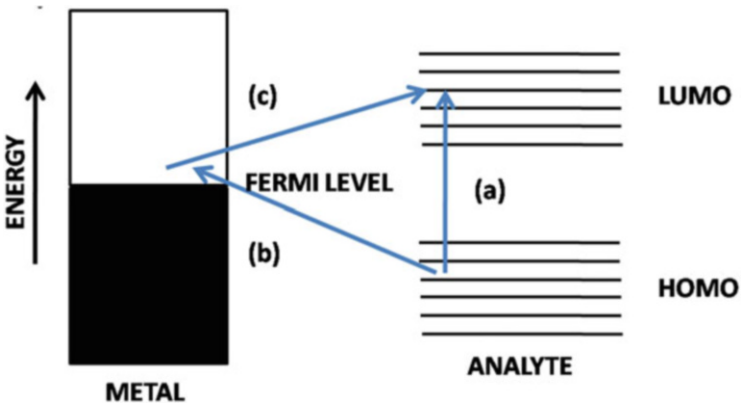

Fig. 20.6 Schematic representation of the chemical enhancement mechanism via charge transfer (inspired by (Ru and Etchegoin 2009). The laser energy can be in resonance with an electronic transition of the molecule-metal complex (right side of the figure (**a**)) or can have an indirect coupling (charge transfer) through the metal (**b** + **c**)

presence of electrolytes such as Cl^- ions. This latter process may alter the intrinsic polarizability of the molecule substantially. A third possibility, although rare, may also arise which involves the photo-driven charge transfer between the analyte and the metal. This can only happen when the differences of the energies of the Fermi level of the metal and the highest occupied molecular orbital (HOMO) or the lowest unoccupied molecular orbital (LUMO) match that of the laser. This may lead to a charge transfer between the HOMO and the unoccupied states above the Fermi level or the LUMO and the occupied state below the Fermi level.

20.2.1.3 Bacterial Detection Using SERS

SERS has been advocated as a sensitive biosensing technique for pathogen detection. It offers several advantages of minimum sample preparation, ease of operation, etc. as seen in conventional Raman spectroscopy. Additionally, it offers sensitivity up to single molecule detection. One of the reasons for the success of SERS for detection of biological samples is suppression of fluorescence background, which is otherwise observed in conventional Raman spectra. Due to the high sensitivity of this technique, low concentration of analytes can be used for SERS studies. This makes it a lucrative technique for clinical and field applications.

Most of the SERS literatures report use of gold (Au), silver (Ag), and/or bimetallic nanoparticles for bacterial detection. The spectra is collected by mixing the bacteria with the synthesized nanoparticles followed by drop casting on a suitable substrate (Jarvis and Goodacre 2004; Kahraman et al. 2007; Premasiri et al. 2005). Few studies have also reported an in situ synthesis of nanoparticles within the bacteria suspensions (Zhou et al. 2014, 2015). Depending upon nanoparticles (size, shape, optical properties), light wavelength, etc., the SERS signal may vary. Therefore, the SERS spectra often appears very different from the bulk Raman spectra of the same system (i.e., bacteria). In addition, the surface charges on the nanoparticles also determine the interaction of the nanoparticles with the bacteria.

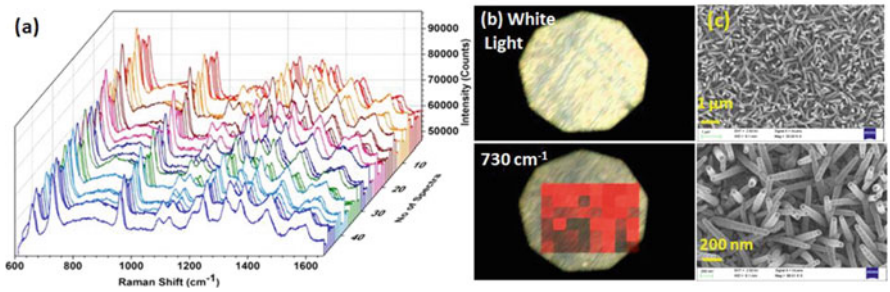

Fig. 20.7 (**a**) SERS of *E. coli* performed on Renishaw inVia Raman microscope using a 3D SERS substrate and 785 nm laser line with 10s acquisition, (**b**) white light image of the substrate and color mapping of 730 cm^{-1} peak, and (**c**) SEM micrograph of the 3D Ag-coated germanium SERS substrate

Most of the synthesized nanoparticles are negatively charged as are the bacterial surface. Therefore, positively charged or neutral nanoparticles can favor strong SERS signatures of bacteria. Recently, Prakash et al. have reported obtaining SERS signals of bacteria using label-free positively charged Ag/Au bimetallic nanoparticles. They have used multivariate analysis for discrimination (Prakash et al. 2019).

Endeavors are being made to obtain effective SERS substrates for detecting biological samples that also provide high sensitivity and are highly reproducible. In this direction, Nayak et al. have developed large area 3D silver nanobuds for SERS applications (Nayak et al. 2014, 2017, 2018). Figure 20.7 depicts SERS spectra of *Escherichia coli* (*E. coli*) bacteria obtained using 3D Ag nanowires using 785 nm laser source acquired for 10s. Note that the spectra are highly reproducible. The band at 656 cm^{-1} is attributed to protein (phenylalanine). According to some reports, this band has also been ascribed to C-S stretching. The band at 730 cm^{-1} is a characteristic signature band for SERS of bacteria in earlier literature reports. The origin of this band may be due to polysaccharide structure, as the outer cell wall of *E. coli* is made of lipopolysaccharide. It has also been attributed to the glycosidic ring mode of *N*-acetylglucosamine (NAG). NAG is one of the components of outer core of both lipopolysaccharide and peptidoglycan. The band at 958 cm^{-1} has been ascribed to C-N stretching and C-O stretching of proteins. The band at 1082 cm^{-1} is assigned for vibrational mode due to symmetric stretching of $O\text{-}P\text{-}O^{-}$ and C-O-C stretching of aliphatic esters, oligonucleotides, and polysaccharides. Other typical bands at 1241 cm^{-1} (amide III), 1324 cm^{-1} (protein), 1456 cm^{-1} (saturated lipids), and 1581 cm^{-1} (lipid) were observed in SERS of *E. coli.*

SERS is a powerful biosensing technique to obtain signals. If the challenges of reproducibility and signal variability are addressed, SERS has the potential to become an extremely useful tool in the field of medical diagnostics and other applications to detect pathogenic bacteria or infectious agents.

20.2.2 Resonance Raman Spectroscopy

Another offshoot of Raman spectroscopy employed for studying bacteria is resonance Raman spectroscopy. In the case of resonance Raman spectroscopy, the frequency of laser used for excitation must fall within the absorption range of the molecule of interest for the spectrum of the molecule to be resonance enhanced (Fig. 20.2). Presuming a mixture of two molecules A and B is excited with a laser which comes within the absorption peak of A but not of B, Raman peaks of A will be selectively resonance enhanced and of much higher intensity than that of B. Due to this, resonance Raman spectroscopy is widely applied for obtaining signatures from specific biomolecules like carotenoids. An advantage of this technique is that different information can be obtained from the same system by changing the excitation source. For example, consider a bacterium which is composed of proteins, lipids, DNA, and carotenoids. From an off-resonance spectrum (with 785 nm excitation), one can obtain information from the entire cell consisting predominantly of protein, lipid, and nucleic acid signatures. It is hard to find specific carotenoid signatures from the cells (bacteria) since they are present in low concentration. However, if the source is changed to green (514 nm) or blue (442 nm), the carotenoid signature gets enhanced as carotenoids absorb near 440 nm. Thus, one can obtain specific signatures only from carotenoid. Similarly, with a deep UV laser, specific signature from DNA can be obtained (Neugebauer et al. 2006) (as DNA has absorbance maxima at 280 nm). Verma et al. have shown application of resonance Raman spectroscopy in discriminating two closely related strains of bacteria (Verma et al. 2019). Bacterial cytochrome has been probed with resonance Raman spectroscopy using a 633 nm laser (Mukherjee et al. 2020a). Thus, resonance Raman spectroscopy helps in obtaining additional information from bacterial system.

20.3 Important Considerations for Obtaining Raman Signatures of Bacteria

In this section challenges confronted in obtaining Raman signatures of pathogens are discussed. The inherent inhomogeneity in a biological system such as bacteria makes the Raman studies complex (Kuhar et al. 2018). The oversensitivity of the Raman technique can be both advantageous and disadvantageous at the same time. With the technological advancements, today we have different forms of Raman spectroscopy including resonance Raman, SERS, ROA, CARS, TERS, and so on targeting at achieving better sensitivity, more detailed 3-dimensional information, or a better spatial resolution depending on the need of the problem and availability of resources (techniques). The multitude of options has advanced the use of Raman spectrometer in different types of samples starting from chemicals to materials to biology and medicine (Singh et al. 2012). Raman spectroscopy can be used to study pure biomolecules in their solid or solution form, inside the cells, tissues, or plasma, and even for direct in vivo applications in a patient. While proper and optimized use of a Raman spectrometer can provide us a lot of information about any biological

system, improper use can lead to less or wrong interpretation and sample damage. The choice of laser wavelength, power, exposure time, substrate, sampling, pre-processing, and post-analysis have a great impact on the outcome of any spectroscopic study. From a beginner's perspective, it is often troublesome and time-consuming to standardize the parameters. There are a few reports in the literature which discuss some of the parameters for biological sample analysis (Butler et al. 2016). More detailed information in this regard will be useful. Additionally, sampling and data handling for relatively newer techniques like SERS or ROA is a matter of concern. Data mining for a large number of human samples is another challenging task. Practically there are five steps involved in the experiment and analysis of vibrational spectroscopy as discussed in the subsequent section (1) sample preparation (sample thickness, sample casting, Raman substrate), (2) instrumental consideration (choice of laser, grating, objective, acquisition, and accumulation), (3) pre-processing (baseline subtraction methods, normalization, and smoothing algorithms) and post-processing, (4) data analysis, and (5) data interpretation (finding the right way). All these processes will be discussed in the next four sections.

The objective of this section is to collate all the different aspects of Raman spectroscopy, starting from instrumentation to sampling to data processing, analysis, and interpretation to get an overall idea of how Raman spectroscopy (and its derivative like SERS and RRS) can be potentially useful for bacterial study and the challenges faced therein.

20.3.1 Sample Preparation

For bacterial samples obtained in clinical settings, decontamination is a prerequisite step as it facilitates safe handling by eliminating their pathogenicity. Decontamination is generally achieved by means of chemical treatment, but the choices get limited when it is desirable to retain the integrity of biological structures and preserve molecules, cells, and tissues at a specific stage for further composition analyses. This is classically obtained by fixation, which also protects the biological components from decay during harsh treatment protocols such as dehydration, washing, and staining. Multiple approaches of sample fixation have been utilized for different applications, and fixatives such as ethanol, paraformaldehyde (PFA), and sodium azide (to a certain extent) have been tested for Raman spectroscopy previously on various bacterial species (Read and Whiteley 2015). Srividya et al. have suggested use of PFA over glutaraldehyde and sodium azide based on two specific parameters: (1) loss of cell viability and (2) retention of Raman signatures (Kumar et al. 2016, 2020).

Another method for pathogen detection can be for obtaining Raman signature of pure cellular components such as DNA (deoxyribonucleic acid) instead of considering the whole bacteria. This method of detection takes care of pathogenicity as well as the effect of sample fluorescence from other biomolecules. Additionally, this also avoids differences due to change in growth conditions.

Choice of proper substrate is important to obtain a good quality Raman spectra (Kerr et al. 2015) of complex samples like bacteria. With the widespread use of Raman spectroscopy from laboratory to clinics, there are a plenty of options available for Raman substrates. For recording spectra, the samples are kept inside a cuvette (for liquids) or on top of a slide (for solid/dried samples). For samples which have high Raman cross-sections, glass/quartz can be used for making this cuvette or slides (substrate, as it is commonly referred to). It is worth remembering that when a laser light is focused onto a sample, the focal volume is always a cone (vs a point, according to the popular notion). If the sample volume is less, then, some portion of substrate also falls within the focal volume, contributing to the overall collected signal. In case of samples having strong signals, this background signal does not play a major role, but in case of samples having weak Raman cross-sections (e.g., bacteria or any biological samples), these backgrounds may mask the signal from the sample. Hence, choosing a proper background-free substrate to cast the sample is important to avoid interference from the substrate.

Since metals are not Raman active, metal slides and foils are often used as substrate. In the literature, we can find metal foils and metal surfaces (Cu, Ni plates) being used as substrates for biological samples in many instances. There are complex sampling protocols in some cases for specific purposes.

Aluminum is one such metal which is used as a substrate for Raman studies. The problem with aluminum slides is their roughness and reflectivity which makes them less useful for bacteria as the bacteria may enter into the pores of the rough substrate. Polishing the substrate or using aluminum foil, which is much smoother, is a cheaper option (Cui et al. 2016).

However, aluminum foils might be problematic if the sample is not thick enough due to its high reflectivity. This is where the need for other sophisticated substrates like commercially available MgF_2 and CaF_2 is realized. These substrates are background free and do not interfere with Raman signal (Fullwood et al. 2013). Although these substrates are more expensive, compared to glass or quartz, they are reusable making them ideal for laboratory use. There is no difference between these two, if the sample is relatively thicker (less effect of background).

It is to be noted that substrate contribution decreases when higher NA objectives (50×, 100× compared to 20×) are used. In high NA objectives, the focal volume is smaller resulting in less background collection.

20.3.1.1 Single Vs. Bulk Bacteria

In case of bacterial study, Raman spectroscopy has reached the level of single bacterial detection (Ganapathy et al. 2014). Many of the bacterial Raman work are reported using single bacteria. However, analyzing the samples in bulk also has certain advantages. Especially considering single bacterial detection often comes at the expense of longer acquisition and higher laser power leading to higher chances of sample degradation. The need for a sophisticated substrate makes the process more complicated. Despite this, the spectra obtained from single bacteria often have low signal-to-noise ratio (SNR) and have a large background that requires involved pre-processing techniques before actual analysis.

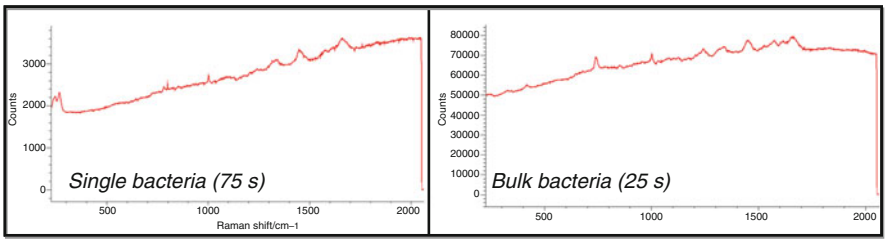

Fig. 20.8 Comparison of the spectral quality of a spectra recorded from a single bacteria and a bulk bacteria

Conversely, for analysis with bulk bacteria, the advantages are (1) easier to obtain spectra (technical reason), (2) detailed spectral information (tiny spectral information often gets masked in the single bacterial detection), (3) less chance of sample damage (due to lower laser exposure), (4) less time-consuming (technical advantage), and (5) biologically relevant (Q sensing).

As can be seen in Fig. 20.8, the spectral quality of the bulk bacteria is much better than single bacteria despite the fact that much longer time has been given for the acquisition of single bacteria. So, if a beginner is confused before starting a project on bacterial Raman analysis on whether to go for single or bulk bacterial analysis, then one may consider the following two aspects. To understand the physiological processes in bacteria (stress response, growth mechanism, antibiotic susceptibility, etc.), bulk bacterial samples can be probed. However, for detection purpose (detection and classification of pathogens, some bacterial borne diseases, etc.), single bacterial analysis is a clinical requirement.

20.3.1.2 Concept and Importance of Biological and Technical Replicates

Another point worth discussing in bacterial studies is the importance of technical and biological replicates (Blainey et al. 2014). When working with bacterial samples, data from only one batch of sample is never reliable. There might be some technical variations in two sets of samples, e.g., one sample being thicker than the other leading to more fluorescence background in Raman spectra or some impurity in the substrate where the sample is drop cast. Though these types of mistakes are undesirable, they are unavoidable in a practical scenario. To account for these heterogeneities, the concept of "technical replicate" is coined. In this, the same analyte is sampled (drop cast) in replicates for spectral measurement purpose.

Apart from that, the error might be in the preparation of sample itself. For example, while monitoring bacterial growth, in one batch of experiment, there might be some experimental error by the user or due to technical difficulties (e.g., more salt concentration in the nutrients while growing bacteria or non-maintenance of the temperature in the incubator). These errors are random and can cause a lot of variation in the obtained data. To account for these problems, it is always advisable to conclude from the average data of independent biological experiments,

known as biological replicate, where the entire experiment is set up from scratch preferably in different days.

20.3.2 Instrumental Consideration

20.3.2.1 Choice of Laser

The advancement of lasers has greatly benefitted the field of Raman spectroscopy. Choice of laser for excitation can lead to obtaining better information from a sample. Theoretically, Raman cross-section is higher for blue laser (due to $1/\lambda^4$ dependence), but due to fluorescence background signal is masked. Red lasers (785 nm, 830 nm) can be used in such cases. Additionally, red laser causes less sample degradation in case of biological samples. So, as a rule of thumb, for a molecule which does not fluoresce (which is not the case for bacterial samples), blue/green laser should be used. Otherwise, red laser is a better option.

Laser bandwidth, laser mode, and need for pulsed laser might be a criterion for few special experiments. For resonance Raman studies, laser of a particular wavelength might be a requirement for mode-selective signal enhancement. In literature different laser wavelengths have been used for bacterial study. Most of the reports uses green (532 nm) or red laser (785 nm).

Impact of Laser Power

Though discovery of high-power lasers was a great blessing for Raman spectroscopy, use of high laser power can have an adverse effect on the sample. Ideally, exposing the sample to less power for a longer time should serve the purpose. But practically, more exposure leads to collection of more background signal and more readout noise. Additionally, the method is time-consuming. Many a times it is safe to use higher power for higher wavelength laser, since samples do not get damaged with red laser, though prior knowledge of sample is important. The spectral quality obtained with a high-power laser (if the sample is not damaged) cannot be matched with a lower-power one, even when the exposure time is increased. Lower-power spectra are often noisy. In order to improve the SNR, one can increase the number of accumulations. However, giving more accumulations might lead to collection of more cosmic rays (as more time is required to collect the spectra).

As can be seen from Fig. 20.9, the spectra obtained with more laser power (Black line) have an improved SNR and less background. This was not the case when half of the laser power was attenuated, even though total exposure time was increased which resulted in collection of more background. When the laser power was attenuated further (10%), the spectral quality was visibly poor even with a four-fold increase in the spectral collection time, which leads to an additional problem of collection of cosmic rays.

Table 20.1 compares different methods of improving SNR and their pros and cons.

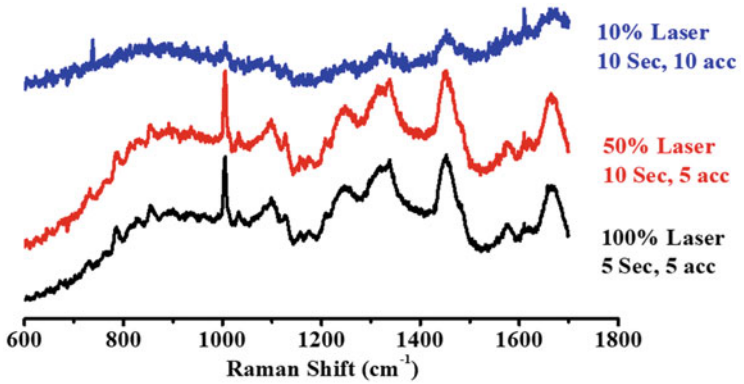

Fig. 20.9 Raman spectra obtained for a bacteria using 785 nm laser with different parameters. Black, Raman spectra obtained with 100% (~30 mW) laser power and 25 s total exposure (5*5); red, Raman spectra obtained with 50% (~ 15 mW) laser power and total 20 s exposure (10*5); blue, Raman spectra obtained with 10% (~5 mW) laser power and total 100 s exposure (10*10)

Table 20.1 Different methods to increase SNR

Methods	Advantages	Disadvantages	When to use
1. Increase in laser power	Not time expensive, does not add noise/ background	May be damaging for the sample	When prior knowledge about sample is available. The user is sure about sample photostability
2. Increase in exposure time	Does not add added noise	Time expensive, may lead to increase in the overall background and saturate the detector	When the entire signal of the sample including the background is low
3. Increase in accumulations	Not damaging for the sample	Time expensive, may add to the read-out noise	When time is not a concern for the overall experiment
4. Averaging over collected data from different points in space	Take cares of sample inhomogeneity	Might be time expensive than option 1 and 2	When the sample is expected to be inhomogeneous

20.3.2.2 Choice of Objective

Microscope objectives are a great addition to spectroscopy especially for biological samples since with the addition of microscope objectives in spectroscopic techniques have opened the door towards giving specific spatial information in a heterogeneous sample. For using objectives in Raman spectroscopy, a major concern has been increased power density in case of higher magnification objectives. One needs to be careful in using 100× objective while using 442 nm or 514 nm laser since the increased power density may be damaging to the sample at these wavelengths.

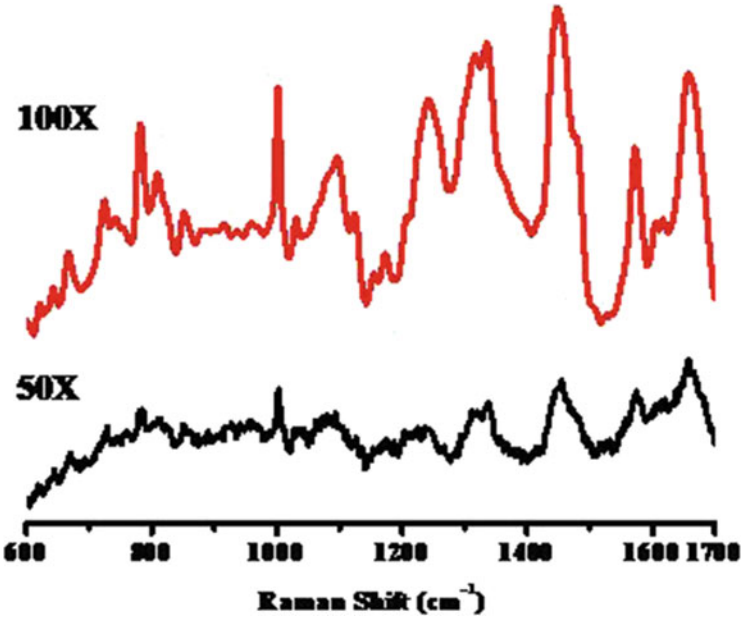

Fig. 20.10 Comparison of spectral background and SNR when the spectra are recorded with different objectives (100× and 50×)

Table 20.2 Spot size in different magnifications of objective[a]

Objective	NA	Spot size (μm)	Resolution (μm)	Focal volume (μm^3)	Power density ($mW/\mu m^2$)
100×	0.9	1.06	0.53	2.01	123.75
100×L	0.75	1.28	0.64	4.17	85.94
50×	0.75	1.28	0.64	4.17	85.94
50×L	0.5	1.92	0.96	21.13	38.19
20×	0.4	2.39	1.20	51.59	24.44
20×L	0.35	2.74	1.37	88.01	18.72
5×	0.12	7.98	3.99	6369.31	2.20

[a]The calculations are done for 785 nm laser where laser power on the sample is 110 mW

However, the chances of damage decrease while using red laser (e.g., 785 nm) since biological sample normally does not absorb in this wavelength. Although the effect of local heating may be prevalent in damaging samples, such cases are rare. Figure 20.10 shows effect of using two different objectives in the spectral quality of a Raman spectrum. Table 20.2 provides a comparative overview of laser power on spot size, resolution, focal volume, and power density of the sample for different objectives. These parameters are calculated using the following formulae:

$$\text{Laser spot size}\,(\mu\text{m}) = 1.22 * \lambda\,(\text{nm})\,\text{NA};$$

where λ = wavelength of excitation and NA = numerical aperture of the objective

$$\text{Resolution}\,(\mu\text{m}) = \text{Laser spot size}\,(\mu\text{m})^2$$

$$\text{Focal Volume}\,\mu\text{m}^3 = 3.14 * \text{Resolution}\,2 * \text{Rayleigh Range}\,(\mu\text{m})$$

where Rayleigh range (μm) = 2*3.14*Resolution2λ (nm)*1000

$$\text{Power density}\left(\text{mW}\mu\text{m}^2\right) = 3.14 * \text{Power at the sample}\,(\text{mW}) * \text{Resolution}\,(\mu\text{m})^2$$

If the sample under study doesn't get damaged, a 100× (higher magnification) objective offers additional advantages apart from obtaining better spatial information: (1) increased power which improves SNR and (2) lesser background (as NA is higher) and thus improved quality of spectra. The effect is more prominent when sample concentration is low (e.g., a thin layer of bacteria).

However, for SERS, if the spatial information is not required, lower magnification objectives are better since it allows less exposure of the sample to laser power and more spatial averaging (which means more reproducible condition). For liquid samples, immersion objective or a microfluidic device to overcome spectral artifacts due to the presence of bubbles and surface tension can be used. This has permitted the analysis of biofluids, such as blood plasma, sputum, saliva, and urine, in diagnostic studies (Mitchell et al. 2014).

20.4 Pre-Processing and Post-Processing

When studying biological systems, spectral data processing is a crucial step in spectroscopic data analysis (Bocklitz et al. 2011). The information one can obtain from the acquired data depends not only on the quality of spectra but also on an individual's knowledge and understanding of data processing and analysis. The incorporation of chemometrics in vibrational spectroscopy has made the technique yield more information and broadened its application making the process more involved and complex.

The raw data obtained from a sample has contributions from some instrumental parameters like laser intensity fluctuation, CCD noise and sampling protocol (sample thickness, substrate used), environment (cosmic rays), or the inherent properties of the sample (fluorescence background), which the user might not be interested in. Thus, an experimental Raman spectrum is a convolution of the signatures from the biomolecules and many external factors as has been shown in Fig. 20.11.

The information from a Raman spectrum can be obtained from three main parameters: peak position, peak intensity, and peak width. However, for a complex biological system, obtaining straightforward information from these parameters is

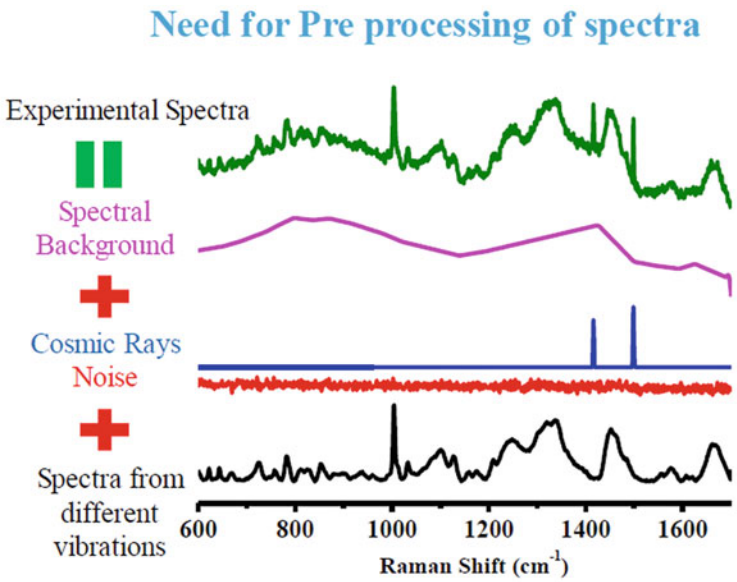

Fig. 20.11 Depiction of spectral pre-processing for meaningful data analysis

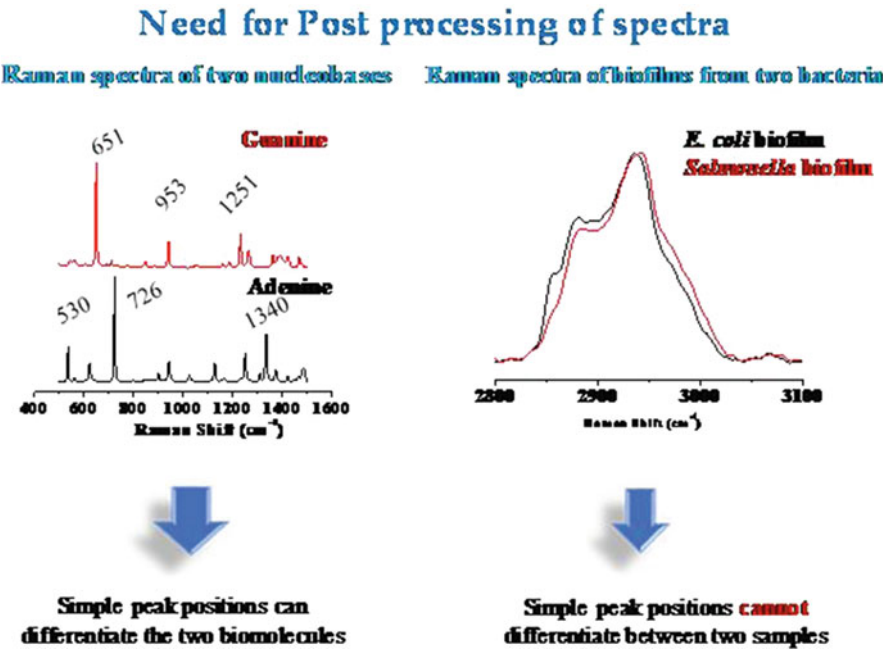

Fig. 20.12 Depiction of spectral post-processing for meaningful data analysis

often not trivial. To garner information hidden in the spectra, different post-processing methods are needed to be employed, e.g., derivative analysis, peak deconvolution, etc. These types of analysis help in better understanding and interpretation of the spectra.

Figure 20.12 demonstrates that while it is easier to differentiate the signatures between two simple biomolecules (two differentiate nucleobases) only based on peak positions, for biofilms from two different bacteria, which are relatively complex system, differentiation only based on peak analysis is not trivial. However, when the second derivative of the spectra of these two biofilms is taken, these differences between the two bacteria biofilms becomes prominent, as can be seen from Fig. 20.13. Thereafter, with the help of curve deconvolution, the individual peaks can be quantified.

20.4.1 Data Analysis

The spectral data analysis is the most crucial part of bio spectroscopic analysis. In case of vibrational spectroscopy, the data analysis is mostly classified into two categories, as described below.

20.4.1.1 Univariate Analysis

Univariate analysis, as the name suggests, involves only one variable at a time for analysis. Peak position, intensity, area, and full width at half maximum (FWHM) are some of the parameters to be evaluated in univariate analysis. Apart from considering only one peak, sometimes, the ratio of two peaks is also considered, which is known as ratiometric or bivariate analysis. Univariate analysis is important for biochemical understanding of the system.

There are different ways of quantifying a peak for univariate analysis—(a) quantification of intensity, (b) quantification of area under the peak, and (c) quantification of area under the fitted curve.

For a very sharp peak like Fig. 20.14a, any of these methods can be used. For a peak like Fig. 20.14b, where the peak is not very sharp and finding the highest position in the peak is not very trivial and/or the highest position varies upon different conditions in the experiment, peak area should be used to quantify the entire population. Here, Amide I peak is shown, which has a contribution from alpha helix (1657 cm^{-1}) and beta sheet (1672 cm^{-1}). The relative amount of these two positions changes according to the course of experiment. However, if the analyst wants to quantify the amide I band, the best method would be taking the entire area under the entire amide I band.

Another advantage of taking area under the curve is that sometimes due to instrumental fluctuations, the peak positions may vary. Due to this, the peak might shift by 1 to 2 wave numbers. For example, if the 1003 cm^{-1} peak shifts to 1002 cm^{-1}, the peak intensity at 1003 cm^{-1} will be less than 1002 cm^{-1}. So, comparing this 1003 cm^{-1} intensity with the other 1003 cm^{-1} intensities where the maxima were originally at 1003 cm^{-1} will be wrong. Taking the entire area

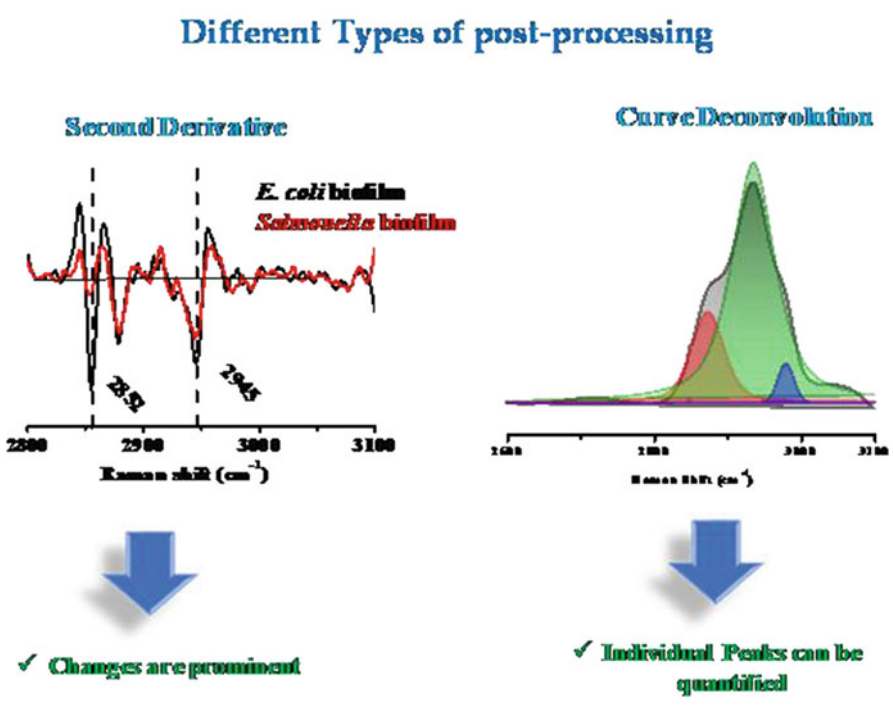

Fig. 20.13 Depiction of spectral different types of post-processing for meaningful data analysis

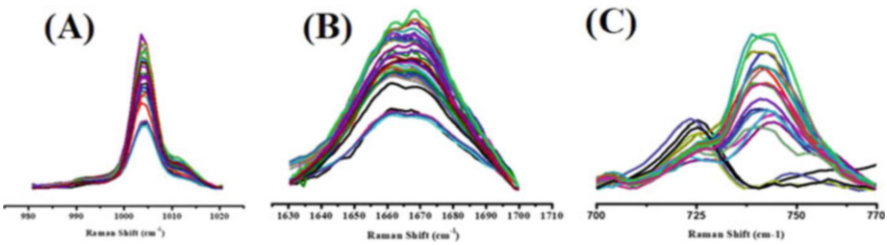

Fig. 20.14 Different peak shapes obtained from Raman spectrum

under 1003 cm^{-1} peak could be of use in these cases. That's why the standard deviation is less when a peak is quantified with area compared to the standard deviation when a peak is quantified with intensity. Another advantage of quantifying with area is that the peak shift due to instrumental changes is taken care of in this case.

Another scenario may arise, such as in the case of Fig. c (Fig. 20.14c), where two or more peaks get convoluted to form one peak. The relative amount of these peaks may change under different conditions of the experiment. For example, the peak shown in Fig. c is clearly a convolution of two peaks (726 in the left and 740 in the right). Taking the intensity of any of these peaks is not a correct way to quantify any of these two peaks. Taking the area under the curve will be inadequate since the range for both these peaks is not clear. In these cases, the peak should be quantified, using curve fitting. Performing the curve fitting analysis can also provide information from the spectra in the higher region where different stretching frequencies from CH_2 and CH_3 get convoluted forming a broader peak. The information for each peak can be obtained after curve fitting the region as shown in the following figure (Fig. 20.15).

20.4.1.2 Multivariate Data Analysis

Multivariate data analysis methods refer to the analysis techniques where more than one variable is used at a time to interpret the data. Spectroscopic data consists of thousands of variables (wave numbers) and measurements (subjects/samples). Multivariate data analysis helps in better handling large dataset and provides a platform towards automation and making it user-friendly. The main aim of multivariate data analysis technique is to interpret the relationship between the variables considering different statistical parameters like mean, median, mode, standard deviation, variance, co-variance, correlation, distance matrix and corresponding eigenvalues and eigenvectors (Geladi 2003; Geladi et al. 2004; Lavine n.d.; Gautam et al. 2015).

There are different kinds of multivariate analysis techniques available which can again be classified into two groups. Unsupervised multivariate analysis like principle component analysis (PCA), multivariate curve resolution (MCR), etc. and works without prior knowledge of the existing groups in the samples. These algorithms reduce the dimension of the entire dataset resulting in the distribution of the dataset along a new axis which is calculated considering the highest variation of

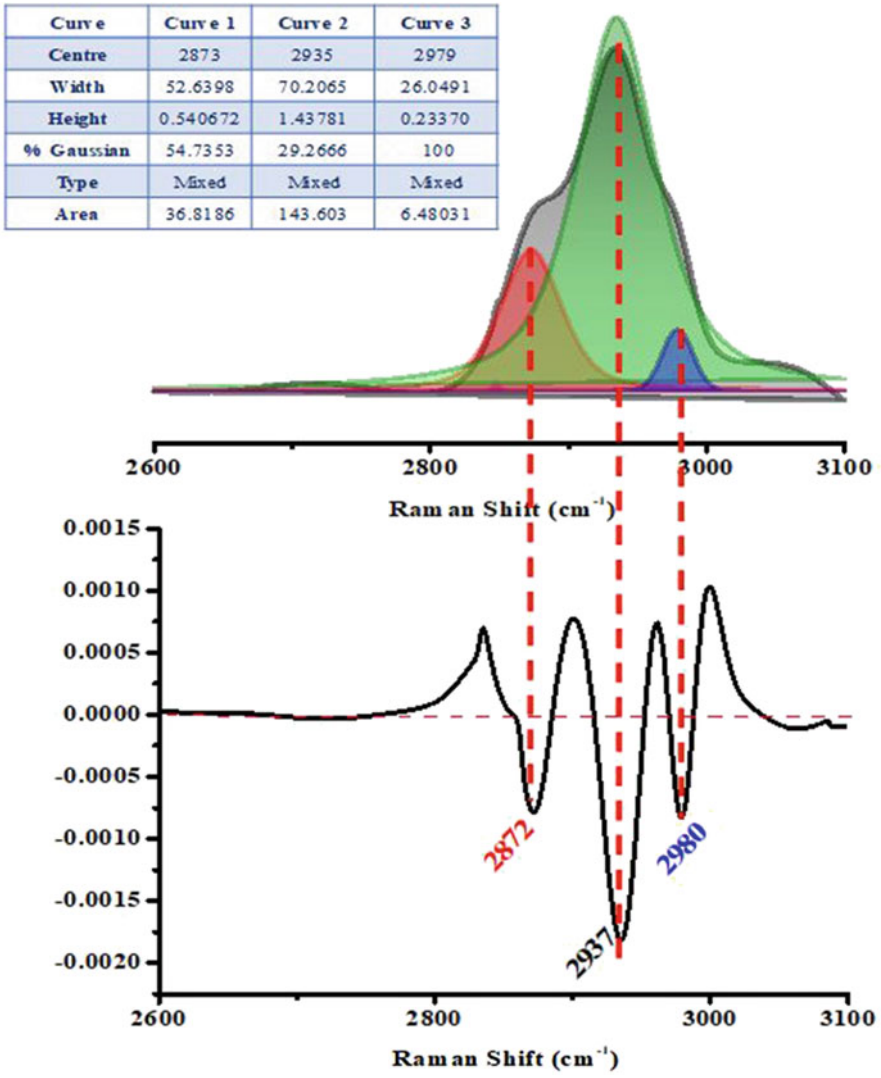

Fig. 20.15 Curve fitting analysis of the higher region (lipid region of the spectra)

the data. PCA separates the samples based on the abstract variables, while MCR tries to look at the phase change among the variables thus separating the data on a more clinically significant basis. PCA has been demonstrated with an example in Fig. 20.16. As can be seen, PCA has been applied to a dataset having 4 different types of bacteria. In this case an unsupervised analysis has been performed, where the software has no prior information about the grouping present in the sample. This

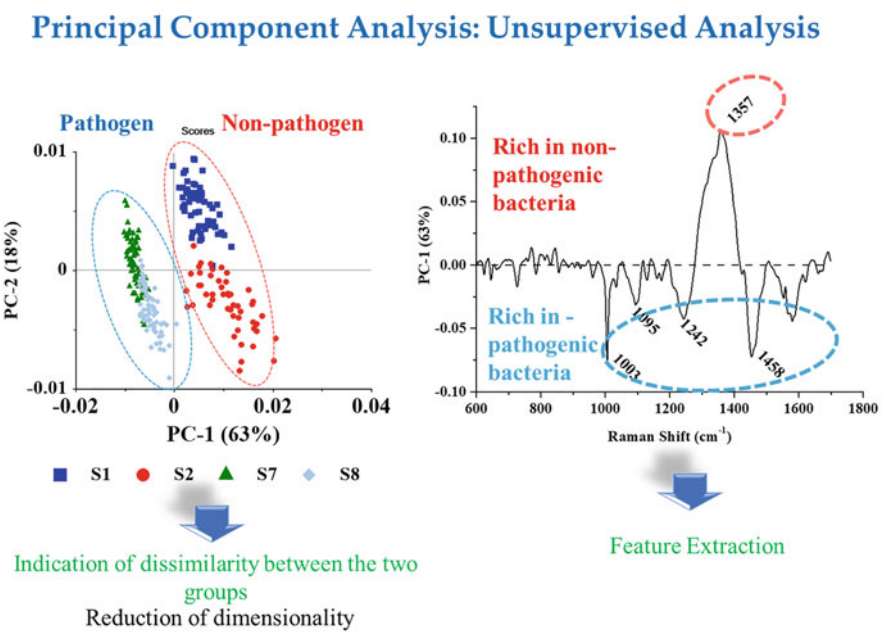

Fig. 20.16 Application of PCA (principle component analysis) in bacterial Raman study

method cannot classify the bacterial sample or predict the grouping of another sample.

On the contrary, in the case of supervised multivariate analysis (Fig. 20.17), like linear discriminant analysis (LDA), and support vector machine (SVM), the software is given the details of the groups in the sample space. The result of this type of analysis is classification and prediction of the groupings in the data. The prediction is reported with a cross validation from blind dataset. That means, with the help of this type of analysis, a model system can be built based on existing data which can be used for predicting the groups or class of the future data. Thus, these techniques help in automation of classification and prediction techniques. The working principle of multivariate analysis is based on complex statistical, mathematical, and computational concepts. The readers may refer to other published articles for further details.

The most decisive part of a spectroscopic study is the data interpretation. Although Raman spectra of molecules can be quite informative, interpretation of these spectra can be complicated due to various reasons including contribution from different biomolecules in the same spectra region. The skill of data interpretation is required at every stage, starting from assigning a peak to a particular component to finding out the reasons for a change associated with each marker. Since a particular vibrational peak can have its origin in different biochemical constituents, assigning the dominant contribution should be based on user's understanding of the system or comparison of the trend of the peaks with other peaks from that constituent. The certain changes in a peak's parameters can also have different implications. For

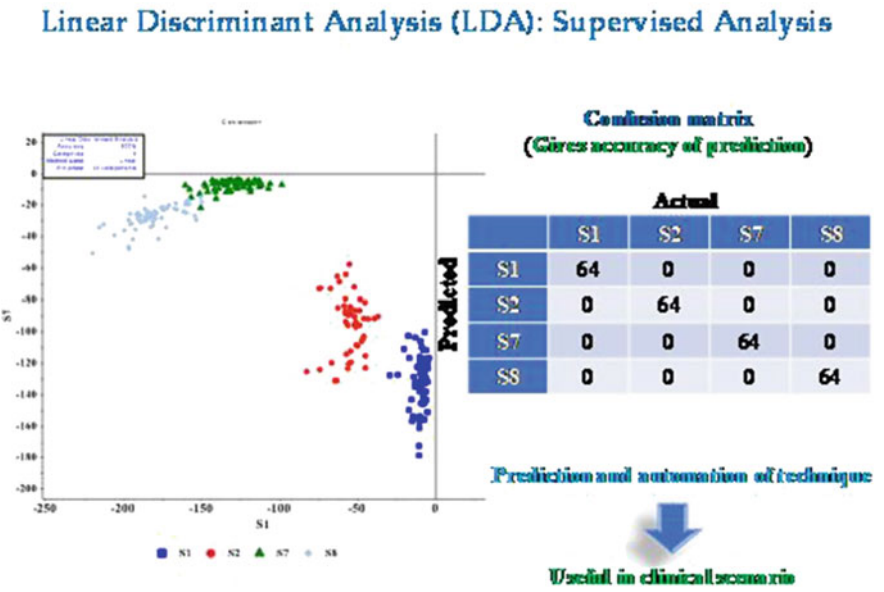

Fig. 20.17 Application of LDA (linear discriminant analysis) in bacterial Raman study

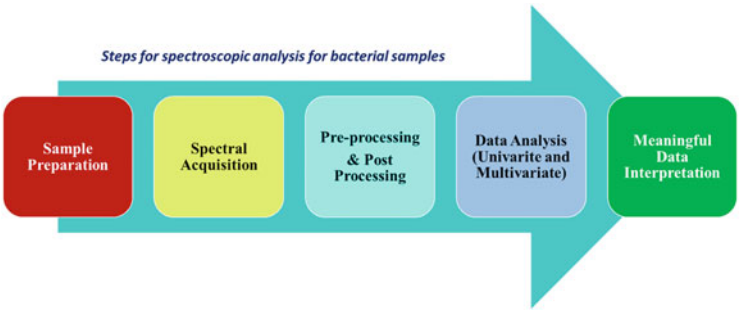

Fig. 20.18 Multistep approach of a bacterial Raman analysis

example, an increase in the peak intensity may arise due to an increase in the amount of that component or a conformational change or a relative decrease in other components. In case of interpreting a multivariate data also, the extracted features might be overestimated. This is when the analytical skill, validation from different techniques, analysis methods, and knowledge on the system of interest make the interpretation accurate.

Figure 20.18 provides an overall flow of the Raman spectroscopic experiment.

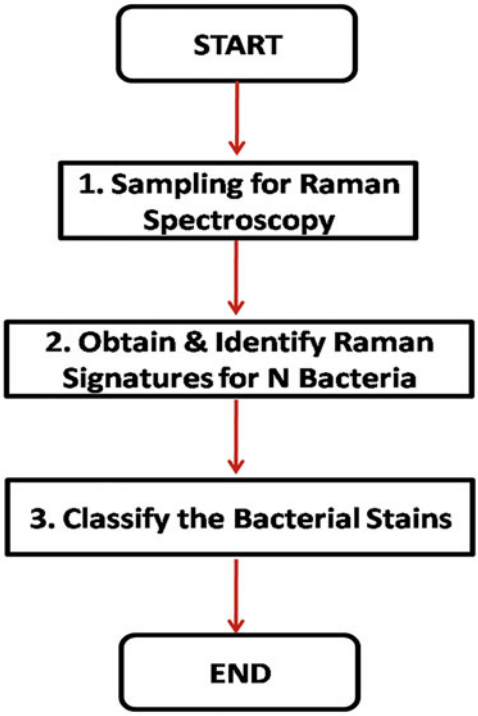

Fig. 20.19 Flowchart representation of the entire process

20.4.2 Instrumentation Aspects

Commercial Raman systems have been divided into two broad categories as (1) the large laboratory instruments (for research purposes, sub-micrometer resolution, high-speed imaging, etc.) and (2) the handheld systems. All or most of the handheld Raman systems have been mainly catered for the identification of materials such as pharmaceuticals, narcotics, bulk material identification, etc. Additionally, the spectral resolutions of the handheld systems vary from 8 cm^{-1} to 20 cm^{-1}. These systems require small, lower-power consumption lasers, miniature monochromators, and detectors (Lieber and Mahadevan-Jansen 2007; Vagnini et al. 2017). However, these off-the-shelf equipment are not suitable for pathogen detection. Most of such product catalogs present the Raman spectra of either solvent or powder materials or pharmaceutical tablets.

In order to develop a portable Raman unit for bacterial identification, the following flowcharts may be considered to depict the overall processes (Figs. 20.19, 20.20, and 20.21). Briefly, the bacterial culture to be tested should be sampled in accordance to the protocols for Raman spectroscopic experiments as mentioned in the earlier sections. Thereafter, the bacterial sample should be subjected to Raman spectroscopic analyses. Optimization experiments need to be carried out to obtain the most suitable laser source, laser power, acquisition time, accumulations, numerical aperture, etc. After the optimization, Raman spectroscopic studies should be

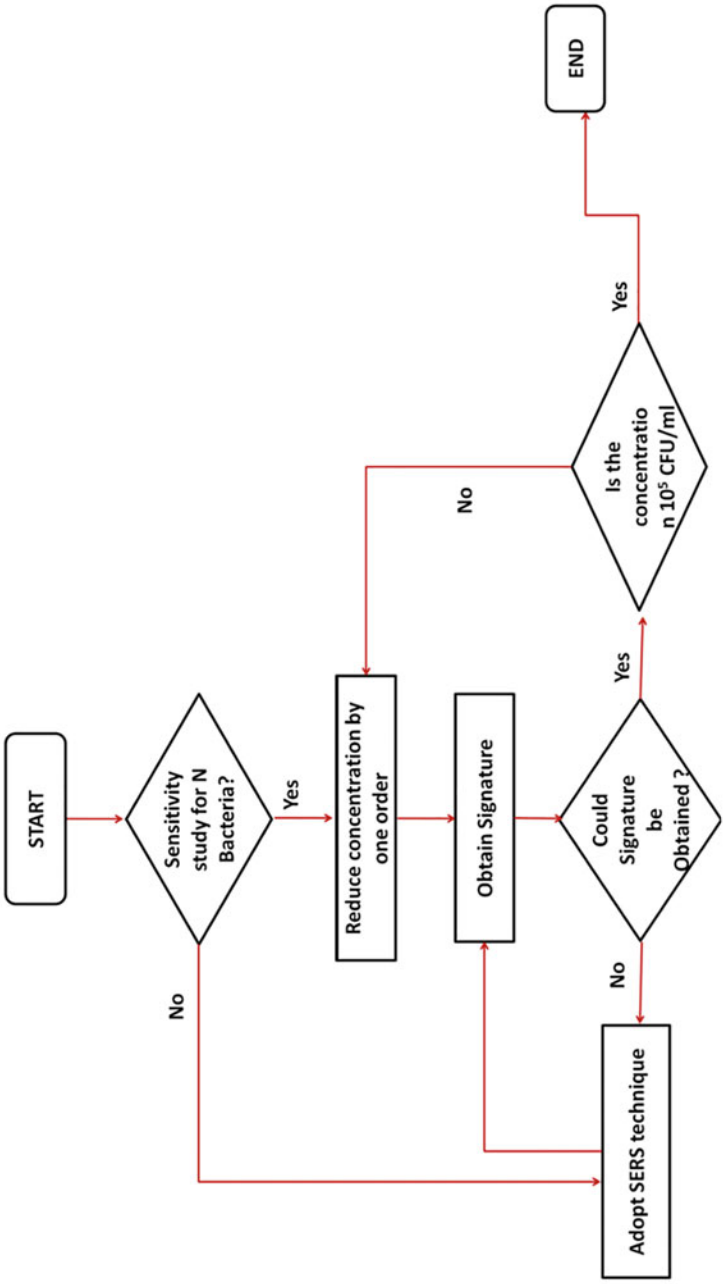

Fig. 20.20 The identification of bacteria using various Raman spectroscopic methodologies

carried out on the bacterial samples. Large number of data should be acquired to ascertain the homogeneity of the Raman signals throughout the sample. Once the signals are obtained at higher concentrations, data should then be processed and subjected to statistical analyses such as univariate and multivariate approaches for classification of the bacteria. This is necessary for the development of Raman spectroscopic equipment for the identification of bacteria. In addition, to obtain Raman spectroscopic signatures at low concentration of bacteria surface-enhanced Raman spectroscopic (SERS) technique as already explained may be a suitable alterative for analysis in field environment such as defense scenarios or for clinical samples (Fig. 20.21).

The preceding sections provide an overview of the Raman spectroscopic process and the most important considerations to obtain Raman signatures of biological systems, especially bacteria. The efforts to develop a portable Raman microscope for pathogen detection have been realized successfully. Figure 20.22 shows a benchtop Raman spectrometer developed by IISc and has been named RAIDER-B for bacterial detection. The right panel shows the spectra of bulk *E. coli* acquired using RAIDER-B. This portable unit has in-house designed optics and spectrometer coupled with fiber-optic probe for bulk bacterial detection.

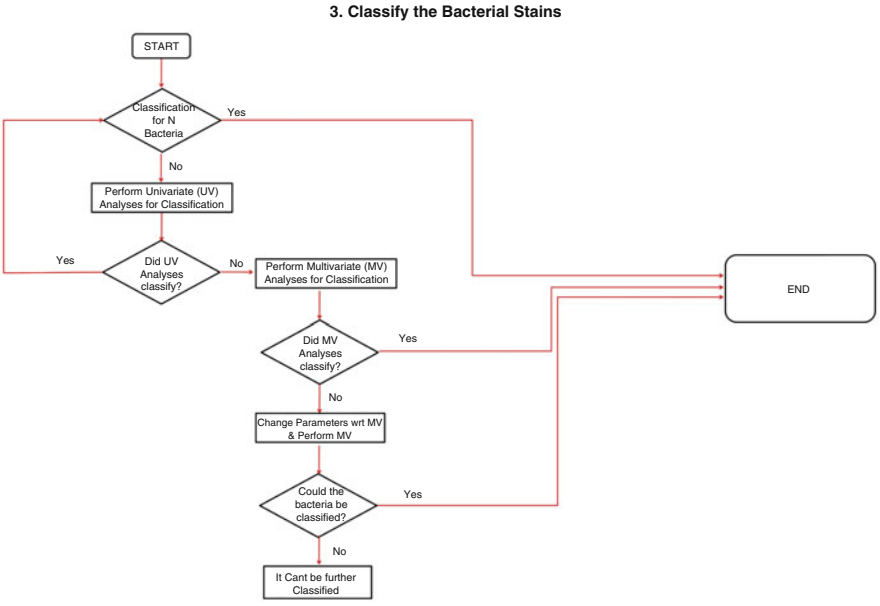

Fig. 20.21 The schematic process for classification of bacteria

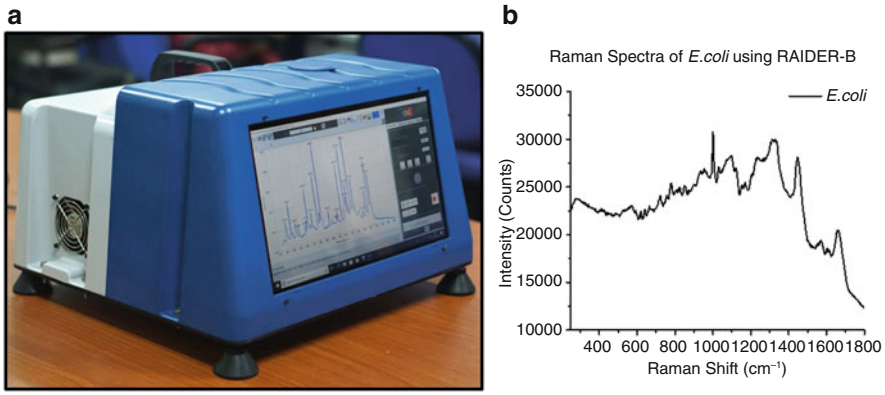

Fig. 20.22 (**a**) A benchtop Raman spectrometer developed in-house called RAIDER-B for bulk bacterial detection, (**b**) Raman spectra of bulk *E. coli* obtained from RAIDER-B using 785 nm diode laser for 10 s

20.5 Summary

This chapter provides a brief overview of Raman spectroscopy applications for bacterial detection. A historical background of the technique has been provided followed by discussion on the recent Raman-based offshoots which have been used for bacterial research. Thereafter, the importance of sample preparation, instrumentation aspects, and pre-processing and post-processing steps to obtain Raman spectra of bacteria for meaningful analysis was shown with the help of some samples. In addition, the concept of multivariate analysis techniques for discrimination and classification of bacterial samples was introduced. Suitable references have been cited for interested readers for further reading. We finally closed this chapter by showing the developed portable Raman system RAIDER-B for bulk bacterial detection. Raman spectroscopy research has stepped into its ninth decade since its discovery. A humble process such as scattering holds in itself immense information of a molecular structure. An understanding of both the fundamental processes and the instrumentation aspects is crucial to enable spectroscopists to develop solutions for biomedical and other applications. Research in the area of Raman spectroscopy has seen tremendous growth. It is envisaged that in the future portable Raman systems will be employed for clinical applications.

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Potential of Magnetic Resonance (MR) Methods in Clinical Cancer Research

21

Naranamangalam R. Jagannathan

Abstract

Magnetic resonance imaging (MRI) is often used as a versatile imaging modality for diagnosis of various diseases in clinical medicine. The technique is noninvasive and has multi-planar imaging capability with high spatial resolution. It offers an excellent soft tissue contrast compared to other imaging modalities. MR images are primarily from the protons of water and the fat present in human tissues. Over the years various forms of MRI technique were developed to study specific disease processes. Apart from routine T1- and T2-weighted MRI, dynamic contrast-enhanced MRI that uses exogenous contrast agents is useful to differentiate between malignant and benign lesions. Diffusion and perfusion MRI are other commonly used MRI procedures for investigating epilepsy, stroke, and various cancers. Another facet of MR is the in vivo magnetic resonance spectroscopy (MRS) that is used for studying the metabolic processes in humans. Both MRI and MRS have become an indispensable radiological tool and are widely used for diagnosis, staging of tumors, and follow-up of diseases during therapy without the use of any ionizing radiation and thus are useful for repetitive measurements. Additionally, these MR methods cover a vast range of applications in medicine from fast noninvasive anatomical measurements to evaluation of tissue physiology, function, and metabolism. In this chapter, the

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potential applications of MRI and MRS in clinical cancer research are reviewed with few examples from author's work on breast and prostate cancers.

Keywords

MRI · Magnetic resonance Spectroscopy (MRS) · Cancer · Diffusion MRI · Dynamic contrast MRI

21.1 Introduction

The last couple of decades have witnessed the growth of plethora of biochemical and radiological tools in clinical medicine, but diagnosis of cancer is still challenging. Detection at an early stage of cancer development and initiation of early and appropriate treatment are important that would significantly improve the quality of life and the overall survival of patients. The traditional diagnostic techniques like X-ray, ultrasonography, and CT are widely used that significantly increase the diagnosis by early detection. However, in many instances, the specificity of these techniques is low and associated with several limitations. In this regard, in vivo magnetic resonance (MR) technique like MR imaging (MRI) is widely used in clinical radiology as a diagnostic modality because of its high sensitivity and specificity to many soft tissue tumors. It has become an essential and necessary component of medical imaging compared to other medical imaging modalities. This is because MRI provides not only anatomical information but also physiologic, metabolic, and functional information. MRI does not use any ionizing radiation; it is noninvasive and has the ability to generate high-resolution images (Stark and Bradley 1998; Jagannathan 2005; Raghunathan 2007). MR imaging is the display of the spatial map of the distribution of hydrogen nucleus (placed in an external powerful magnet) present in tissues/organs using magnetic field gradients. It provides exquisite details (morphological picture, anatomical information) of the anatomy of the organ scanned. Further the ease of obtaining excellent soft tissue delineation of different regions within an organ is its greatest advantage. Recent advances in high-field MRI scanners with improved RF coil technology, parallel imaging, etc. are possible to have a spatial resolution of 100μm or less that enables visualization of finer details of the anatomy of the imaged organ.

Though MRI is a useful investigational tool to examine the tumor extent and the pathology, it fails to provide the biochemical or metabolic information that are normally seen in malignant progression or regression of the tumor. In this regard, in vivo MR spectroscopy or MRS has the potential to probe the biochemistry of living systems that is of diagnostic importance (DeCertaines 1992; Danielsen and Ross 1999; Mukherji 1998). Compared to high-resolution NMR that is used to study the structure of molecules, in vivo MRS of organs and tissues is more complex but provides information on different metabolites (biochemicals) present in a particular region of tissue or organ. In vivo localized MRS in humans basically uses the MR images acquired earlier for positioning the region of interest (ROI) within an organ. From an in vivo MR spectrum, it is possible to determine the relative levels or the

concentration of endogenous metabolites that are detected. This facilitates information on normal and abnormal status of the tissues, thus facilitating the understanding of the various processes of metabolism in tissues/organs. In short, in vivo MRS acts as a unique method for probing the biochemistry of living systems.

In a clinical situation, the diagnostic information is obtained from MRI, MRS, or a combination of these methods. Both these methods are widely used to monitor the progression of tumors as well as the treatment response of tumors to various therapeutic interventions and to further our understanding of the metabolic processes involved. In view of these advantages, both MRI and MRS have found widespread application in clinical, experimental (preclinical), pharmaceutical, and biological research. Thus in vivo MR methods act as an attractive tool which provides the link between clinical and preclinical applications (Jagannathan 2005). This chapter briefly reviews the potential role and applications of various in vivo MR methods like MRI and MRS in the study of cancer (especially breast and prostate) with few examples from author's work carried out in the last two decades.

21.2 History

The history of nuclear magnetic resonance (NMR) dates back to 1938 when Isaac Rabi first demonstrated that when a beam of molecules sent through a magnetic field, they could emit radio waves at specific frequencies. For this work the 1944 Nobel Prize in Physics was awarded to him. Later, in 1946, Bloch, Purcell, and Pound observed the phenomenon of NMR in bulk materials (solids and liquids), and for their discovery they were jointly awarded the 1952 Physics Nobel Prize. Till 1970s the NMR technique was used mostly for chemical and physical analysis by physicists and chemists. During mid-1970s the Fourier transform (FT) NMR method was discovered by Richard Ernst, and this was followed by two-dimensional (2D) NMR. Ernst was awarded Nobel Prize for his discovery of FT and 2D NMR in 1991. With the advent of FT and 2D NMR, NMR became a sensitive and powerful tool for the determination of three-dimensional (3D) molecular structure and characterization of variety of compounds including macromolecules. These initial applications opened up a new avenue for the potential use of NMR in biology, and for his seminal contribution to NMR of macromolecules, Prof. K. Wuthrich received the Nobel Prize in 2002.

However, the most useful and widely used application of NMR is the discovery of in vivo magnetic resonance imaging (MRI) that achieved unparalleled level of success as an important tool in medicine and experimental preclinical research. The first medical application was made by Damadian (1971). He showed that the MR relaxation times (T1 and T2) are different for malignant and normal breast tissues and reported that NMR could be used to distinguish a cancerous tissue from a healthy one.

In 1973, Paul Lauterbur, a chemist from State University of New York at Stony Brook, USA, demonstrated that it is possible to use NMR to create an image of two tubes containing water (Lauterbur 1973). This is the first MR image produced with

the use of a gradient magnetic field that demonstrated that it is possible to generate a 2D image of objects. Lauterbur named it as "zeugmatography." Around the same time Peter Mansfield, a physicist from University of Nottingham, UK, was working ways to image objects faster, say in minutes, and came up with a method of line scan imaging. For their discovery both Lauterbur and Mansfield were jointly awarded the 2003 Nobel Prize for MRI. In 1976, Mansfield and Maudsley produced an MR image of human finger (Mansfield and Maudsley 1976). Later Andrew et al. in 1977 showed that it is possible to image a human hand (Andrew et al. 1977). Hinshaw et al. in 1999 demonstrated an MR image of wrist (Hinshaw et al. 1977).

During the initial days of the development of MRI, the technique was named as nuclear magnetic resonance imaging (NMRI). But when it started to be used routinely in medical field for diagnosis of various diseases, the word "nuclear" was dropped from the literature. This is mainly to alleviate the unfounded fears of radiation hazards that are associated with the word "nuclear" in the mind of general public. Today MRI is an important state-of-the-art imaging tool in diagnostic radiology and clinical medicine.

Ever since its development in the 1970s, MRI has proven to be a versatile and safe diagnostic imaging modality. It uses a strong magnetic field (0.5 T or more) and radio frequency (RF) waves to produce detailed images from any organ of the human body that is placed in the magnetic field. Hydrogen (which consists solely of a proton) has the most frequently imaged nucleus in MRI because it is present in abundance in biological tissues and also because of its high gyromagnetic ratio which gives a strong MR signal. An MRI scanner is a large cylindrical magnet of tube of length of approximately 1.5 m with a diameter of 50–70 cm. The patient lay (either supine or prone position) on a table attached to the scanner which then slides and takes the patient (especially the organ to be imaged) to the center of the magnet bore (tunnel). For example, if MRI of a brain is required, then the brain of the patient is placed inside a cylindrical RF coil and is taken to the center of the magnet. And during MRI scanning, this RF head coil produces RF waves which manipulate the position of water hydrogens (protons) within the brain. Once the RF is stopped, the emitted energy (MR signal) is picked up by a powerful receiver. The MR signal is then sent to a computer that performs FT of the time domain MR signal to a frequency domain black and white image of the brain. These images are converted into 2D or 3D pictures that will help a radiologist to interpret the image and look for any abnormalities. Thus, in MRI, the hydrogen nuclei that are present in tissues create a signal that is processed to form an image of the human body in terms of the density of those hydrogen nuclei in a specific region.

21.3 Salient Features of Various In Vivo MRI and MRS Methods

The strength of MR imaging is its versatility and its wide range of applications to diagnose various diseases and abnormalities in a noninvasive manner (Edelman et al. 1995). One of the advantages of MRI is the ease of imaging any orthogonal planes (axial, coronal, or sagittal) as well as any desired oblique plane/s. In view of the

ability to give excellent soft tissue contrast, MRI is used to visualize even small structures like the cranial nerves. Other applications include determining the extent of the disease, stage of the tumor, information on function, and metabolism and assessing the tumor response to treatment. To evaluate these, there are several variants of MRI technique that were developed over the last two decades or more, that address and solve specific disease process.

21.3.1 Routine T1- and T2-Weighted Imaging

The most commonly used MR imaging protocol is the routine T1- and T2-weighted imaging that is useful for soft tissue delineation of anatomic structures and to identify pathology. In most malignant tumors and lesions, the relaxation times T1 and T2 are long in comparison to normal and benign tissues. Thus, tumors and other pathologies show a good contrast on a T2- weighted imaging (T2WI) as shown in Fig. 21.1a. For example in the brain, the T1- and T2-weighted images are useful to clearly differentiate between white and gray matters. Similar distinction is possible in other body organs like breast, liver, prostate, extremities, and other musculoskeletal systems.

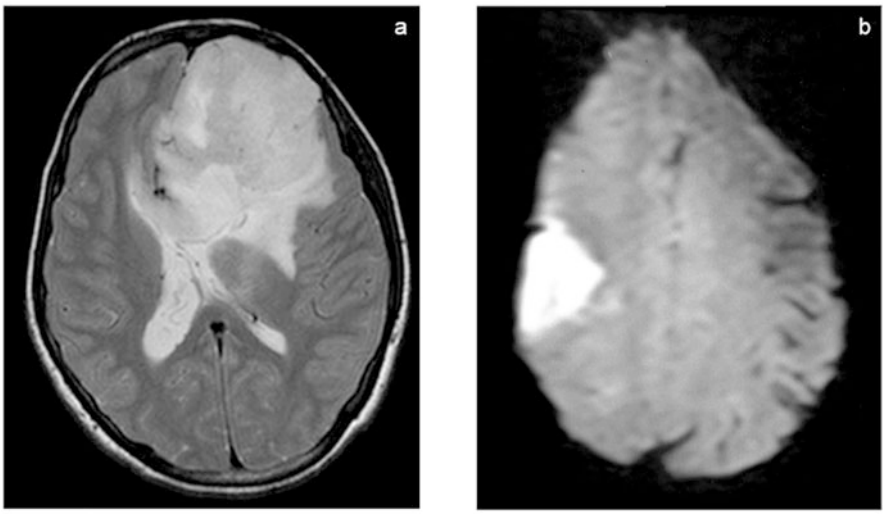

Fig. 21.1 (**a**) T1-weighted MR image in the axial plane of a patient suffering from mixed glioma. (**b**) Diffusion-weighted MR image of a patient who had brain stroke and the infarct region is seen clearly as a hyperintense area

21.3.2 Diffusion-Weighted Imaging (DWI)

It is known that during development, aging, and pathologic processes, there is change in the microstructural composition and architecture of the tissues. These changes seen in tissue microstructure and organization would lead to changes in the diffusion of water protons within the tissues. This molecular diffusion results from random translational Brownian motion of the molecules, for example, the motion of hydrogens in water. In normal, malignant, and benign tissues, the cellularity and other tissue properties vary widely, and this induces changes in the diffusion of water molecules in these tissue types.

In this direction, diffusion-weighted imaging (DWI) is another variant of the convention MRI method wherein MR signals mainly arise from the movement of water molecules, that is, mapping the microscopic motion of water protons in tissues (Le Bihan 1995). In addition to the cellularity and other tissue properties being different in different tissue types, the water molecules interact and collide with various macromolecules and membranes that are present in them. This therefore limits the diffusion of water protons, which can easily be monitored by DWI. Thus, in biological tissues, the diffusion coefficient values are quoted as apparent diffusion coefficient (ADC). During initial days of the development of DWI, the method was used for early detection and characterization of cerebral ischemia and thus best suited for stroke management (Ozscunar and Sorensen 2000; Molko and Chabriat 2001). Figure 21.1b shows the DW MR image of a patient who had stroke, and the infarct region is seen clearly as a hyperintense area. DWI has also been demonstrated to be useful for other organs of the body. In breast and prostate cancers, the method is routinely used for the differentiation between benign and malignant lesions and to monitor the response of the tumor to various treatments. In the liver DWI is used for the demonstration of metastatic disease and to assess the response of the tumor to treatment. Today, DWI is potentially a powerful probe in clinical medicine that is widely used for characterizing the effects of various disease processes and to monitor the diffusion process that may serve as a potential biomarker (parameter), which is of great relevance in diagnosis.

Further in tissues, the water proton diffusion is not isotropic (same) in all directions (anisotropic) because of the local environment being different (tissue heterogeneity), which greatly influences the diffusion. For example in the brain, in areas of white matter, a greater diffusion is seen along the myelin sheaths than across them. Hence, the diffusion of water molecules in tissues is anisotropic, which leads to different ADCs in each direction. This implies that diffusion is a 3D process leading to the molecular mobility not being same in all directions. Thus, diffusion is a tensor with the diffusion rate of water protons being different in different directions, and hence diffusion tensor mapping becomes more useful. In MRI, diffusion imaging is one of the important advances that enable to have a different contrast than regular T1W or T2W imaging. It also provides characterization of the tissues at the cellular level that may imply differences in function.

In 1994 the diffusion tensor imaging (DTI) technique was first introduced, and the significant application was in the brain. In comparison to routine MRI, DTI is an

advanced MRI method in which the MR signals arise mainly from the water proton movement in tissues. Many researchers have utilized DTI to determine the brain network and try to understand the anatomy and several brain functions. Since the diffusion of water molecules in a tissue is anisotropic, DTI can be used to determine the nerve cell organization in the brain (Molko and Chabriat 2001; Ozscunar and Sorensen 2000). The premise is that the water protons move faster along the axon fiber instead of moving upright to the fiber direction. This may, as discussed earlier, be due to the obstructions along fiber orientation that are in comparison less to restrict the water movement. DTI can thus produce a different image contrast to visualize important brain structures that depend on the axonal orientation and anisotropic diffusion of water protons.

21.3.3 Perfusion-Weighted Imaging (PWI)

Blood flow is the main source of supply of oxygen and nutrients to tissues that is mainly achieved through perfusion, which is one of the most fundamental physiological parameters. Any abnormality associated with the blood flow would alter perfusion that would lead to several vascular disorders. It is known that there is a close coupling between blood flow, metabolism, and function which can be determined through measurements of various perfusion parameters. Thus determination of perfusion characteristics is of direct diagnostic value and would serve as biomarkers for many physiological and pathophysiological disorders and functions.

In this context perfusion MRI (PWI) is a sensitive MR tool to monitor the microvasculature that can be of use in several clinical applications (Le Bihan 1995; Ozscunar and Sorensen 2000). Tumor classification, tumor grading, stroke region identification, epilepsy, characterization of other diseases, and monitoring the response to tumor therapy are some of the important applications of PWI. Perfusion MRI is carried out in two ways. First is with the use of an exogenous contrast agent and later without the use of an contrast agent. In the first approach, the use of a dynamic injection of contrast agent provides a better sensitivity with higher spatial resolution and is therefore widely used in many clinical indications. From post-processing of the PWI data, the perfusion maps are obtained, and different parameters like blood volume, blood flow, mean transit time, and time to peak are estimated.

The second approach is without the use of any exogenous contrast agent to measure the perfusion in tissues through arterial spin labeling method. In this experiment, first the pre-saturation pulses are used to saturate or invert the water proton magnetization in arterial blood in larger vessels. Thereafter after a certain delay, the inverted spins reach the capillary and diffuse out into the tissue water content of the imaged slice. Later, the scan is repeated with no inversion of the spins of the arterial blood, and the two images are subtracted from each other. The resultant difference signal presents the information on the amount of blood delivered from the arterial blood during the delay which is directly proportional to the local perfusion. Arterial spin labeling has a better accuracy of quantification, and in

general PWI measurements are minimally invasive and do not use any radiation and radioisotopes.

For identification of brain regions that were affected by cerebral ischemia, both PWI and DWI are highly useful. Brain regions with compromised cerebral blood flow can be seen well in PWI, while the affected tissues are seen in DWI. PWI is also being used in other organs like breast, prostate, etc.

21.3.4 Dynamic Contrast-Enhanced MRI (DCEMRI)

Both oxygen and nutrients are the metabolic need that are high in cancer and especially so in aggressive tumors. Angiogenesis is the process by which this is achieved, and in fact angiogenesis is a hallmark of cancer development. For quantitative estimate of tumor biology through microcirculation, DCEMRI is often used. This method can also be used to monitor the response of cancer following therapy (Gilbert and Ahearn 2009). DCEMRI uses a fast imaging protocol that is repeatedly used over the imaging volume during the intravenous injection of a contrast agent. It is basically a serial 3D MR acquisition method carried out before, during, and after a bolus of contrast media, typically via the antecubital vein. Normal injection rate used is of the order of 2–4 mL/s followed by a saline flush. Following the bolus intravenous injection, there is a rapid increase of the contrast in the imaging volume leading to high signal enhancement in both artery and tumor. Thereafter the contrast agent is washed out with leakage into the interstitial space.

During this process, the contrast present in vessels and in the extracellular space basically shorten the relaxation times (either T1 or T2 or both), leading to a rapid MR signal enhancement on T1-weighted imaging (T1WI). The basic principle being that leaky vessels are formed due to neoangiogenesis allows faster extravasation of contrast agents administrated, leading to rapid local enhancement of the MR signal intensity. The plot of MR signal intensity change from a ROI and as a function of time (time–intensity curves) gives a semiquantitative parameter that is useful in diagnosis. Peak enhancement of the contrast, initial slope that shows the rapid arterial enhancement, time to peak, the area under the concentration–time curve, and washout rate of the contrast agent are all useful parameters that aid diagnosis. The increase in MR signal intensity is proportional to the amount of contrast administered during the DCEMRI procedure, and it also measures the perfusion in tissue microstructure that directly reflects the physiological properties of tissues. The signal thus measured on DCEMRI represents a combination of both perfusion and permeability. For the differentiation of malignant and benign tumors, DCEMRI is a routinely used.

21.3.5 In vivo MR Spectroscopy (MRS)

Between MRI and MRS, the significant technical difference is the use of magnetic field gradients during the acquisition of MRI signal, while for MRS it is essential to

have a homogeneous magnetic field over the ROI to observe the chemical shift differences of biochemicals (metabolites) and hence no requirement of magnetic field gradients during signal acquisition. The growth of both MRI and in vivo MRS over the years has been simultaneous.

During initial days of development of in vivo MRS, the focus is to acquire spectra of isolated tissues/organs or from the surface regions from intact animals. However with the availability of gradients for MRI, localization methods were developed that facilitated obtaining spectra from single volume (VOI or ROI) from organs or tissues. In vivo MR spectrum from a particular VOI is by the use of MR images that are acquired earlier in all three planes and using frequency selective RF pulses applied during the application of a gradient. Thus MRI had a considerable impact on the development of various in vivo MRS methodologies. It is possible to get a MR spectrum from a single volume (or voxel), known as single voxel spectroscopy (SVS), or from multivolume, known as multiple voxel MR spectroscopy or MR spectroscopic imaging (MRSI). Both 2D and 3D acquisition of MRSI of an organ are possible, and since localized spectra from many locations are acquired simultaneously, MRSI is the preferred choice in most clinical cases.

As discussed earlier, MRS is used to detect and estimate the concentration of different metabolites or biochemicals that are present in a particular ROI of the tissue or organ. The concentration of these metabolites or their relative levels provide information on the normal and pathological status of the tissues. Like MRI, MRS also being noninvasive, it is possible to use this method repetitively to monitor the tumor response to various therapeutic modalities in patients, study of the efficacy of drugs, and to understand the various metabolic processes (Danielsen and Ross 1999; Mukherji 1998).

In vivo MRS can be carried out using different nuclei that include proton (^{1}H), phosphorus (^{31}P), carbon (^{13}C), lithium (^{7}Li), and fluorine (^{19}F). But most MR spectra reported in clinical medicine are from hydrogen nucleus (^{1}H) for the reasons mentioned earlier. While the basic idea of in vivo localized ^{1}H MRS is to acquire a spectrum from a particular VOI with optimal sensitivity, the detection of metabolites with lower concentrations in the presence of high tissue water content (concentration) is a major hindrance. Water concentration in tissues is approximately 50 M compared to the millimolar concentration of other biochemical/metabolites. Thus, there is a requirement to suppress water from the VOI from which MRS is to be acquired. There are several methods available to achieve water suppression and in certain cases the suppression of both the water and the lipid peaks (DeCertaines 1992; Danielsen and Ross 1999; Mukherji 1998).

21.4 Important Observations from MRI and MRS Studies of Breast and Prostate Cancers

The application of various in vivo MRI and MRS methods to study cancer has seen exponential growth in the last two decades. Vast amount of literature exist on various cancers, and it is not possible to review all of them in this chapter. Hence, only a few

representative examples on the diagnosis and on the use of these methodologies in assessing the tumor response to treatment in breast and prostate cancers are presented from author's work.

21.4.1 Breast Cancer

One of the major health concerns worldwide in women is the breast cancer, which is highly prevalent and leads to considerable mortality. Thus to increase the survival rate and the quality of life of these patients, early diagnosis and treatment is essential. In this regard, MRI plays a crucial role in diagnosis. Generally it is better to use field strength of 1.5 T or more to acquire breast MR images, and also the use of a dedicated breast coil enables to achieve sufficient high spatial resolution (Mann et al. 2008; Sardanelli et al. 2010; Newell et al. 2018).

Routine breast MRI protocol includes T2WI and DWI followed by contrast-enhanced T1-weighted imaging in axial plane (Kuhl et al. 1999; Westra et al. 2014). In fact, of the various MRI procedures used, DCEMRI forms the backbone of breast MRI protocol since it is a sensitive method to discriminate breast cancer from benign lesions. Further, it provides high-resolution morphological information and functional information on the angiogenesis of breast tumors.

T2WI with fat suppression is normally used for diagnosis of cysts, while imaging without fat suppression is better to view the breast lesion morphology. High MR signal intensity seen on T2WI generally arises due to benign lesions such as breast masses like apocrine metaplasia, myxoid fibroadenoma, fat necrosis, and lymph nodes. Also T2WI may increase the specificity for the differentiation of benign and malignant breast lesions. Further, the malignant invasive breast cancers typically manifest as a focal hypointense mass with an irregular shape and irregular or spiculated margins, on a T2WI. Figure 21.2 shows the comparison of the

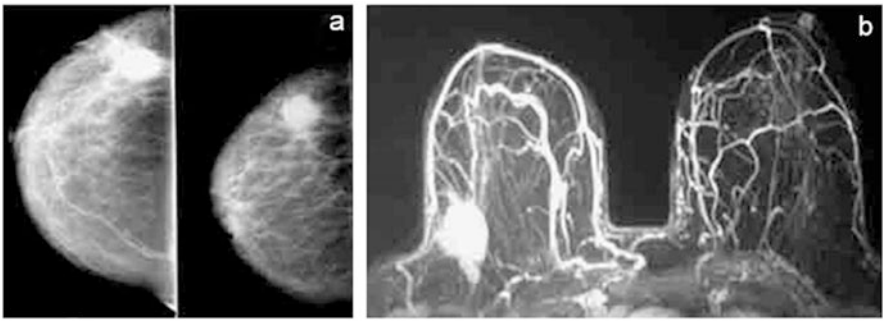

Fig. 21.2 (**a**) Mammogram of the right breast of a patient with invasive ductal breast cancer showing a spiculated, dense mass in the upper-outer quadrant. (**b**) Post-contrast MR image of the same patient, showing the enhancing mass with irregular margins and increased vascularity suggestive of invasive ductal carcinoma. (Reprinted from Sharma et al. (2010) with permission from John Wiley & Sons Inc.)

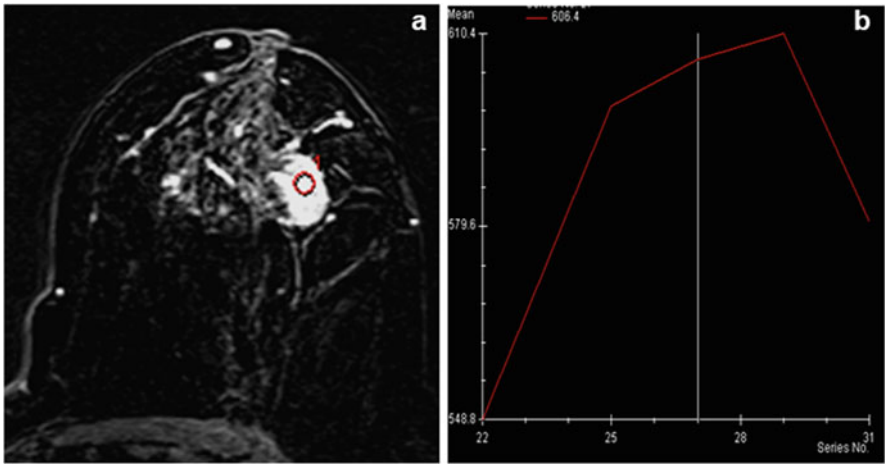

Fig. 21.3 (**a**) Subtracted gadolinium-enhanced T1-weighted axial MR image from a locally advanced breast cancer patient showing homogenous enhancement. (**b**) The signal intensity–time curve from the circular region of interest shown in (**a**) that shows early peak enhancement with rapid washout, strongly suggestive of malignancy. (Reprinted from Sharma et al. (2010) with permission from John Wiley & Sons Inc.)

mammogram of a breast cancer patient with the post-contrast MRI of the same patient showing the enhancing mass with irregular margins (Sharma et al. 2010). Smooth borders along with gentle lobulation are characteristics of benign lesions.

In T1W DCEMRI of breast, normally a contrast of 0.1 mmol per kilogram of body weight is given with a pressure injector at a flow rate of 2 mL/s. Later it is flushed with a bolus of saline of approximately 20 mL. It is a useful procedure for the identification of mammographically occult primary tumors in patients presenting with malignant axillary nodes (Sharma et al. 2010). Maximum enhancement is seen after 1–2 min after the contrast administration in malignant breast lesions, while normal breast tissues and benign lesions enhance progressively thereafter. Figure 21.3 is the axial T1W contrast-enhanced image of a locally advanced breast cancer (LABC) patient, along with the washout curve characterizing malignancy. The typical persistent enhancing curve of a benign breast lesion from DCEMRI is shown in Fig. 21.4 (Sharma et al. 2010).

Due to increase in cell density in cancers, there is decreased water diffusion, and DWI is another useful MR procedure in breast cancer diagnosis. Further, DWI can be acquired in short time and require no contrast agent. It is useful to distinguish between benign and malignant breast lesions. Malignant breast lesions show low signal intensity in an ADC map (see Fig. 21.5). As discussed earlier, ADC is a quantitative measure of the diffusivity of the water molecules in tissues, and the ADC values in cancers are generally low (range, 0.8 to 1.3×10^{-3} mm^2/s) compared with those of benign lesions (range, 1.2 to 2.0×10^{-3} mm^2/s) and normal breast tissues (range, 1.6 to 2.3×10^{-3} mm^2/s) (Sharma et al. 2016; Shi et al. 2018).

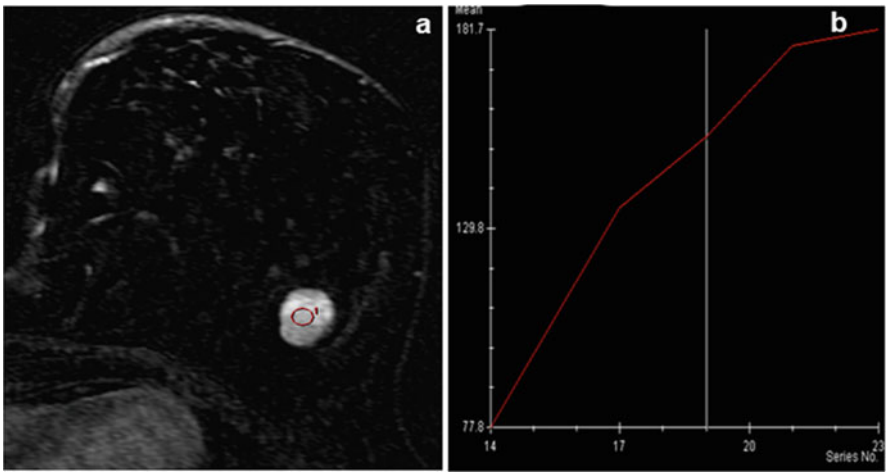

Fig. 21.4 (**a**) Subtracted axial gadolinium-enhanced T1-weighted axial MR image from a patient with a benign breast lesion. The lesion shows homogenous enhancement. (**b**) The signal intensity–time curve obtained from the circular region of interest as shown in (**a**) which shows a progressive enhancement that is suggestive of a benign lesion. (Reprinted from Sharma et al. (2010) with permission from John Wiley & Sons Inc.)

The last two decades have witnessed extensive research on the use of in vivo ^{1}H MRS in breast cancer (Sharma et al. 2011). Generally, the ^{1}H MR spectrum of breast is acquired without and with water and fat suppression. A MR spectrum obtained without water or fat suppression is useful for estimation of the water and fat content of the breast tissue. However, in a spectrum obtained with water and fat suppression, a signal from tCho biochemical is seen. From breast MR spectra, one can derive parameters like W–F ratio and concentration of tCho peak. It is possible to determine both qualitative and quantitative estimate of the concentration of tCho.

A dominant peak from protons of fat present in the breast tissues with less contribution from water is the characteristics of breast MRS ^{1}H spectrum and is shown in Fig. 21.6. The T1W image of the right breast from a volunteer is shown in Fig. 21.6a, while Fig. 21.6b is the corresponding ^{1}H MR spectrum obtained without water suppression from the VOI shown in Fig. 21.6a. Peak seen at 1.3 ppm corresponds to $[-(CH_2)_n-]$ protons of the fat of breast tissues, while that seen at 0.9 ppm is from the terminal methyl protons of glycerides (Sharma et al. 2011). The methylene protons α to carboxyl in the glyceride chain and the diallylic CH_2 protons are observed at 2.2 ppm and 2.7 ppm, respectively. The water peak is seen at 4.7 ppm, while other small peaks are as assigned in the figure. The intensity of the major fat peak at 1.3 ppm (along with the peak at 0.9 ppm) and that of water seen at 4.7 ppm are used for the estimation of W–F ratio. Higher W-F ratio is observed in malignant compared to benign and normal breast tissues (Jagannathan et al. 1998). Figure 21.7 shows T2W fat-suppressed axial MR image of a breast cancer along with ^{1}H MR in vivo spectrum obtained with and without water and fat suppression. A tCho peak at 3.22 ppm is seen clearly as shown in Fig. 21.7c (Sharma et al. 2011).

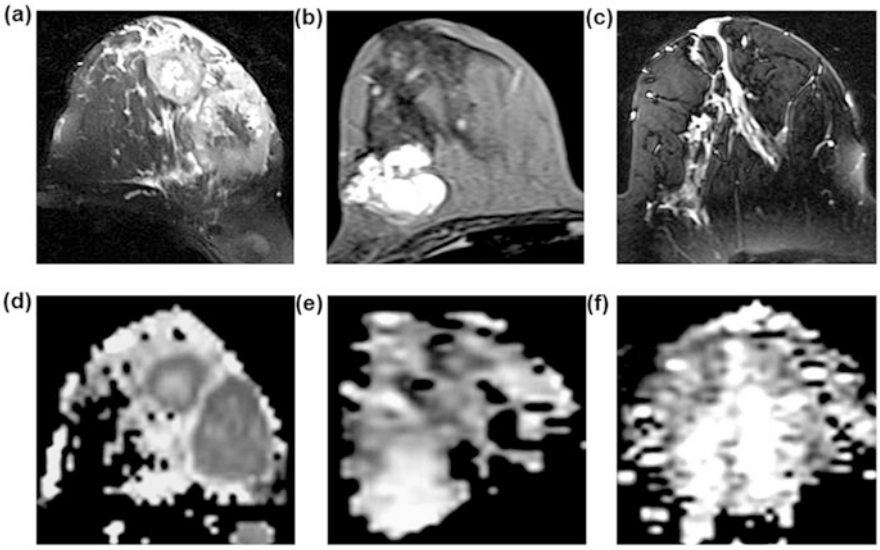

Fig. 21.5 T2-weighted MR images of (**a**) a 28-year-old patient with infiltrating ductal carcinoma, (**b**) a 25-year-old patient with benign fibroadenoma, and (**c**) a 30-year-old volunteer with healthy breast tissue. The respective ADC maps obtained are shown in (**d**–**f**). (Figure as originally published in reference Sharma, U. Sah, R.G. Agarwal, K. Parshad, R. Seenu, V. Mathur, S. R. Hari, S. & Jagannathan, N.R. (2016). Potential of diffusion weighted imaging (DWI) in the characterization of malignant, benign and healthy breast tissues and molecular subtypes of breast cancer, *Frontier in Oncology*, 6:126. doi: https://doi.org/10.3389/fonc.2016.00126)

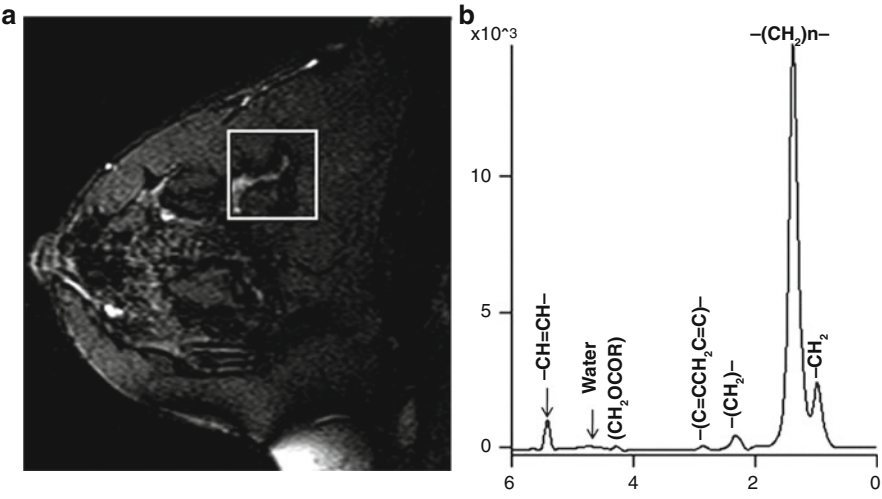

Fig. 21.6 (**a**) T2 weighted MR image from the normal breast of a volunteer (35 years old) showing the voxel position from which the ^{1}H MR in vivo spectrum (**b**) was obtained without water and lipid suppression. (Reprinted from Sharma et al. (2011) with permission from John Wiley & Sons Inc.)

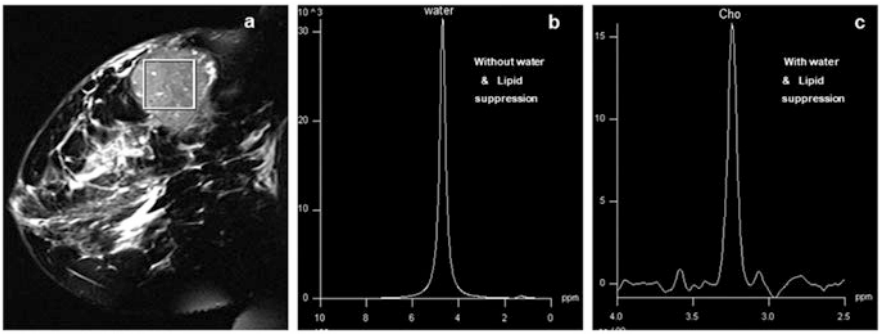

Fig. 21.7 (**a**) MRI of a patient with locally advanced breast cancer showing the voxel position from which the single-voxel (SV) ^{1}H MR in vivo spectrum (**b**) was obtained without water and fat (lipid) suppression. (**c**) MR spectrum obtained with the suppression of water and fat (lipid) resonances. (Reprinted from Sharma et al. (2011) with permission from John Wiley & Sons Inc.)

In clinics, the utility of breast MRS is for the differentiation of malignant from benign and normal breast tissues. Initial breast MRS studies used the W–F ratio as the diagnostic marker. Low W–F ratio was observed in normal and benign breast tissues, while in malignant tissues an elevated W–F ratio is seen (Jagannathan et al. 1998). Subsequent studies have focused on the use of both qualitative and quantitative estimate of tCho peak for differentiating different breast tissue types (Jagannathan et al. 2001). A meta-analysis of existing MRS data till 2001 was carried out by Katz-Brull et al. (2002). They analyzed 100 malignant and 53 benign lesions and reported a sensitivity of 83% and a specificity of 85%. Their data also showed that ^{1}H breast MRS sensitivity was found to be dependent on the tumor size, while the absence of tCho in some studies has been attributed to motion-related artifacts and software-related problems. The fat content of breast was reported to be involved in the development and progression of breast tumors (Agarwal et al. 2018).

Monitoring tumor response to therapy is necessary for treatment planning especially in nonresponders. This would enable physician to initiate appropriate change in patient treatment management. In addition to routine MRI, DCEMRI can be used to evaluate the response of the tumor by measuring its size and the amount of contrast enhancement. The method is also useful for evaluating the residual disease after treatment. Further, post-therapy MRI is useful to evaluate the need for a re-excision or a mastectomy. Similarly DWI is highly useful to monitor the response of patients undergoing neoadjuvant chemotherapy (NACT). ADC, volume, and diameter after each cycle of NACT were measured in clinical responders and nonresponders, and comparison of the percentage in these parameters was calculated (Sharma et al. 2009). The data showed a significant change soon after the first chemotherapy cycle in ADC between responders and nonresponders in comparison to volume and diameter. This result indicates that DWI is useful to assess the early response to therapy (Sharma et al. 2009).

Similar to MRI, MRS has also been investigated by researchers as a useful procedure for therapeutic monitoring of tumors. Both W–F ratio and tCho

parameters derived from breast MRS have been found to be useful parameters to assess the treatment response of the breast tumors to therapy. Our study showed that W–F ratio reduced following NACT which is a useful noninvasive indicator to assess the clinical outcome to therapy (Jagannathan et al. 1998). A significant decrease of W–F ratio was seen in responders that is consistent with the tumor size reduction seen after NACT.

In 2001 our group reported a sequential monitoring of tCho in 14 LABC patients over the complete six cycles of NACT. Patients were monitored prior to therapy and after 1, 3, and 6 weeks of NACT using single-voxel ^{1}H MRS. In ten out of 14 breast cancer patients, tCho was observed prior to initiation of therapy. Seven showed no tCho, or its level was significantly reduced in these ten patients. This observation indicated tumor response to NACT that is in line with the clinical and histological findings (Jagannathan et al. 2001). In another study we evaluated the role of MRSI by measuring the changes in signal-to-noise ratio of tCho peak in LABC patients both before and after each cycles of NACT (Sharma et al. 2010). The signal-to-noise ratio of tCho reduced significantly after the I NACT in responders compared to nonresponders. Recently we reported that MR multiparametric approach is extremely helpful to assess the tumor response to treatment in a more objective way. Our data showed that tCho, tumor ADC, and tumor volume (see Fig. 21.8) are useful parameters in assessing the tumor response of LABC patients undergoing NACT (Sharma et al. 2018).

21.4.2 Prostate Cancer

Cancer of the prostate (CaP) is primarily a disease related to elderly men and is rare before 40 years of age. Generally during the initial stages, the tumor grows slowly and mostly confined to the prostate gland. Hence majority of CaP patients may not require any treatment, or they may require minimal treatment. However there are certain types of CaP that are more aggressive and also spread quickly. CaP is common in Western countries, and age, race, ethnicity, family history, and the hormonal status are some of the risk factors. However, the incidence of CaP in India is considerably lower than in the Western countries and the incidence being more common in urban population (Hariharan and Padmanabha 2016). Most CaP are adenocarcinoma arising from prostatic epithelium.

MRI is useful to visualize the prostate anatomy with excellent soft tissue contrast that would facilitate assessing the local extent of the cancer. For improved MR image quality, the use of an endorectal coil with a pelvic phased-array coil is preferred, and most CaP MR studies were carried out initially at 1.5 T scanner. However recent studies use 3 T MR scanner without the use of endorectal coil. Studies have shown that the image quality obtained at 3 T without endorectal coil is comparable to that obtained at 1.5 T with endorectal coil (Jagannathan et al. 2008; Kumar and Jagannathan 2014). T2WI in axial plane gives excellent depiction of prostate zonal anatomy compared to T1WI. The peripheral zone (PZ) of the prostate on T2WI shows higher signal intensity than the central gland of the prostate. Most cancer

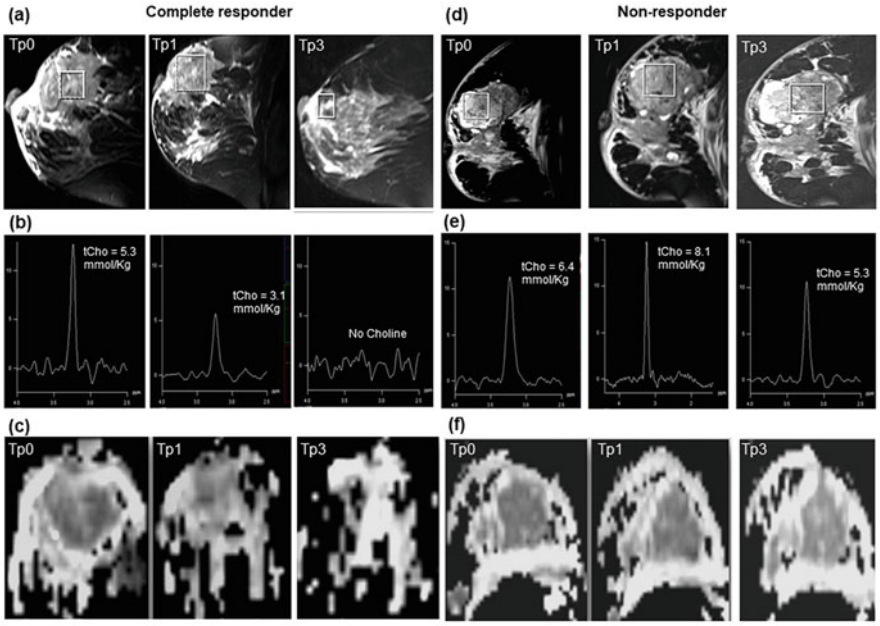

Fig. 21.8 Representative example of T2W sagittal MR images (**a**) showing the voxel location and the corresponding proton MR spectra acquired (**b**) from the voxel shown in (**a**) of a complete responder acquired, at I, II, and III NACT, respectively, while (**c**) shows the corresponding ADC maps. The representative example of a patient who was a nonresponder is shown in figures **d** to **f**: (**d**) T2W sagittal MR images showing the voxel location and (**e**) the corresponding proton MR spectra, and (**f**) the corresponding ADC maps acquired, at I, II, and III NACT, respectively. (Figure as originally published in reference Uma Sharma, Khushbu Agarwal, Rani G. Sah, Rajinder Parshad, Vurthaluru Seenu, Sandeep Mathur, Siddhartha D. Gupta and Naranamangalam R. Jagannathan (2018). *Frontier in Oncology*, 15 August 2018. doi: https://doi.org/10.3389/fonc.2018.00319)

arises from prostate PZ with characteristics low MR signal intensity on a T2WI. However the limitation of T2WI has been in the detection of cancer located in the central gland of the prostate since cancer and normal tissue both show similar low MR signal intensity in this region. Figure 21.9a shows the T2W MR image of prostate from a volunteer while (b) that obtained from a patient with CaP. The tumor is observed as a hypointense area (Fig. 21.9b) in the PZ of the prostate (arrow), the site of the tumor. Literature reports suggest that the sensitivity and specificity show wide variation of T2WI using the endorectal coil (Kumar et al. 2018).

Due to the sensitivity and specificity being limited on T2WI for cancer detection, the role of DCEMRI has been explored to improve the contrast in the images for tumor detection (Kumar et al. 2018). The peak contrast enhancement of CaP relative to the surrounding normal and benign tissues can be used as a parameter for

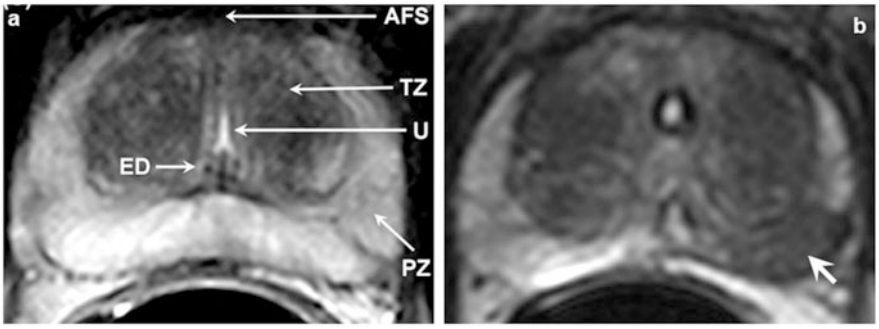

Fig. 21.9 (**a**) The appearance of normal prostate on axial T2W image: *AFS* anterior fibromuscular stroma, *CZ* central zone, *ED* ejaculatory ducts, *PZ* peripheral zone, *TZ* transition zone, *U* urethra. (**b**) Prostate cancer lesion (white arrow) appearing in the left peripheral zone in axial T2W image. (Reprinted from Kumar et al. (2018) with permission from Elsevier)

localization of cancer (Kumar et al. 2018). DCEMRI performed at 1.5 T and 3 T showed improvement in detection and characterization of CaP.

DWI of the prostate is the most commonly used procedure after T2WI for prostate cancer detection and is generally carried out in the axial plane. Data from our group revealed that CaP patients showed significantly lower ADC value compared to benign lesions and normal prostate tissues of volunteers (Kumar et al. 2007a, 2018; Jagannathan et al. 2008). Lower ADC seen in malignant prostate tissues is due to the altered tissue structure because of cell proliferation associated with malignancy. This loss of glandular histology leads to decrease in the interstitial space, which restricts the diffusion. Many CaP studies have shown that DWI of prostate significantly improves the accuracy of tumor detection beyond that achieved with T2WI alone.

Since CaP is multifocal nature, prostate MRS is generally performed using ^{1}H multi-voxel MRSI. The MR spectrum obtained from normal PZ and from the malignant portion of the PZ of CaP patient is shown in Fig. 21.10 along with the T2W image of the prostate (Kumar et al. 2007b). Metabolites such as Cho, creatine (Cr), and citrate (Cit) are the three major peaks that are generally seen in a ^{1}H in vivo MR spectrum of the PZ of normal prostate. These three dominant metabolite peaks appear at 3.2, 3.0, and 2.6 ppm, respectively, in the MR spectrum (see Fig. 21.10c). A peak at 3.1 ppm due to polyamines (mainly from the biochemical spermine) has also been documented. Decreased Cit and high Cho are characteristics of CaP from PZ of the prostate (see Fig. 21.10c).

The interpretation of the MR spectrum of CaP is based on the use of metabolite ratio of Cit/Cho or [Cit/(Cho + Cr)] or [(Cho + Cr)/Cit] or [(Cho + Cr + PA)/Cit]. The studies carried out during the late 1990s documented that (Cho + Cr)/Cit ratio was found to be a specific marker for CaP (Kurhanewicz et al. 1996a, b). The role of ^{1}H MRSI in segregating patients who are likely to show cancer of the PZ of the prostate on biopsy has also been demonstrated (Kumar et al. 2009).

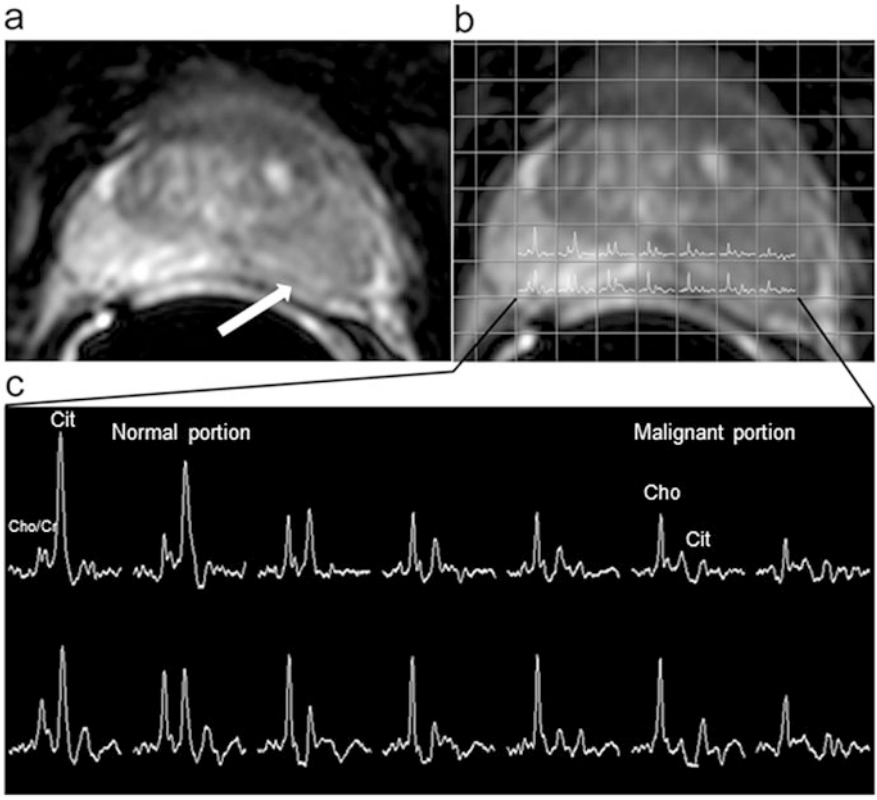

Fig. 21.10 (**a**) High-resolution T2W axial image of prostate of a patient whose PSA level is 5.8 ng/mL. (**b**) Spectral map of the same patient showing the MRSI grid on the above image. (**c**) The expanded spectral pattern showing the variation of the citrate and choline signals from the normal and abnormal portions from the peripheral zone as indicated. (Reprinted from Kumar et al. (2007b) with permission from John Wiley & Sons Inc.)

The Cho peak seen in vivo is a composite peak that arises from three choline-containing compounds, namely, free choline, glycerophosphocholine, and phosphocholine. These compounds participate in the biosynthesis and degradation of phospholipids that are essential elements of cellular membranes. Thus, the observation of high level Cho containing compounds indicates the rapid proliferation of malignant cells in CaP.

Generally, high levels of Cit and zinc are seen in normal prostate and BPH tissues in comparison to all other tissues of the body (Costello and Franklin 2000). Cit level being higher in prostate gland is due to limited activity of enzyme m-aconitase that is responsible for converting Cit to isocitrate in Kreb's cycle (Costello et al. 2005). However, in malignant prostate tissue, the Cit level is considerably lower of the order of less than 1000 nmol/g compared to the normal levels from 8000 to

15,000 nmol/g (Costello and Franklin 2000). This is the reason that in vivo MRS showed reduced Cit in CaP as compared to normal and benign prostate tissues.

Both MRI and MRSI can be used longitudinally to monitor the time course of tumor response to therapy as well as the mechanism of therapeutic response. Identification of residual or recurrent cancer facilitates early intervention that provides opportunity to initiate additional treatment, if required. Routine MRI investigations carried out after therapy in CaP patients basically rely on the changes in the morphology of the tumor and measurement of reduction in tumor size and volume (Westphalen et al. 2008). Kurhanewicz et al. showed the potential of MRSI following cryosurgery in prostate cancer patients and demonstrated the presence of recurrent local disease after therapy (Kurhanewicz et al. 1996b). Further, Parivar et al. in their study showed that MRSI is superior to TRUS and MRI in evaluating the local recurrence after cryosurgery (Parivar et al. 1996).

21.5 Summary

Cancer is a dreadful disease, and both men and women are affected equally with considerable morbidity and mortality. Cancer detection at an early stage is important and key to plan appropriate treatment. This would facilitate initiation of appropriate treatment at an early stage that would save many lives. Though various diagnostic modalities are available in clinical radiology like ultrasound, CT, etc., the use of MR methods has revolutionized the area of diagnosis. MR being noninvasive has many advantages in clinics for monitoring longitudinally the disease progression, regression, and therapy response. This chapter briefly reviewed the potential of various in vivo MR methods that are normally used in clinical cancer research and the potential role of these procedures in breast and prostate cancers. The examples given in this chapter on breast and prostate cancers clearly demonstrate that in vivo MR methods are well established in clinical cancer research that includes a wide range of applications from fast noninvasive morphological assessment to study the tissue physiology and metabolism.

In clinical medicine, the future of in vivo MR methods looks incredibly bright. In the last three decades of MRI as a diagnostic modality, the method has gone through lots of improvements in generating ultrafast high-resolution MR images with thinner slices that would enhance detection of small sized lesions and to study several biological functions noninvasively. Further, it is used to guide interventional procedures. With the advances in the newer design of open magnet MR systems, the patient access becomes easy to carry out such MR guided interventional procedures using fully MR compatible needles and catheters. With the advent of high-field strength MR scanners operating at 7 T or more, the detection sensitivity would greatly improve, and also it would increase better chemical shift dispersion especially for MRS. Such developments in both hardware and software methods would virtually push MR to its limitless applications to accurately diagnose very small lesions at an early stage that would provide variety of appropriate treatment options for patients.

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Microfluidic Point-of-Care Diagnostic Devices: A Contemporary Relevance

22

Shristi Handa and Anupreet Kaur

Abstract

Rapid increasing population and outbreak of various diseases globally have intended an essential demand for ideal diagnostic platform that can serve the purpose of point-of-care (POC) diagnosis. Therefore in this context, microfluidic technology is developing as a promising tool in various fields of biomedical engineering such as therapeutics, pharmaceutics, and diagnostics. Owing superior competences in manipulating fluids at microscale, it offers robust and versatile platform for construction and fabrication of devices such as organ-on-chips with precise control on the geometric, structural, and composition like parameters. The article provides the outline of the designing of diagnostic devices using microfluidic platform, emphasizing on the basic concept of fabrication and its various research developments. Further, the review also covers the discussion in the existing and emerging clinical application. Finally, an outlook has been talked over on the future direction of advancing microfluidic system for its development as effective diagnostic tool for developing countries.

Keywords

Microfluidics · Point-of-Care devices · Biomedical applications · Diagnostics · Nanocomposites

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22.1 Introduction

To revolutionize the point-of-care (POC) diagnostics, microfluidic devices have turned out to be advertised as a long key candidate. This field of research enables one to address some of the important issues. Microfluidics is a technology that processes or manipulates the flow behavior of the small volume of fluids, through the fabrication of microminiaturized devices consisting of channels and chambers, which are of the dimensions in the order of ten to hundreds of micrometer. Several applications can be envisaged in various sectors of biotechnology, in synthesis of chemical, and in analytical chemistry, to name a few (Ahn and Choi 2007; Mark et al. 2010). Therefore, the precise manipulation of molecular interactions offers to process the samples on a lab-on-a-chip platforms (Figeys and Pinto 2000; Gupta et al. 2016). Since the inception in the 1980s, microfluidics has emerged as significant trend in bioinstrumentation. The salient features offered by microfluidic devices such as the processing of small volume samples quickly, flexibility of multiplexing, automated analysis, cost-effectiveness, disposability, portability, easy design, and novel principles and phenomena increased the demand in the biomedical field (St John and Price 2014). To modernize, microfluidic technology has come up with the paper bio-diagnostics technology that contains a great potential of advancement in healthcare sector. These paper-based point-of-care devices hold potential of application in remote area where there is scarcity of resources or poor healthcare settings to detect various biological analytes of interest. Moreover in the future one can anticipate the integration of POC devices with the Internet of Things (IoT) to generate healthcare systems that are more competent and cost-efficient. In today's time there are various glucose sensors and POC system that involve the incorporation of miniaturized small, simple, and smart sensors. Subsequent innovation and development in technology led to the buildout of devices such as dip stick, which involve self-contained testing based on the phenomenon of lateral flow movement of the sample to the detection site. Therefore the dip stick devices hold application for rapid pregnancy testing, detection of cardiac disease, and quick detection of HIV-1. These microfluidics devices have the greater potential to move beyond conventional benchtop methods and reach toward bring the trend of small handy POC devices (Kozel and Burnham-Marusich 2017; Gale et al. 2018), (Sia and Kricka 2008).

The review focuses on the discussion of development of new technologies, architecture, being applied for developing biosensing devices that are cost-effective, efficient, and comprise of advance technology to regulate the parameters such as greater controllability, high throughput, better reliability, and a large range of diagnostic applications. Further, given is the insight of development of the new material such as using paper for fabrication of POC applications as well as designing new chips. Authors have tried to concentrate on the recent trends and advances in this field in the current article.

22.2 Microfluidic Point-of-Care Devices

Rapid and precise measurements offered by reliable microfluidic systems for measurement of smaller quantities of sample make it feasible to advance chip-scale microfluidic POC devices to precise laboratory setups (Choi et al. 2011; Jung et al. 2014). Using various microfluidic systems, devices have been established for the application in different biological and chemical prospects. Like using conventional microfluidic systems which involve continuous flow regimes, fabricating micron-sized channels is mainly through use of soft lithography methods (Fiorini and Chiu 2005). Further enhancement in the technology involved the development of the sensors using droplet-based microfluidics with the advantage of reduced sample size as well as creation of isolated reaction sites.

Due to intrinsic characteristic of systems, various generations of the droplet-based microfluidic system were developed after fundamental generation of the droplet-based microfluidic systems such as digital microfluidic, making it a suitable choice for employing additional sensing units (Fig. 22.1) (Teh et al. 2008; Sánchez Barea

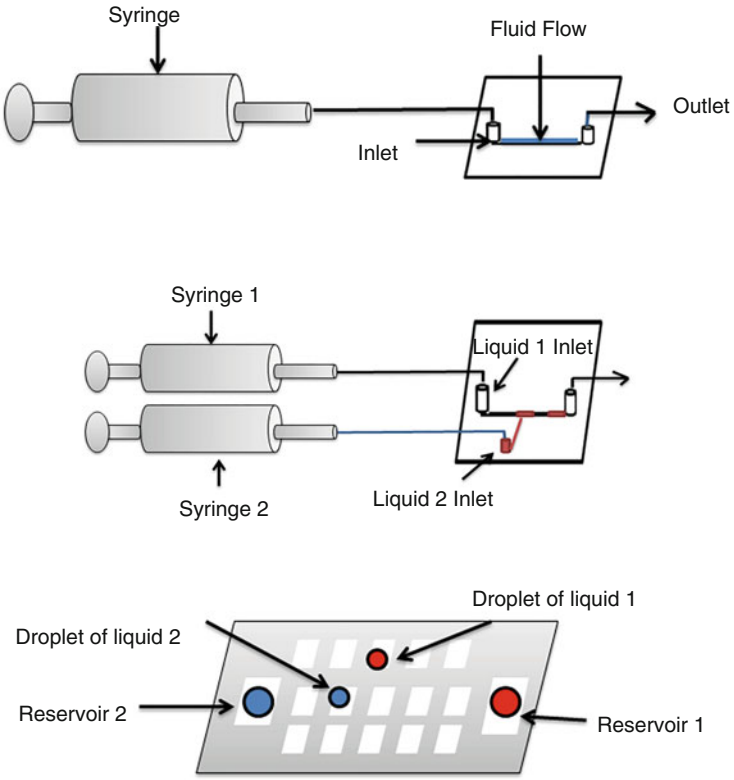

Fig. 22.1 Schematic of three different microfluidic systems: (**a**) continuous, (**b**) drop-based, and (**c**) digital

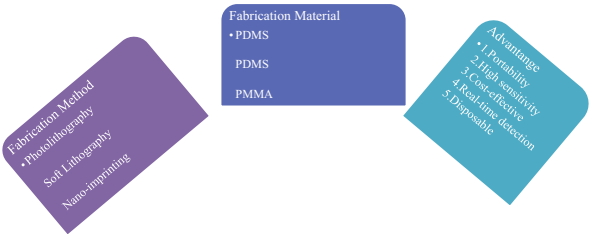

Fig. 22.2 Different fabrication methods and materials used to fabricate microfluidic devices for biosensing application

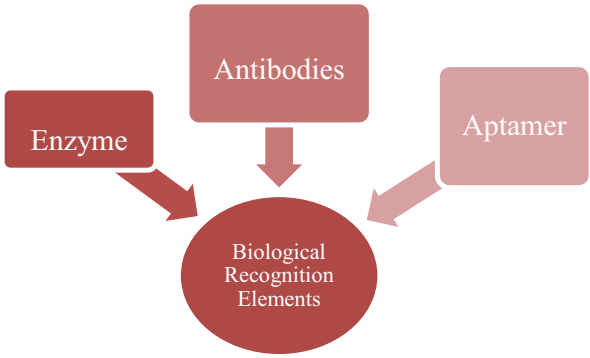

Fig. 22.3 Schematic of the biobased entities used in microfluidics

et al. 2019). In particular, in recent time, numerous biosensing applications have been reported. The operating and sensor actuation methods of the different types of microfluidics have been illustrated in Fig. 22.2.

Using microfluidics as a platform for designing and fabricating biosensors requires careful addressing to geometrical parameters as dimensions, volume, materials, and methods with a scope to improve biocompatibility of the device.

Commonly used substrates are glass and silicon (Tiwari et al. 2020). Recently, as alternate various polymer materials such as polymethylmethacrylate (PMMA), polydimethylsiloxane (PDMS), and paper are being used as substrate for fabrication of the microfluidic devices due to their superior chemical, physical, and mechanical properties offered by the polymers as well as other factors such as low cost, readily availability, or ease of manufacturing have led to gain the popularity among substrates (Tsao 2016).

22.3 Fusion of Microfluidics with Biosensor Technology

A microfluidic system enables the integration of biosensors with a lab-on-a-chip (LOC). This fusion of biosensors with microfluidics approach has been demonstrated as one of the significant demands these days. Nevertheless, contemporary biosensors are based on various approaches of microfluidics, and the biological recognition elements are being used in the biosensors that are listed in Fig. 22.3.

22.3.1 Enzyme-Based

One of the widely used approaches for detection is enzymatic reaction scheme. It possesses the ability of immobilization and amplification of the signal through their catalytic properties, generating colored products. Ali et al. (2013) designed microfluidic biochips that comprised of a nanocomposite (carbon nanotube and nickel oxide nanoparticles) fabrication and surface functionalization by two enzymes (cholesterol oxidase (ChOx) and cholesterol esterase (ChEt)). The chip was used to quantify the substrate based on the observed chronoamperometric change. Gu et al. (2014) illustrated the use of microfluidic electrochemical sensor comprising a Pt-black microelectrode and enzyme for the detection of glucose. The sensor measured the changes and fluctuation in the electrochemical current using electrochemical impedance and cyclic voltammetry as a result of formation of H_2O_2 as by-product on the oxidation of β-D-glucose. Nosrati et al. (2016) using paper as substrate developed μPADs that evaluate male fertility on the basis of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide (MTT) colorimetric assay. It was observed that the presence of the enzyme diaphorase flavoprotein in human sperm sample forms a complex with MTT that was seen in the form of color change from yellow to purple formazan. Srinivasan et al. (2003) using the integrated system of optical absorbance demonstrated the identification of various metabolites present in the body using glucose oxidase as recognition element. It was also found that the development system could successfully be used for the detection of molecules such as amino acids like glutamate and sugar molecules such as pyruvate, lactate, etc.

22.3.2 Antibody-Based

To detect and examine breast cancer (Lee et al. 2007), a continuous microfluidic biosensor based on SPR phenomena integrated with antibodies was developed. The automated lab-on-a-chip developed by these groups of researchers offered the advantage of fabricating multiple channels for the carrying microarray detection of the sample. The biosensor also involved a temperature control setup composed of microheater and temperature sensor to overcome and to measure temperature change during experimentation, since SPR is a temperature-sensitive technique. Owing to its high selectivity, rapid sensing provided by the integrated system allows its application such as detection of various biomolecule interaction like protein-protein, protein-enzyme, etc. For capturing and detection of microorganism such as *Escherichia coli* in drinking water, Golberg et al. (2014) designed a biosensor using droplet microfluidic approach. The biosensor involved the selective capturing and isolation of the microorganisms using fluorescence antibody assay and lastly analyses using automated fluorescence microscope. Therefore the system comprised of fluorescently labelled anti *E. coli* antibodies conjugated on magnetic beads. For the detection of avian influenza antibody, Choi et al. (2012) developed a digital microfluidic field-effect transistor-based biosensor. The mechanism of the devices

was based on the measure of amount of current being drained from the field-effect transistor biosensor upon the binding of the antigen to the antibodies, thus providing an advantage of label-free detection. FET-based sensor incorporated with digital microfluidics showed a great prospective toward detection of biomolecules without the need of bulky equipment.

22.3.3 Aptamer-Based

The simplicity of structure and ease to inculcate in biosensors make aptamers as a preferable biological element over antibodies. Upon binding with the target aptamers, optical or electrical signal is yielded without the requirement of secondary labeling, thereby reducing tedious washing steps and moreover reducing the time and cost of processing. An illustration for an aptamer-based lab-on-a-chip sensor for the continuous detection of living cells was fabricated (Zhou et al. 2013). The fabricated devices are comprised of glass substrate, gold electrodes, and polydimethylsiloxane (PDMS) layers. The PDMS layers were intended in a form such that the first layer is comprised of microchannels and the second layer casted pneumatic control. The association between the cell-secreted protein and the aptamer was measured using square wave voltammetry (SWV) assessments. Decline in the redox signal was observed as a direct relation to the amount of the sample analyte. To monitor damages to organoid such as cardiac organoid, Shin et al. (2016) demonstrated an organ-on-a-chip microfluidic aptamer-based electrochemical biosensor. The chip measured the response secretion of creatine kinase by the cardiac organoid upon the drug exposure, which was in agreement with the behavior of beating of heart and cell viability analyses. Wei et al. (2015) developed simple rapid POC device based on colorimetric apta sensor for the simultaneous detection of cocaine, adenosine, and lead ions. As a flow controller and regulator, a target-responsive hydrogel was used. The hydrogels act as regulatory element for the "signal on-off" readout. The observation could be made without any auxiliary equipment, and entire assay can be completed within few minutes.

22.4 E-Health Microfluidics

For real-time monitoring, fabricating the device using the integration of fusion of technology as information and communication technologies (ICTs) has opened a wider scope in biosensing technology (Yager et al. 2006; Ajami and Teimouri 2015). This emerging field of technology brings about a revolutionary change motivating the field of usage of chip-based sensing as well as wearable sensing devices, thus bringing a better alternate to carry diagnostic approaches in the progressing world (Guk et al. 2019; Mondal et al. 2021). Best examples of wearable sensors are glucose monitors that have been presented in market. It helped to change the background of glucose monitoring in patients who suffer from diabetes. The glucose monitor usually comprise of a microneedle; electronic parts such as potentiostat, wireless

Table 22.1 Summary of microfluidic devices to detect various analytes and diseases

Detection substrates	Device	Method
Breast cancer biomarker	Microfluidic immuno-array devices	Electrochemical amperometric
Glucose	Microchip device, wearable microfluidic devices	Electrochemical amperometry
Escherichia coli	Lens-free interferometric microscopy	Impedance spectroscopy
Urine analysis	Paper-microfluidic lab-on-a-chip	Colorimetric

transmitter, etc. for signal acquisition and wireless transmission; and at last wearable components (Huang et al. 2007). For detection of sodium in sweat, Anastasova et al. (2017) designed a wireless potentiometric sensor for electrochemically record glucose, and for lactate level in sweats (Martin et al. 2017), an epidermal microchip using lithographic and screen-printed technology has been fabricated . A fully integrated wearable device with potentiometric sensors for the detection of Na^+ and K^+ sensors has also been designed for sweat analysis (Gao et al. 2016). The excess of concentration of creatinine in the urine is symptoms of kidney disorder (Sununta et al. 2018). The method involved the collection of the urine samples, followed by dilution and centrifugation. Further, few microliters of alkaline picrate reagent (indicator) were pipetted into the detection zone of a μPAD on one side, followed by the addition of the centrifuged solution of creatinine into the sample zone. A complex formation between creatinine and picrate resulted in the visible color change from yellow to orange. Presence of different enzymes, protein, lipids, and electrolytes makes tears as a potential candidate for detection of diseases. Thus through using antibody-free paper microfluidic devices, one can detect the concentration level of lactoferrin. The methodology involved is an enzymatic reaction that involves the complex formation between terbium chloride hexahydrate and lactoferrin, resulting in emission of fluorescence. Additionally various electrolytes can be analyzed, thus acing as indicative for various eye conditions (Yamada et al. 2014; Yetisen et al. 2017). These devices are fabricated in such a manner that the channels exhibit a rapid wicking time. To monitor breathing pattern, Güder et al. (2016) developed a paper-based respiration sensor. The sensor detects measurable change in the electric signal sensed by the electrode directly attached into a medical mask caused by inhalation and exhalation, leading to change in humidity. Types of microfluidic devices to detect number of analytes and diseases have been summarized below in Table 22.1.

22.5 Conclusion

To improve the global public health, the development in diagnostic technologies is an important factor. This significant improvement in the health can only be achieved if there is an adequate coordination between the diagnostics developers and the communities as well. One can believe that soon the introduction of

microfluidics-based home care devices could bring potential to perform assays with higher sensitivity, specificity, and reproducibility of results at par with the results produced by central laboratory analyzers. Such devices in the hands lay a platform for people to perform routine testing or to rapidly detect the presence of an infectious agent. Nonetheless, the potential benefits often associated with the use of microfluidic devices for e-health diagnosis still pose various challenges such as cost factor, sensitivity, etc. Lastly, it can be speculated that with the increased interest toward community of the global health, the surge in the usage of customized microfluidic diagnostic devices can be anticipated.

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Fluorescent Quantum Dots (QDs): A Theranostic Tool for Breast Cancer Management

23

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Abstract

Bioimaging is one of the most sought-after technique that has been developed recently to allow the visualization of cellular mechanisms and thus aid in accurate diagnosis as well as treatment of a disease. Quantum dots (QDs), owing to their unique features of photostability, high quantum yield, and size tunable behavior, are equipped with one of the most contemporary biosensing properties. They possess the ability to emit light at a particular wavelength depending upon size, shape, and material used for their synthesis. Nanocarrier decorated QD conjugates are being evaluated since the past decade for their ability of controlled release, smart, and targeted delivery using suitable ligands thus offering a platform particularly suited for diagnostics and therapeutics in cancer therapy. Breast cancer ranks second in the category of occurrence and mortality rates among all the cancers prevalent in women worldwide. The onset of breast cancer and its progression is a complex phenomenon which alters the normal functioning as well as growth of the healthy cells. Clinical stage of breast cancer, its size, and lymph node metastasis.

Autophagy markers in tumor and tumor microenvironment are the conventional prognostic indicators used to assess the disease intensity. Specific labelling of QDs with ligands corresponding to specific breast cancer biomarkers (viz., HER2, ER, and PgR) is the currently proposed alternative for evaluation of breast cancer progression. This chapter will consolidate the literature available for

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QD-based theranostic applications particularly for better management of breast cancers.

Keywords

Breast cancer · ODs · Biomarkers · Theranostics · Tumor microenvironment

23.1 Introduction

Optical imaging was first introduced by Jobsis in the year 1977 where he demonstrated cerebral circulation in cats by near-infrared radiations (Jöbsis 1977). Well-established imaging techniques including ultrasound, magnetic resonance imaging (MRI), and computed tomography (CT scans) exhibit real picture of actual molecular events occurring during disease progression and other biological mechanism (Hillman 2007). Moreover these techniques are inexpensive, simple, and well accepted by medical fraternity but require trained personnel for its proper function. However these conventional imaging tools are lesser sensitive, use too laborious methods, and are time-consuming (Allam et al. 2020; Lim et al. 2018). Cancer cell imaging is also done with abovementioned techniques which are often associated with poor diagnosis. Therefore cancer detection techniques require an upgradation for more precise monitoring of tumor growth. To address the need of cancer therapy in terms of sensitivity and specificity, nanotechnology comes with the benefits of nanosize, precise diagnosis, and biocompatibility. Nanotechnology has revolutionized the field of cancer treatment along with the application of theranostics. Theranostic is the term applied for combination of therapy and diagnostic capabilities of a delivery approach. This provides cancer treatment along with real-time monitoring of cancer regression. Theranostic platform promises maximum drug exposure of tumor cells and minimum peripheral side effects which are mainly due to ligand-mediated transport of nanoparticles (NPs) at the cancer site (Sun et al. 2018).

Breast cancer is the second most prevalent cancer after lung cancer with highest mortality rates in humans (Azamjah et al. 2019). The initiation and progress of breast cancer is dependent upon multiple factors and is a quite complicated process. Various indicators like tumor size, pain, and inflammation at the site and presence of metastasized lymph node are the signs used to anticipate the prognosis (Kabel 2017; Takalkar and Advani 2018). In addition to these, nuclear proliferative markers, namely, ER, PR, HER2, and Ki-67, first identified by (Gerdes et al. 1984) are reported to foresee the chemotherapeutic benefits of the therapy (Miller et al. 2018). The ligand specific to the markers is conjugated with the NPs to form theranostic tool for breast cancer.

The light in the UV-visible range does not get penetrated deep into the organ tissues. However the light gets superficially absorbed and scattered to generate a strong fluorescence (Slominski et al. 2018). Near-infrared wavelength, on the other hand, reaches deeper into the breast tissues and thus generates significant signal-to-

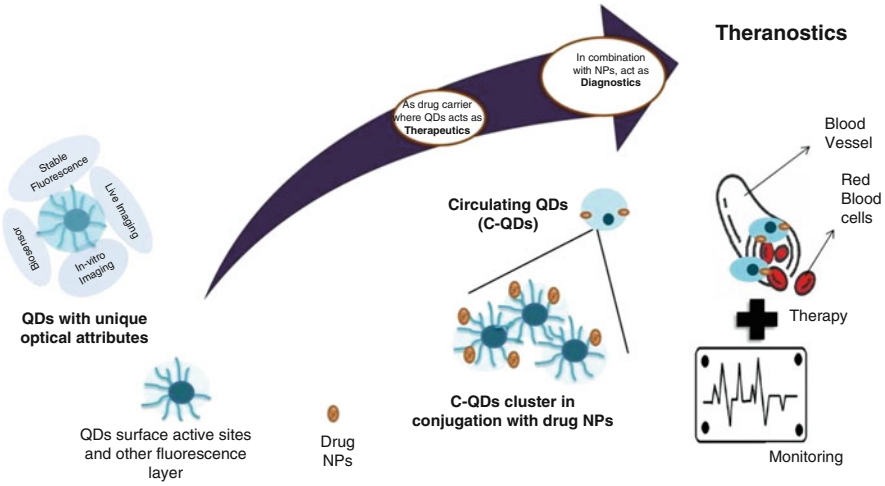

Fig. 23.1 Unique attributes of QDs and its application as a theranostic tool

noise ratios in response. Thus use of optical imaging with a particular wavelength range is dependent upon specificity, sensitivity, and cancer site characteristics to be achieved (Mambou et al. 2018; Recinella et al. 2020). Fluorescent labels like proteins and organic dyes are being used to label and differentiate the cellular structures. Among the optical imaging technologies, fluorescence imaging using fluorescent proteins and dyes offers high sensitivity for breast cancer detection. Fluorescent organic dyes have several drawbacks as well, namely, photobleaching over a period of time and broad range of absorption spectrum hindering their use in the long-term imaging.

Hence, to overcome the limitation of photodegradation of organic dyes, the semiconductor nanocrystals called as quantum dots (QDs) have been developed and found to have immense potential to detect the onset of cancer with precision as well as accuracy. These nanoparticles offer long-term imaging due to their property of aqueous solubility and biocompatibility. Further fluorescent NPs, namely, (QDs), silicon, and gold NPs, are used nowadays with proven potential in acquiring the biological information both in in vitro and in vivo animal models (Gubala et al. 2020; Li et al. 2018; Muñoz-Rosas et al. 2018).

QDs are nanomaterials typically belonging to family of group III-IV and most commonly carbon, gold, and silicon elements with unique contrast properties (Wegner and Hildebrandt 2015). These nanomaterials can be easily conjugated with ligands possessing affinity to a particular biomarkers, namely, peptides and nucleic acids, to impart site specificity to the nanocargoes (Guan and Zhang 2020; Liu et al. 2017; Pei et al. 2014). Figure 23.1 highlights the unique optical behavior exhibited by QDs and its applicability in diagnosis and treatment of cancer. This forms the basis for the biological fluorescent assays essential of disease detection, treatment, and reporting of other intracellular events.

23.2 Breast Cancer and Conventional Treatment Strategies

Breast cancer treatment comprises multidisciplinary approaches ranging from breast-conserving radiation surgery to removal via mastectomy (Tang et al. 2016). The conventional modalities of treating breast cancer include surgery, chemotherapy (CTx), and radiation therapy (RT) which forms the main skeletal of the breast cancer treatment plans. From this clinicians choose the type of treatment plan to be executed based on the stage of cancer, patient health, and co-morbidities if present. Hormone therapy is an effective treatment for breast cancer and may be given before surgery to shrink a tumor and help prevent a cancer recurrence and death from breast cancer when used either by itself or after chemotherapy (Sayed et al. 2019). However, due to limitations of conventional therapies, St. Gallen Guidelines in the year 2011 and then 2013 were adopted to treat the cancer with the use of systemic adjuvant therapies which were based on individual ER/PR, HER-2, and Ki-67 profiling (Harbeck et al. 2013; Senkus et al. 2013).

Breast cancer therapy is a big challenge for physicians with respect to tumor resistance and metastasis. In order to overcome the revolting problem, the selected chemotherapeutic drugs were given at maximum tolerated dose (MTD), which comes under the MTD regimens but often presents acute or chronic systemic toxicities (Kareva et al. 2015)**.** Therefore the use of low doses of chemotherapeutic agents was used frequently and was equally efficient as MTD along with lesser toxicity issues that have been documented in various reports, for example, metronomic delivery (Paluchowski et al. 2016). Adjuvant CTx is used in majority of patients depending on patient's survival benefits and after individual molecular profiling. Among various standard CTx regimens, combination of an anthracycline and a taxane was mostly advocated. In several reports amalgam of doxorubicin and cyclophosphamide called as AC for four cycles then paclitaxel for another four called as AC-T is documented. One more widely discussed routine is AC followed by a taxane for straight 12 weeks with four cycles of docetaxel every third week is being used for treatment (Almeida and Rosa 2018; Fujii et al. 2015). However, most chemotherapeutic drugs have a fairly narrow range for dose safety and effectiveness; therefore, the dose was decided upon by stage of breast cancer, tumor size, and its metastasis that have been exhibited in various reports (Anampa et al. 2015). Hitherto, the present novel CTx strategies revolve around the novel nano-platform where a suitable nanocarrier is conjugated with ligands such as peptides, antibodies, and aptamers. The ligand selected for selective delivery of therapeutic drug to cancer cells specifically binds to a receptor that is overexpressed at the target site (Toporkiewicz et al. 2015). Various ligand receptor approaches are depicted in Table 23.1, highlighting the different anticancer drugs investigated for their chemotherapeutic action when used as such (Sect. 4) and when administered in a nanoplatform (Sect. 5).

Nanotechnology has made possibility of combining multiple therapeutic agents with various MRI-based contrast and fluorescent NPs to serve the purpose of imaging/diagnosis or as a theranostic platform.

Table 23.1 List of anticancer drugs used for treatment of breast cancer as a chemotherapeutic agent compared when administered in a nano-platform

S. No. I	Drug category II	Anticancer drug III	Mechanism IV	As a chemotherapeutic agent V	In a nano-platform VI	Remarks
1.	Nitrogen mustards	Cyclophosphamide	Cross-linker for genetic material thereby inhibits protein synthesis	Untch et al. (2010) reported that trastuzumab in combination with epirubicin/ cyclophosphamide is a feasible and potentially effective regimen for patients with HER2-positive MBC	Tiash and Chowdhury (2016) reported cyclophosphamide-loaded NPs did not accumulate in the liver and resulted in reduced tumor volume	Bio-distribution pattern inside different organs of the body was better for nano formulations
2.	Platinum complexes	Cisplatin	Cross-link with the purine bases on the DNA, interfering with DNA repair mechanisms, causing DNA damage, and subsequently inducing apoptosis in cancer cells	Byrski et al. (2012) reported a partial/ complete clinical response achieved in 16 of 20 (80%) patients who received cisplatin chemotherapy as a single agent	Wan et al. (2019) documented use of PTX and cisplatin co-loaded micelles for improved chemotherapy of breast cancer and potential for the clinical translation	Sustained release to serum, improved pharmacokinetics and increased tumor distribution with nanoformulation
3.	Folic acid analogs	Methotrexate	Inhibition of DHFR enzyme, leading to Partial depletion of Cofactors required for the synthesis of new thymidylate and purines Nucleotides	Soriano et al. (2011) suggested oral administration of low doses of cyclophosphamide and methotrexate, prolonged survival of a subgroup of patients with metastatic breast	de Oliveira et al. (2018) reported nanoformulations Of methotrexate and its diethyl ester derivative with antiproliferative activity	No cytotoxicity to non-tumoral cells, with increased expression of folate receptors with nanosystem

(continued)

Table 23.1 (continued)

S. No. I	Drug category II	Anticancer drug III	Mechanism IV	As a chemotherapeutic agent V	In a nano-platform VI	Remarks
				cancer with low toxicity		
4.	Selective estrogen receptor modulators (SERMs)	Raloxifene	Blocking coactivator Binding to the activating function 2 domain of the ER, thereby blocking transcription of the ER-regulated Gene	Taurin et al. (2013) demonstrated raloxifene suppressed tumor growth in two relevant mouse xenograft models, mediated by decreased EGFR protein levels and their altered localizations within endosomes	Ağardan et al. (2015) demonstrated raloxifene dimethyl-β-CD cochleate formulations resulted in reduction of breast tumors	Slow drug release and penetration-enhancing effect of cochleates showed similarities with composition of cell membranes
5.	Taxanes	Paclitaxel	Causes cell death due to chromosome disaggregation on multipolar spindles	Andre et al. (2010) concluded that the combination therapy of everolimus plus paclitaxel and trastuzumab is well tolerated along with higher rate of tumor response	Chowdhury et al. (2018), showed nano-paclitaxel uptake was more than 95.52 + 11.01% in 6 h	Enhanced drug availability for tumor sites, suppression of metastasis as well as preventing drug resistance as seen in nanoformualtions of taxanes
6.	Anthracyclines	Doxorubicin	Bind to DNA-associated enzymes, intercalate with DNA base pairs, target multiple	Bandyopadhyay et al. (2010), showed doxorubicin with a TGFβ inhibitor has the potential to reduce the	Cabeza et al. (2015), demonstrated greater drug loading values and a slower drug release	Nanoformulations showed 21.4% tumor growth inhibition, reduced therapeutic dose

			molecular targets to produce a range of cytotoxic effects	dose and consequently the toxic side effects of doxorubicin, with improved efficacy	profile with nano systems	
7.	Aromatase inhibitors	Anastrozole	Reduction of estrogen concentration within ER+ breast Cancer cells	Mehta et al. (2012) reported significant improvement in overall survival by more than 6 months	Shavi et al. (2015) demonstrated long circulation and sustained delivery properties with nanosystems	Showed greater tumor Growth inhibition, 3.33- and 20.28-fold increase in AUC values when nanosphere compared to pure drug
8.	Bisphosphonates	Zoledronic acid	Gamma–delta T cells effectively targeted and killed zoledronic acid-treated ER+ breast cancer cells	Rachner et al. (2010) communicated apoptosis with zoledronic acid via activation Of caspase-3 and − 7 and by upregulating TNF-related apoptosis-inducing ligand	Choi et al. (2013) reported zoledronic acid liposomes have longer circulatory, targeted delivery	Study showed 50% reduction in inhibitory concentration (IC50), increased anticancer effect with liposomes
9.	Pyrimidine analogs	Gemcitabine	Phosphorylated by deoxycytidine kinase to dFdCTP is Incorporated into DNA, inhibiting DNA replication and inducing apoptosis	Park et al. (2013) demonstrated paclitaxel/gemcitabine resulted in better progression-free survival and overall survival	Papa et al. (2013) showed more potent cytotoxic and apoptotic agent in sensitive breast cancer cells, it more potently inhibited cell migration in the resistant cell line	Acted as a more potent inhibitor of migration, inhibited tumor growth while increasing mice survival with nanoformulations in comparison to pure drug

MBC metastatic breast cancer, *NPs* nanoparticles, *DHFR* dihydrofolate reductase, *ER* estrogen receptor, *EGFR* epidermal growth factor receptor, *TGF* transforming growth factor β, *dFdCTP* difluorodeoxycytidine

23.3 QDs as a Diagnostic Tool for Breast Cancer

Fluorescence techniques are apt in producing reliable, fast, and reproducible detection of molecular level changes occurring during the disease process with the help of specific ligands. A n ideal diagnostic tool has (1) an easily excitable behavior which is distinguishable from other biological elements, (2) solubility in body fluids and different buffers, (3) stability under physiological conditions, (4) least/no toxicity profile, and (5) stable and detectable fluorescence after ligation (Maguire et al. 2014; Mukherjee et al. 2015). Striking feature that differentiate fluorescent dyes (FDs) from QDs is the toxicity reported with the FDs which is dependent upon the concentration used (Prabhakar et al. 2015). For brighter fluorescence, higher concentration of FDs is used which become toxic to other healthy tissues, whereas QDs are generally regarded as safe. This is because of a stable and bright fluorescence even at lower concentration of QDs (Bruna et al. 2019; Lim et al. 2015). Another quality attribute associated with QDs is stability against photo-bleaching, and these can be excited for multicolor emission spectra from a single excitation source wavelength. These distinctive properties are well suited for real-time dynamic analysis at molecular levels and are in sync with the modern biomedical diagnostics which require higher sensitivity fluorescence probes. They can serve as fluorescent agents for the detection of deep tissue tumors, thereby enabling a surgeon to excise the entire tumor, as verified in real time through fluorescence imaging (Yan et al. 2019). QDs are functionalized to detect the genomic aberrations of breast cancer genes that will give a more precise picture for the causes of breast cancer. For detection of cancer biomarkers, different QD-based assays have been designed successfully, in which QDs provide stable optical characteristics beneficial for multicolor bioassays. In addition, they can be used for development of nanobiosensors which can open several prospects toward identification of a large number of molecular markers for various diseases (Pericleous et al. 2012).

Recently researchers have documented bright and stable QDs for tracking of single protein. These fluorescent nanoparticles can be used as tracers for evaluation of entire sample population of protein. The study proved the role of erB1 and erB2 receptors (from the family of HER1 and HER 2 signaling proteins) in aggressiveness of the breast cancer disease (Jin et al. 2011).

Recent research advances in nanotechnology for diagnostics have minimized the toxicity of QDs, and they are successfully used in in vitro and in vivo imaging. The detection of breast tumors and cancer cells in vivo has been possible because of the encapsulation of QDs into polymeric nanoparticles and linking them with targeting ligands (Zhang and Clapp 2011). QD-antibody conjugates were successfully used in monitoring and diagnosis of breast cancer, whereas near-infrared (NIR) QDs were found to minimize the absorption and scattering of light by the breast tissues thus rendering them suitable in deep tissue analysis. New synthesis of QDs may provide new types of bioconjugates of QDs to biomolecules, which leads to a variety of applications to many challenged research areas. QDs with narrow emission wavelength ranges are very suitable for monitoring multiple cellular targets simultaneously and still remain the best-known probes for imaging as an alternative

to traditional fluorophores in breast cancer diagnosis. Table 23.2 summarizes the different sets of fluorescent nanomaterials investigated by the researchers in diagnosis of breast cancer.

23.4 QDs as Theranostic Platform for Breast Cancer

The QDs qualify for the theranostic tool on basis of their nanosize as it allows them to cross biological barriers and through the organs including blood-brain barrier, blood-intestinal barrier, and dermal barrier (Zayed et al. 2019b). Further they get easily excreted through the route of renal filtration, thus rendering them less toxic. Size is increased significantly by addition of surface functionalities required for target ability of the developed nano system. Certain lacunas like poor drug payload, improper targeting, and altered release of conjugated therapeutic drug are also reported (Wang and Chao 2018).

Very recently a novel work on graphene quantum dot (GQD) from our lab proved the theranostic abilities of GQDs where in-house synthesized biosurfactant showed excellent internalization into the MCF-7 cells when conjugated with the ligated GQDs (Bansal et al. 2019). Another work on GQD-based nanocarrier labelled with Herceptin (HER) and β-cyclodextrin (β-CD) was documented as a promising theranostic candidate for the treatment of breast cancer. To achieve enhanced anticancer activities, each component plays multiple critical functions. Ligand specificity with HER-2 is for localization of GQDs at HER2-overexpressed breast cancer cells, whereas β-CD is the carrier for anticancer drug, doxorubicin (DOX), adjusted mainly by host-guest chemistry. The blue-color emission of the GQDs allows for the diagnostic effects of the nanosystem. In the presence of acidic environment of cancer cells, GQD complex gets eroded releasing DOX in a controlled fashion to stop the further growth of tumor. It serves as a multifunctional (due to DOX and GQD itself) synergistic cancer killing strategy along with diagnosis (Ko et al. 2017). Figure 23.2 illustrates the conjugation of QDs with NPs and the use of this theranostic platform for organ specific therapy and monitoring the exact status of the cancer spread.

Semiconductor nanocrystal quantum dots (QDs) integrated with biomaterials have proved to overcome issues faced in breast cancer therapy by using biodegradable chitosan (*N*-acetyglucosamine) for tumor-targeted drug delivery. The drug-loaded chitosan-encapsulated ZnO:$Mn2^{+}$ QDs represent a potential platform to deliver tumor-targeted drugs and monitoring of cancer simultaneously (Yuan et al. 2010). The doping with low concentrations of manganese increases the bandgap energy of ZnO with a consequent increase in luminescence. Further, to increase the efficiency of QD, its solubilization in aqueous medium with fully deacetylated chitosan prepared by thermochemical deacetylation is favored. Subsequently, the low-molecular-weight, fully deacetylated chitosan can be conjugated with folic acid for in situ encapsulation of water-dispersible ZnO:Mn^{2+} QDs. The cellular apoptosis of tumor cells were enhanced by the drug-loaded, biofunctionalized chitosan-encapsulated QDs. Moreover, by using drug-loaded, biofunctionalized

Table 23.2 Different types of QDs synthesized and their use as diagnostic probes in breast cancer

No	QDs synthesized	Method of preparation	Characterization	Remarks	Ref.
1.	CdSe/CdS/ZnS QDs	Cd (ClO4)2 + mercaptosuccinic acid into 100 ml water into three-necked flask under inert atmosphere. Hydrogen telluride (H2Te) gas passed. QDs produced upon refluxing at 100 C	Hydrophilic QDs showed 79% quantum yield; cellular uptake for MCF-7 cell lines was via receptor mediated endocytosis	QDs conjugated with anti-HER2 antibody showed superior sensitivity in comparison to immunohistochemistry assay	(Singh et al. 2016)
2.	CdSe/CdZnS QDs	TOPO+ HAD+ SA into a *25 mL three-necked flask* heated at *200 °C* under an *argon atmosphere*	DLS and FCS (650 nm) hydrodynamic diameters: 6.9 ± 0.5 nm; quantum yield:0.39; apparent MW: 300 kDa	Colloidal stability of QDs prepared by SMCC coupling, red-emitting QDs most effective and quantitative for imaging of HER2 expression in the breast cancer cells	(Tiwari et al. 2009)
3.	Ag_2S QDs	DDW + selenium+$NaBH_4$+ $AgNO_3$+ GSH+ NaHSe at *85 °C* under *nitrogen atmosphere*.	With Ag2S QDs for 4 h, the cells exhibited 92% viability, at 98 cells/mL for MCF-7 cell line	Photoelectric active interface was used to detect glucose and MCF-7 cancer cell and showed the good sensitivity and specificity	(Zhang et al. 2014)
4.	CdTe/CdS QDs	$CdCl_2$ 2.5H2O+ thioacetamide +thioglycolic acid+ DI water +NaOH+NB 20 min + heat at *100 °C* 3 h	Apoptotic bodies and chromatin condensation: Hoechst staining; DNA ladder profile in the exposed cells: DNA fragmentation assay; apoptosis in a dose-dependent manner	QDs controlled or Repressed cancerous cells induced cell death in MDA-MB468 and MCF-7 Breast cancer cell lines	(Naderi et al. 2018)
5.	ZnS:Mn/ZnS QDs	$Zn(OAc)_2 2H_2O$+ MPA + water+ NaOH+NB 30 min + reflux 7 h + rotating evaporator(50 °C, 15 mm hg)	Photoluminescence quantum yield of 22%, TEM:4.4 ± 0.7 nm;X-ray photoelectron spectroscopy, and DLS:9.5 nm	The receptor-mediated uptake of ZnS:Mn/ZnS QDs by FR^+ T47D breast cancer cells was demonstrated by TPCM. MTT assays demonstrated that the cytotoxicity of QDs correlated with their concentration, that is, cell viability	(Geszke et al. 2010)

				decreased with increasing QD concentration	
6.	PEGylated graphene QDs	EDC + SulfoNHS+GQD-COOH+ 10% amine-PEG-amine diluted with PBS & Filter	TEM: 4.2 ± 0.8 nm; UV–vis absorption spectra: Blue shift; FRET assay: Highest quenching efficiency	FRET assay was successfully used for EpCAM protein detection with a logarithmic linear detection range from 3 nM to 54 nM and a limit of detection of 450 pM on MCF-7 cells	(Shi et al. 2017)
7.	CdSe/ZnS QDs	PBS + anneal 95 C for 5 min and slowly cooling+TCEP in 100 fold excess+20-fold molar excess of DNA+ super filtrated+ dissolved in PBS and stored at 4 °C	Hydrodynamic size:27 ± 7.2 nm; no apparent cytotoxicity of either QDs aptamer conjugate or QDs alone	Recognition of QD-aptamer nanocomplex to breast cancer cells was demonstrated using confocal microscopy, and the viability of QD-aptamer bioconjugate bound cells were not affected within 24 h on MDA-MB-231cell line	(Lian et al. 2012)
8.	Surface passivated (PEG200) carbon QDs	Citric acid+ urea+PEG+ 180 °C for 5 min + centrifuged at 6000 rpm for 30 min + cellulose acetate filter +ultrafiltration via dialysis tube for 30 min at 5000 g + lyophilized	TEM:7–10 nm; FTIR; XPS survey: Ndoping; Raman spectra: Amorphous nature; UV–vis absorption: Red shift	Due to the heat generation capability of QD, MCF-7 cell apoptosis was enhanced, and the power of doxorubicin in inducing cell death was increased	(Ardekani et al. 2017)
9.	PAMAM*-derivatized QDs	PAMAM G5 dendrimer in methanol solution+ tetramethylammoniumhydroxide +shaken for 15 h at 30 °C+ ethyl acetate	IC_{50}:1.549 μM; NOAEC:0.320 μM; TLC: 3.427 μM for MCF-7 cells	Fluorescence microscopy images demonstrated that the designed PAMAM-derivatized QDs nanoparticles show great potential in the areas of cellular imaging and targeted therapy	(Akin et al. 2012)

(continued)

Table 23.2 (continued)

No	QDs synthesized	Method of preparation	Characterization	Remarks	Ref.
10.	IO QDs in the nanoparticles of PLA-TPGS	THF + PLA-TPGS+ sonicated at 25 W + centrifuged at 10,500 rpm for 15 min	Size:325.8 nm; zeta potential:37.3 mV; encapsulation efficiency:45%	MRI and fluorescent imaging have both confirmed the ability of nanoparticle formulation system to passively target tumour tested on MCF-7 cells	(Tan et al. 2011)

TOP tri-n-octylphosphine oxide, *Cad-pent* cadmium 2,4-pentanedionate, *SA* stearic acid, *DLS* dynamic light scattering, *FCS* fluorescence correlation spectroscopy, *SMCC* succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, *DDW* doubly distilled water, *DI* deionized water, *NB* nitrogen bubbling, *MPA* 3-Mercaptopropionic acid, *TPCM* two photon confocal microscopy, *EDC* 1-Ethyl-3-(3-dimethyl-aminopropyl) carbodiimide, *SulfoNHS* N-hydroxysulfosuccinimide, *GQD-COOH* carboxylated graphene quantum dot, *TCEP* Tris(2-carboxyethyl)phosphine hydrochloride, *NOAEC* no observable adverse effect concentration, *TLC* total lethal concentration, *PAMAM* amine-terminated polyamidoamine, *IO* iron oxides, *PLA-TPGS* f poly (lactic acid)-d-a-tocopheryl polyethylene glycol 1000 succinate, *HFP* (O-(7-azabenzotriazole-1-yl)-N,N,N'N′-tetramethyluronium hexafluorophosphate, *FRET* fluorescence resonance energy transfer, *EpCAM* epithelial cell adhesion molecule

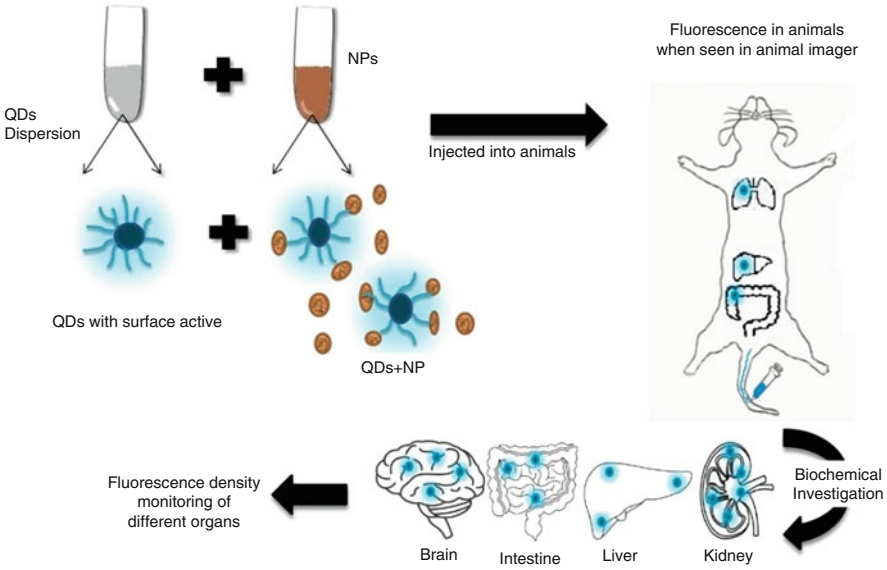

Fig. 23.2 Schematic representation of QDs and nanoparticle preparations, their conjugation, and in vivo experimentation for intensity monitoring of nanoparticles

chitosan-encapsulated QDs, the time taken for tumor cells to go into an apoptotic state can also be monitored by confocal laser-scanning microscope. Thus, this information will determine the dosage necessary for targeted drug delivery. A novel quantum dot (QD) – aptamer(Apt) – doxorubicin (Dox) conjugate [QD – Apt(Dox)] was used as a targeted cancer imaging, therapy, and sensing system for malignant breast tumor (Dou et al. 2017). Table 23.3 contains the investigations carried out for the theranostic platform by different researchers for better management of breast cancer along with the studies carried out to prove the effectiveness of the developed system.

23.5 Conclusion

QDs represent the latest nanomaterials to be used as fluorescent agents and have proved their potential for molecular level imaging for cancer in general and breast cancer in particular. Clinical application of these fluorescent probes is not limited to in vivo imaging but is extended toward identification and selection of breast cancer biomarkers. They can be exploited for recording the clinical responses toward the cancer therapy with a condition of higher specificity and enhanced sensitivity. Undoubtedly, QDs will continue to become an imperative tool when it comes to cancer biological research. The elucidation of molecular mechanisms of protein binding, signaling, and regulation of various oncogenes responsible for breast cancer will be further studied using these nanomaterials. Being a double-edged sword, they

Table 23.3 List of QDs as a theranostic tool for breast cancer management

Ref.	QDs Synthesized	Application	Carrier vehicle	Performance studies	Remarks
(Zayed et al. 2019a)	Hybrid au-SiO2/QDs	Tumor targeting and inhibition of Release into the systemic circulation	PHEA-LA-PEG-FA polymeric micelles	Cellular internalization of fluorescent nano-probes within MCF-7 cells at 24 h and higher decrement in cellular viability upon photo-thermal treatment	Hyperthermic effect of QDs synthesized along with chemotherapeutic killing of doxorubicin
(Muthu et al. 2012)	Docetaxel-loaded QDs	Greater targeting and multifunctional QDs	D-alpha-tocopheryl polyethylene glycol 1000 succinate mono-ester liposomes	IC50 value was 0.23 ± 0.05 μg/ml after 24 h culture with MCF-7 cells and internalization of QDs by MCF-7 cells	Folate receptor targeting QDs and cytotoxic activity of docetaxel
(Bwatanglang et al. 2016)	Mn:ZnS QDs	Nontoxic nature of the compositeand better binding affinity	Folic acid–chitosan nanocomposite	No toxicity to MCF-7 and MDA-MB-231up to a 500 μg/mL concentration. Enhanced the internalization and binding affinity of the nanocarrier toward folate receptor-overexpressed cells	Specific targeting of folate-expressed cancer cells, highly intense fluorescent emission from $Mn^{2+}$and encapsulation with anticancer drug
(Hua et al. 2013)	Nucleolin aptamer AS1411-CdTe QDs	Enhanced detection sensitivity and extension to other cells	Monodispersed silica nanoparticles	Detection sensitivity was enhanced, detection limit of 201 and 85 cells mL^{-1} on MCF-7 cells	Affinity interaction overexpressed on the surface of MCF-7 cells
(AbdElhamid et al. 2018)	Cadmium telluride QDs	Enhanced cytotoxicity and superior In vivo antitumor efficacy	Anionic CD44-targeting chondroitin sulfate and cationic low-density lipoprotein	Traced their internalization and on both MCF-7 as well as MDA-MB-231 cell lines	Enhanced cytotoxicity and superior in vivo antitumor efficacy
(Kalangi et al. 2012)	CdTe QDs	High selectivity and distributionin vitro as	3-Mercaptopropionic acid in the presence of 1-ethyl-3-	Stability of drug conjugated QDs, free from aggregation	Tagging of a tracer CdTe QDs to 5FU showed the

		well as In vivo	(3-dimethylaminopropyl) carbodiimide	suggested no major changes in QDs size After conjugation	entry of chemotherapeutic into the nucleus of MCF-7 cancer cells
(Hasanzadeh et al. 2018)	Thiolated grapheme QDs	High loading of CA 15–3 antibodies on CysA/au NSs/GQD resulted in development of immunosensor	Gold nanospears	Antigen detection as low as 0.11 U/mL increases the number of binding events. The proposed immunosensor showed activity against MCF-7 cells	Displayed good sensitivity and specificity
(Lian et al. 2012)	CdSe/ZnS QDs-aptamer bioconjugate	Targetability of the developed nanosystem with aptamer	Poly(ethylene glycol)-phospholipidsmicelles	Strong red fluorescence in MCF7 and MDA-MB-231 cells, specificity, binding capability to cancer cells.	No cytotoxicity of either QDs aptamer conjugate Or QDs alone at tested concentrations, good Biocompatibility and suitable for biosensing
(Bae and Chung 2014)	CdSe/ZnS QDs	Conjugation of ligands targeting over-expressed cell surface receptors	Perfluorooctylbromide and Perfluoro-15-crown ether nanoemulsion	Cell viability studies in SKBR3 cell, MCF-7 cell, and MDA-MB 468 cell	Selectively bind to the target-protein, low toxicity because of lower concentration of Cd^{2+} or Se^{2-} ions
(Rana et al. 2019)	CdSe/ZnS QDs	Coating of L-GSH for QDs enhances detection and cytotoxicity	N-dimethyl formamide, dimethyl sulphoxide nanoprobes	Significant growth inhibition and cell death and max fluorescence (safe dose 5 pg/μL)	Less reactivity with healthy living cells, Serve as a favorable tool for in vivo imaging
(Rejinold et al. 2013)	CdTe/ZnTe QDs	Fibrinogen-coated-QDs had bifunctional, imaging, an therapeutic effect	Fibrinogen and $CaCl_2$	Targeted attachment and retention of these QDs on the α5β1-integrins of MCF-7 cells	Significant localization and retention of QDs and cytotoxicity of paclitaxel
(Roshini et al. 2017)	ZnO QDs	ZnO QDs facilitate tracking them during chemotherapy	N-methylpyrrolidone	Cytotoxicity to MCF-7 and metastatic MDA-MB-231 breast cancer cells (10 and	Destabilize cancer cells by using its acidic tumor microenvironment

(continued)

Table 23.3 (continued)

Ref.	QDs Synthesized	Application	Carrier vehicle	Performance studies	Remarks
				15 μg/ml), induced nuclear fragmentation and apoptosis. Cell cycle arrest at the G0/G1 phase	
(Su et al. 2014)	Carbon QDs	Enhanced the conductivity and enlarged the specific surface area	Mesoporous silica nanoparticles	Detection limit of 230 cells mL^{-1} on MCF-7 cells	High sensitivity, specificity, and good stability of QDs
(Fakhroueian et al. 2014)	ZnO QDs	Anticancer activity against breast and colon cancer cell lines	Polymers, oily fatty acids, PEG, and organosilane	IC50 values were determined as 10.66 and 5.75 μg/ml for MCF7 and HT29	Low toxicity in normal cells (MDBK)
(Xiao et al. 2010)	CdSe/ZnS QDs	PEG modification blocks Nonspecific QD delivery into cells	Polyethylene glycol	Cellular uptake high in MCF-7 cells, no detectable decrease in cell viability	QDs localized within lysosomes
(Sanwlani et al. 2014)	CDSe QDs	Toxicity produced with elemental ions of QDs	Trioctylphosphine	Enhanced levels of Cd^{2+} and Se^{2-} ions cause oxidative stress to the cell, generating OH^- and O^- as byproducts, to form metal ions leading to apoptosis	Apoptosis via the generation Of ROS and modulation of GST
(Aswathy et al. 2012)	ZnS:Mn QDs	Cell-specific drug or bioactive component Delivery to target cells	Zein nanoparticles (NPs)	Cytotoxicity study on L929, murine fibroblast cell line and MCF-7, human breast cancer cell line	Biocompatibility of zein QD NPs. Viable cells decreased with the increase in concentration Of DL zein QD

(Alibolandi et al. 2016)	MSA-capped QD	QD-NPs accumulated in the tumor via the EPR effect	PEG-PLGA nanopolymersomes	Doxorubicin sustained for 12 days, preferentially accumulated in 4 T1 and MCF-7 cells in vitro	Higher cytotoxicity of doxorubicin and sustained release of QDs
(Ardekani et al. 2017)	Carbon QDs	Combined chemotherapy and photothermal therapy	PEG200, urea, and citric acid	Drug loading capacity0.98 w/w, 88% cell viability, death of 78% of the MCF-7 cells	Intense green luminescence of QDs, cytotoxicity of doxorubicin
(Ko et al. 2017)	Graphene QDs	DOX released in a controlled manner inhibiting proliferation of cancer cells	Herceptin (HER) and β-cyclodextrin (β-CD)	Viability of MCF-7 was >95% up to 500 μg mL − 1 indicating low cytotoxicity of QDs	Inhibit proliferation of cancer cells and cytotoxicity of doxorubicin.

Hybrid gold-silica/CdSe-CdS QDs, *5-FU* 5 fluoro uracil, *conc* concentration, *ROS* reactive oxygen species, *GST* glutathione S-Transferase, *PLA-TPGS/TPGS-COOH NPs* poly(lactide)-vitamin E TPGS copolymer and vitamin E TPGS-carboxyl NanoParticles, *MSA* mercaptosuccinic acid

will be used not only to study the molecular mechanisms of breast cancer but also in drug discovery, newer treatment strategies, and real-time monitoring of breast cancer. The research fraternity inclined toward these fluorescent nanomaterials is of the opinion that the future of QDs in cancer cell biology is as luminescent as these tiny particles.

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Molecular Mimicry: Unravelling the Role of Autoantibodies in Autoimmune Diseases and Infertility

24

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Abstract

Molecular mimicry, proposed almost half a century ago, has been successfully recognized as a mechanism by way of which microorganisms evade or subvert the immune response of the host. Although originally devised to escape from the host immune response, this phenomenon turned sides when the similarity between host and microbial protein triggered an immune response against the host antigens. Such triggering role and its pathogenetic importance have been studied for various autoimmune diseases. However, such is not the case for infertility, which appears rather overlooked by this field of research. Here, the available literature on the possible role of molecular mimicry as a trigger of autoimmune disease as well as infertility has been reviewed.

Keywords

Autoantibodies · Mimicry · Autoimmune diseases · Antisperm antibodies · Infertility

24.1 Introduction

The human body is persistently exposed to concurrent multiple insults—either from normal microbiota or the microorganisms present in the environment which enter mainly via the skin, respiratory tract, and digestive organs (Abt and Artis 2013; Spasova and Surh 2014). Although the former have evolved mechanisms to live in symbiosis with each other and with the host, yet, the peril relates to the latter, which possess a diverse collection of mechanisms by which they replicate, spread, and

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threaten the normal functions of host, indicating the existence of a fragile balance between host and microorganisms (Chaplin 2010). Therefore, in response to the need for survival against the onslaught of a myriad of microbes, the host sets off an array of defense processes (Paludan et al. 2020).

The host defense, mediated by immune system, upon trigger, responds by establishing barriers and activating different classes of innate resistance and adaptive immunity (Goldszmid et al. 2014). Therefore, it becomes imperative that the immune cells and cell products must communicate with each other in a sequential and harmonious manner for the optimum functioning of the immune system (Ganapathy et al. 2017). In fact, the beauty of immune system lies in two facts: first, that it evolves with time and second, the precision with which it detects the structural features of the pathogens and marks them as distinct from host cells (i.e., ability to discriminate between self and non-self-antigens), a property which is essential to permit the host to eliminate the threat without damaging its own tissues (Mousa et al. 2017).

On the other hand, microorganisms have also devised strategies to decoy or inhibit the immune system of the host, primarily by maintaining a highly coevolved relationship with the immune system and secondarily by expressing antigens similar to those of the host (Hooper et al. 2012; Koga 2018). The latter condition is referred to as "molecular mimicry," a property of the pathogen to share antigenic determinants with the host. Various microorganisms generate a range of "mimics" which bear a resemblance to host components, both structurally and functionally (Mousa et al. 2017).

24.2 Molecular Mimicry: The Concept

Molecular mimicry is defined as the property of a given microorganism to share antigenic determinants with the host (Fujinami et al. 2006). Those microorganisms which mimic the host antigens have the capability of evading immunity since self-tolerance mechanisms abolish or anergize the autoreactive T cells. On the other hand, these foreign antigens, mostly from invading microbes, activate immune cells to produce antibodies. These pathogen-specific antibodies not only react with microbes but also bind to host self-antigens, which will subsequently result in inflammation and tissue damage. Basically, it is an erroneous recognition of the microbial antigen which triggers the immune response and induces substantial costs for the host, depending on the molecule/organ that is the target of the misoriented immune reaction (Sorci et al. 2013). Hence, molecular mimicry, which was initially thought to be a tactic employed by all sorts of microbes to avoid immune reaction of the host, has gradually emerged as a phenomenon by which microorganisms can stimulate destruction of host tissues, a situation termed as "autoimmune response." This microbe-induced destruction of host depends on the degree of structural resemblance between microorganism and host epitopes and/or the repeated activation of autoreactive T cells during infection. This highlights the two-faced property

of the immune system, where one side protects against infectious diseases and the other side exposes to the risk of misdirected immune reaction.

24.3 Historical Perspective

The earliest reports of mimicry appeared in the 1862. At that time, mimicry was confined to just camouflaging, in order to amalgamate with the environment, that is, one animal species advertently resembled in shape and color to its surrounding environment thereby bestowing itself with an advantage in the form of protection from predation by imitating the appearance of another obnoxious animal species. Such a mimicry was termed as "Batesian mimicry" (Bates II 1862). However, biological advancement revolutionized this evolutionary concept and established that mimicry occurs in living beings at a molecular level as well (referred to as molecular mimicry by Damian), that is, antigenic sharing can happen between microorganisms and host tissue which could be considered as a means of avoidance from the immune response of host. The concept, definition, and usage of molecular mimicry have changed over the years from the time it was first used by Damian in Damian 1964 as a microbial defense to avoid host immune response to relatively recently wherein it presents itself as an altogether new concept, that is, the "shared" antigenic determinants of a microorganism might elicit an autoimmune response and hence cause damage to the host (Rose and Mackay 2000). This occurs when an adequately high grade of similarity occurs between a protein of microbe and human and the immune system is unable to discriminate between them, thereby generating an immune response against the microbial antigen for defensive purposes. Owing to the mimicry, such an immune response can turn against the self-antigen and persist for an indefinite period even post the resolution of the initial infection.

24.4 Mechanism of Molecular Mimicry

The phenomena of mimicry are thought to have arisen through three mechanisms, namely, lateral or horizontal transfer of genes, convergent or parallel evolution, and by mimicry of host repetitive proteins via independently evolved peptide repeats.

24.4.1 Lateral or Horizontal Transfer of Genes

Horizontal gene transfer events have been reported between two prokaryotic species, from prokaryotes to eukaryotes and from eukaryotes to prokaryotes. The phenomenon is well documented in case of transfer among prokaryotes, whereas the one involving transfer from prokaryotes to eukaryotes is critical since the source of the prokaryote remains the dividing line. Eventually, eukaryotes are also capable of transferring genes to bacteria, although not well elaborated, yet they are anticipated to occur quite frequently. The examples reported for this category include genes

encoding proteins involved mainly in metabolic pathways since it allows recipients to adapt to new ecological niches (Pallen and Wren 2007). An outstanding study revealed that *Coxiella burnetii,* the causative agent of Q-fever in humans, encodes two sterol reductases, CBU1158 and CBU1206, which have similarity with the reductases found in eukaryotes. These mimics play a part in the development of the cholesterol-rich *Coxiella* parasitophorous vacuole which facilitates pathogen to survive inside the host cell. CBU1206 exhibited 46.2% amino acid similarity with Erg4, the Δ24 sterol reductase mutant of *Saccharomyces cerevisiae*. The closest homologue of CBU1206 was from *Naegleria gruberi*, a free-living soil amoeba, displaying 51.7% amino acid identity, suggesting that *C. burnetii* acquired CBU1206 sterol reductase via horizontal gene transfer from amoeba. A similar experience has also been projected for *C. burnetii*'s putative Δ7 sterol reductase, CBU1158. Since these enzymes are very uncommon in prokaryotes, hence, they are contemplated to have arisen in *Coxiella* by lateral transfer from some eukaryotic source (Gilk et al. 2010). Similarly, *Legionella pneumophila*, a pathogen responsible for causing Legionnaires' disease, also contains 44 uncharacterized genes with many distinct eukaryotic motifs and different G + C content, further lending support to the fact that they were acquired through horizontal gene transfer and may serve to amend host cell functions to the advantage of the invading pathogen (Cazalet et al. 2004; de Felipe et al. 2005).

24.4.2 Convergent or Parallel Evolution

Over the course of time, coevolutionary forces have generated microbial proteins which either bear structural resemblance to the host proteins or bear a resemblance to small sequence fragments of host proteins without any evident homology between the pathogen and host proteins, suggesting that even small regions of similarity, and not essentially the wholesome sequence of a protein, are adequate to prompt autoimmunity (Stebbins and Galan 2001; Sikora et al. 2005; Elde and Malik 2009). For example, an effector protein ($EspF_U$), secreted by enterohemorrhagic *Escherichia coli* (EHEC) type III secretion system into human host cells, triggers polymerization of actin filaments by interacting with host Wiskott-Aldrich syndrome protein (WASP) (Sallee et al. 2008). Host function's exploitation is accomplished through slight structural similarity of the host WASP autoinhibitory helix; however, they lack any detectable sequence similarity. By stimulating actin polymerization, $EspF_U$ mediates adherence of EHEC to epithelial cells of the host, a step vital for its virulence.

Besides structural similarity, functional similarity is also exemplified through many instances. An example in this context is that of invasin*,* an effector protein of *Yersinia*, which imitates the integrin-binding surface of fibronectin. Both of these proteins, of which fibronectin is the natural ligand, compete for β1 integrins present on host's M cells. This phenomenon of mimicry evokes reorganization of cytoskeleton, thereby allowing the microbe to gain access into the host cell (Hamburger et al. 1999). Also, since *Yersinia* YopH mimics human tyrosine phosphatases, only then it

is capable of dephosphorylating various human proteins including adaptor proteins such as p130Cas/BCAR1 (breast cancer antiestrogen resistance 1), which eventually hampers phagocytosis (Stebbins and Galan 2001). Similarly, in the case of *Legionella*, the *Legionella* effector, RalF, owing to its mimicry with human guanine-exchange factors (GEFs), acts as a GEF in the host and recruits ADP-ribosylation factor (Arf) to maneuver host vesicular trafficking (Nagai et al. 2002).

24.4.3 Mimicry of Host Repetitive Proteins Via Independently Evolved Peptide Repeats

In this case, distinct progenitor repeats in the pathogen's genome are amplified so as to form repeat proteins that possess the similar repetitive architecture but with different sequences for each repeat unit (Doxey and McConkey 2013). As an analogy to this, β-trefoil proteins, which also include virulence-associated subfamilies (i.e., ricin toxins), have also displayed distinct repeat amplifications while preserving the similarity in the overall structure (Broom et al. 2012). The similarities that have been detected cannot be attributed to an overall homology between the complete proteins, but rather they are a consequence of similarity of the repetitive architecture. Repetitive host proteins, for example, collagens, adhesins, and leucine-rich repeat proteins, signify principle targets for this evolutionary mechanism of pathogen mimicry.

The above mechanisms highlight two basic facts: first, that even molecules with different overall structures can behave in a similar way and second, that even small regions of sequence similarity, and not overall homology, is sufficient to elicit molecular mimicry (as in the case of immune epitope mimicry, which cannot be recognized using a standard homology detection approach). Hence, it can be summarized that molecular mimicry operates at four basic levels: (1) mimicking both sequence and 3D structure of a protein, (2) mimicking only structure without any sequence similarity, (3) mimicking sequence of a short motif–motif mimicry, and (4) mimicking structure of a binding surface without sequence similarity–interface mimicry.

24.5 Molecular Mimicry in Autoimmune Diseases

Of all the mechanisms which can trigger immune reaction and lead to autoimmunity, namely, cryptic antigens, epitope spreading, bystander activation, and polyclonal activation, molecular mimicry has been projected as the front-runner (Fujinami et al. 2006). In a way, it has indeed been involved in the pathogenesis of various autoimmune disorders including rheumatoid arthritis, Guillain-Barré syndrome, diabetes, myocarditis, Lyme neuroborreliosis, Graves' disease, inflammatory bowel disease, etc. Even though the direct evidence which links microbial agents with these autoimmune diseases is limited, yet molecular mimicry has been cited as one of

the potential triggers for these diseases (McClain et al. 2005). In this context of the various instances of mimicry that have been reported in literature, the most famous examples from bacteria, parasites, and viruses have been enlisted below:

Streptococcus pyogenes, which encodes M-protein that bears similarity with antigens of human myocardial tissues (e.g., myosin, vimentin, tropomyosin, and laminin) {Acute rheumatic fever and rheumatic heart disease} (Galvin et al. 2000) and human brain tissues (e.g., tubulin, dopamine, and lysoganglioside) {Pediatric autoimmune neuropsychiatric disorder associated with streptococcal infections, PANDAS} (Cunningham 2012).

Helicobacter pylori, the primary inducer of gastric and duodenal pathologies, produces α-carbonic anhydrase and CagA protein which mimics human α-carbonic anhydrase (Guarneri et al. 2005) and human platelet glycoproteins, GPI, and GPII {Immune thrombocytopenic purpura} (Takahashi et al. 2004) alongwith IL-33 (Buzzelli et al. 2015), respectively. Similarly, the plasminogen-binding protein (PBP) of *H. pylori* shows homology with ubiquitin-protein ligase E3 component n-recognition 2, an enzyme highly expressed in the pancreatic acinar cells {Autoimmune pancreatitis} (Frulloni et al. 2009). Moreover, mimicry has also been demonstrated in this case by simultaneous expression of Type 1 and Type 2 Lewis blood group antigens (Le $^{x/y}$) by lipopolysaccharides of both *H. pylori* and host gastric epithelial cells (Appelmelk et al. 1996).

Campylobacter jejuni, a convict in the case of peripheral neuropathy termed as Guillain-Barre syndrome, possesses lipooligosaccharides which resemble peripheral nerve gangliosides of humans (Yuki et al. 1990). *Salmonella* encoded effector proteins, namely, SopE2 and SptP, also mimic effector molecules of signaling pathways of eukaryotes, that is, G-nucleotide exchange factors (GEF) and Rho GTPase-activating proteins (GAPs), respectively (Schlumberger and Hardt 2005). *Listeria monocytogenes* displays mimicry between its surface proteins, Internalin A and ActA, and host's mucosal lymphocyte-1 antigen {HML-1} (Braun et al. 2000) Wiskott-Aldrich syndrome family of proteins, respectively (Cossart 2000).

Borrelia burgdorferi's flagellin exhibits cross-reactivity with HSP60 of peripheral nerve axon of humans {Lyme neuroborreliosis} (Sigal et al. 2001). On the other hand, outer surface protein A of *Borrelia* displays cross-reactivity with human leukocyte function–associated antigen 1 (LFA-1, CD11a/CD18, or integrin α_L ß$_2$) {Lyme arthritis} (Alaedini and Latov 2005). An explicit example of mimicry has also been witnessed in the case of *Yersinia enterocolitica* whose outer membrane protein (ompF) displays homology with host's thyroid-stimulating receptor (Hargreaves et al. 2013). A functional similarity has also been observed between *Yersinia pseudotuberculosis's* invasin and the integrin-binding surface of fibronectin (Stebbins and Galan 2001). *Clostridium botulinum*'s neurotoxin A (Btx-A) demonstrates amino acid sequence homology with host's thyroid-stimulating receptor {Autoimmune thyroid disease} (Gregoric et al. 2011). Also, *Escherichia coli* and humans share pyruvate dehydrogenase complex, PDC-E2 {Primary biliary cirrhosis} (Fussey et al. 1990). Further, a homology between peptides found on human MHC β-chain and *E. coli* (dnaJ) has also been recognized (Albani et al. 1992).

The numerous parasites and viruses that have been found to mimic human cells include *Trypanosoma cruzi*, which mimics various human antigens, namely, ribosomal proteins (Bonfa et al. 1993); neuronal cells and myocardial cells (Gironès et al. 2001); *Schistosoma mansoni*, which mimics human granulocytes (Van Dam et al. 1996); *Onchocerca volvulus*, which mimics neutrophils (Gallin et al. 1995) and calcium binding proteins (McCauliffe et al. 1990); *Plasmodium falciparum*, which mimics intermediate filament proteins (Lawler and Hynes 1986) and adhesion molecule {Thrombospondin} (Kobayashi et al. 1986); Cytomegalovirus and Coxsackie virus share an enzyme glutamic acid decarboxylase, GAD65, with host {Type 1 diabetes} (Hiemstra et al. 2001; Dotta et al. 2007); Herpes simplex virus mimics human acetylcholine receptors {Myasthenia gravis} (Dyrberg et al. 1990); and Epstein-Barr virus mimics myelin basic protein {Multiple sclerosis} (Lomakin et al. 2017), lupus autoantigens {Systemic lupus erythematosus} (McClain et al. 2005) and type 2 collagen (Davies et al. 1999).

The frequency of occurrence of shared peptide sequences along with the flexibility inherent in immune recognition suggests that molecular mimicry might be omnipresent in biological systems. On the basis of circumstantial evidence, it would not be inappropriate to say that molecular mimicry has stayed an attractive justification in the context of autoimmune diseases for a considerable period of time. However, challenges will always be there for the researchers in the field of molecular mimicry to prove that the phenomena of molecular mimicry can also be a mechanism in causing infertility (Acharya et al. 2010).

24.6 Molecular Mimicry as a Mechanism of Infertility

Cross-reactivity has been hypothesized to play a fundamental role in infertility, that is, an immune response evoked against the invading microorganism generates antibodies that might be produced in response to an epitope that bears similarity to one present on spermatozoa, thereby leading to the formation of antisperm antibodies (ASAs) (Berwary 2017). In this regard, several reports have surfaced up suggesting the presence of cross-reactive antigenic determinants between spermatozoa and bacteria which might be one of the possible triggering mechanisms for the induction of antisperm antibodies (ASA) in men and women (Kurpisz and Alexander 1995; Eggert-Kruse et al. 1993; Lenzi et al. 1991). Molecular biology approaches have also demonstrated the existence of sequence homologies between genes encoding bacterial enzymes and mammalian sperm proteins (Kalaydjiev et al. 2007). Some of the instances have been cited below:

Chlamydia trachomatis, a sexually transmitted bacterium which is responsible for the most damaging genital tract infections, interferes seriously with human reproduction. It has been associated with the production of ASAs in both men and women. In fact, the non-symptomatic men under fertility investigation with a previous history of infection showed a marked increase in the incidence of ASAs than the noninfected controls (Witkin and Toth 1983). Similarly, in females, incidence of chlamydial antibodies with residual inflammatory adnexal lesions was markedly

higher as compared to those with normal adnexae (Gump et al. 1983). It has been proposed that it acts as an initiator through inflammatory process and leads to the formation of antibodies against the bacterial membrane carbohydrates that could cross-react with sperm surface carbohydrates (Witkin and Toth 1983; Akande 2002; de Barbeyrac et al. 2006; Al-Daghistani and Fram 2009; de Jesús De Haro-Cruz et al. 2011). Moreover, the 60 kDa chlamydial heat shock proteins (HSP) have also been demonstrated to bear an amino acid homology with their human counterparts, further accounting for the mimicry between the two at the molecular level (Paavonen and Lehtinen 1994).

Ureaplasma urealyticum is an inhabitant of the human lower genital tract, and its infections are considered to be sexually transmitted which occur more frequently during fertile ages. Its implication in the development of ASA-induced infertility has been evident by the molecular mimicry between its urease subunit, UreG, and human nuclear autoantigen sperm protein (hNASP) as established by enzyme immunoassays and western blotting (Shi et al. 2007).

Helicobacter pylori is mainly associated with various gastric diseases, owing to its tropism for the gastric mucosa. However, the investigation of its role in extragastric diseases, particularly infertility, shows its possible mimicry with spermatozoa. The association was strongly supported by the ability of sera to *H. pylori* whole antigens to cross-react with the tails and the pericentriolar area of human spermatozoa (which are rich in tubulin). Moreover, a linear homology has also been observed at amino acid level between β-tubulin and three *H. pylori* proteins, flagellin, VacA, and cytotoxin-associated gene A (CagA) (Figura et al. 2002).

The homology between *S. aureus* and spermatozoal proteins has been determined on the sequence similarity between the two. The proteins which showed homology between the two include Glyceraldehyde-3-phosphate dehydrogenase, L-lactate dehydrogenase C, protein deglycase DJ-1, sperm acrosome membrane-associated protein-4, and UDP-N-acetyl hexosamine phosphorylase (Anas et al. 2016).

E. coli cells of serotypes 08, 09, and 086 also contain heterogenetic antigens similar to the cellular antigens of human spermatozoa (Popivanov et al. 1981). On the similar grounds, ASAs showing cross-reactivity with *E. coli* and *Salmonella typhi* have also been reported (Kurpisz and Alexander 1995) thereby providing evidences of mimicry between prokaryotes and eukaryotes.

24.7 Concluding Remarks

Sufficient evidence, however, has accumulated recently to establish an important and emerging role for molecular mimicry in autoimmune diseases and infertility. Further in-depth mechanistic studies on microbiota–autoimmunity interplay in autoimmune diseases and infertility are urgently needed and underway to explore novel and precise diagnostic biomarkers and develop disease and patient-tailored therapeutic strategies.

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Strategies of Vaccine Development

25

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Abstract

The history of vaccines dates back to the fifteenth century, and since then, various vaccines have been discovered that have significantly reduced the incidence and mortality associated with several infectious diseases. Today, we have vaccines available not only against a number of infections but also other diseases such as cancers. The process of vaccine development is a long and complex procedure, with several mandatory steps to ensure that the vaccine is able to confer adequate protective efficacy across different populations with minimal side effects for mass immunization. Starting from the simple microbial attenuation and inactivation methods, the process of vaccine development has evolved tremendously, leading to recombinant subunit vaccines and various vector-based platforms capable of expressing selective antigens from different microbes. Further advancements in manufacture of nucleic acid vaccines and antigen delivery systems have enabled us to develop vaccines against a variety of pathogens. The chapter discusses the existing and recent developments in the field of vaccinology and elaborates novel strategies in the development of vaccines against emerging pathogens like the SARS-CoV-2.

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Infectious diseases · Vaccines · Recombinant vaccines · Antigen delivery system · Vaccinology

25.1 History of Vaccine Development

During the course of evolution, humans have been exposed to several pathogens, against which our immune system has the inherent ability to mount an effective immune response to prevent establishment of a disease. The initial immune response towards any invading pathogen is retained in the form of memory, often termed as "immunity" that prevents subsequent reinfection from the same or similar pathogens. However, development of this immunity comes at a risk in the form of mortality or morbidity associated with the primary infection. Therefore, in order to prevent the occurrence of infection or disease on one hand and at the same time develop immunity against the causative pathogen, we have now agents that are popularly known as "vaccines." Today, the field of vaccinology has evolved tremendously, and we have vaccines available not only against a number of infections but also other diseases such as cancers.

The history of vaccines dates back to fifteenth century, with ancient records from China and Turkey revealing practices aimed to induce immunity against locally prevalent infections (Lombard et al. 2007). One of such procedures involved inoculation of dried crusts of small pox pustules into the small cuts in the skin, a procedure termed as "variolation." However, in the modern science, the first documented attempt of vaccination was made by an English physician, Edward Jenner, during the year 1796. Jenner improvised the variolation technique further. Initially, he had observed that milkmaids who had contracted cowpox, a mild and self-limiting disease, became immune to the fatal smallpox disease. He proposed that inoculating people with fluid obtained from cowpox pustules might protect them from acquiring smallpox. He inoculated an eight-year-old boy first with the fluid obtained from a cowpox pustule, and a few days later, he was deliberately infected with the smallpox virus. Expectedly, the child did not develop any smallpox symptoms. This experiment provided the scientific basis of vaccination, due to which Edward Jenner is known as the discoverer of vaccine in the era of modern medicine.

The principle of vaccinology was developed by Louis Pasteur, a French chemist and microbiologist, during the late 1870s. Pasteur made his first important discovery in the field of vaccinology during 1879, while working on chicken cholera, which came as a chance observation when he observed that cultures were no longer pathogenic while retaining attenuation at the same time with the passage of several generations. He then inoculated healthy chickens with such an attenuated chicken cholera pathogen strain and demonstrated that the inoculated healthy chickens became resistant to the virulent strain of cholera. He termed this attenuated strain as "vaccine" (from the Latin word *vacca*, meaning "cow") in honor of Jenner's experiment on cowpox inoculation. He further hypothesized that "attenuated"

microorganisms can be administered in humans to prevent them from various infectious diseases. The success of this experiment was a beginning for Pasteur in the field of vaccinology, and he dedicated his whole life in understanding microbial origins of disease. During this process he invented vaccines for anthrax and rabies that were causing several life-threatening infections in those times.

Since then, various vaccines have been discovered that have significantly reduced the incidence and mortality associated with several infectious diseases. And this process is still ongoing as there are several pathogens like tuberculosis, malaria, HIV, HCV, leishmania, etc., where a successful vaccine is still elusive. After the emergance of SARS-CoV-2, the field of vaccinology has witnessed a major revolution, with several research groups and pharmaceutical industries are working intensively and relentlessly, using various approaches to launch a successful vaccine to curb the COVID-19 pandemic that has gripped the entire world. Table 25.1 depicts the chronological discovery of major vaccines developed against various infections.

25.2 Attributes of a Successful Vaccine

The process of manufacturing a vaccine for mass immunization is a long and complex procedure, with several mandatory steps to ensure that the vaccine is able to confer adequate protective efficacy across different populations with minimal side effects. Therefore, scientists and manufacturers have to ensure that the developed vaccine meets the essential characteristics, some of which are listed as follows:

- *Safety:* The vaccine should demonstrate safety across all age groups and populations, including infants, elderly, and immunocompromised subjects. For any new vaccine candidate, the safety must be monitored for years together, and data must be available to regulatory agencies
- *Efficacy:* The vaccine must be capable of eliciting a high level of long-lived protective efficacy (humoral, cell-mediated, and mucosal immune responses) in all age groups
- *Dosage:* The vaccine should confer long-lived protection through a single dose only or at the most with one booster dose. The protective effects of the vaccine should be observed after the administration of the first dose itself
- *Timing of protection:* The vaccine should be capable of stimulating protection as early as possible, preferably, within 2 weeks of administration
- *Administration:* An ideal vaccine should be administrable preferably through either oral, nasal, or transcutaneous routes. Additionally, it should be administrable concomitantly (coadministered) with other vaccines
- *Manufacturing:* The vaccine should be easy to manufacture and scalable for mass immunization in large population by relatively uncomplicated and economical processes and with adequate quality control
- *Storage:* The vaccine should require less stringent storage conditions

Table 25.1 Chronological order of the important vaccines discovered against infectious organisms

• *1796*: Edward Jenner documented first vaccine for smallpox, by using cowpox
• *1885:* Louis Pasteur successfully tested an injection of a live rabies vaccine into a 9-year-old boy who was bitten by a rabid dog, and this experiment created a lot of controversy
• *1896:* Development of first killed vaccine against cholera and typhoid by Wilhelm Kolle and Richard Pfeiffer with Almroth Wright, respectively
• *1897:* Bacteriologist Waldemar Haffkine developed a killed vaccine for the bubonic plague, which he first tested upon himself before its public use
• *1921:* The Bacillus Calmette–Guérin (BCG) vaccine was administered for the first time in humans for protection against tuberculosis. This strain was isolated after subculturing 239 times during 13 years from a virulent strain on glycerin potato medium by Albert Calmette and Camille Guérin. While the efficacy of BCG against pulmonary tuberculosis appears to be variable, it has a high protective efficacy against tuberculous meningitis in small children. Besides, BCG has shown protection in urinary bladder cancer and autoimmune diseases like type-1 diabetes and possibly COVID-19, by virtue of its training effect on the immune responses (trained immunity)
• *1923:* The toxin from diphtheria bacterium was chemically inactivated to form a toxoid which was used as a "toxoid" vaccine
• *1926:* Development of a killed vaccine using the whole *Bordetella pertussis* bacterium that confers protection from whooping cough
• *1927:* The tetanus "toxoid" vaccine was developed that was later on combined with the toxoids of diphtheria and pertussis and administered as a triple vaccine known as DPT to the children
• *1954:* Jonas Salk developed a killed polio virus vaccine that dramatically brought down the paralysis cases in merely a period of 8 years
• *1961:* Albert Sabin developed the live oral polio vaccine (OPV) which was easy to be administered and successfully limited the polio disease
• *1963:* FDA approved and licensed measles vaccine
• *1964:* Development of measles vaccine based on a live attenuated measles virus. Initially, up to 30 abdominal injections were required which were quite painful. In 1980, a newer version was developed which required only five intramuscular injections
• *1967:* Licensure of mumps vaccine based on live attenuated mumps virus strain, which significantly reduced its annual incidence
• *1970:* A live attenuated rubella vaccine was licensed. In 1971, measles, mumps, and rubella vaccine combination to be given as single shot was created by Maurice Hilleman at the Merck Institute for Therapeutic Research.
• *1970s and 1980s:* Meningococcal, pneumococcal, and haemophilus influenza type b (Hib) vaccines were developed using parts of the bacterial layer (subunit vaccine). These vaccines helped protect against three fatal diseases, that is, meningitis, blood infections, and bacterial pneumonia
• *1986:* A recombinant hepatitis B vaccine was licensed and approved by FDA. This vaccine was prepared by inserting the hepatitis B surface antigen into a yeast vector, leading to production of only the noninfectious viral subunit. This vaccine is still in use
• *1990:* A highly immunogenic inactivated vaccine against the hepatitis A virus was developed
• *1995:* The vaccine against the varicella zoster virus that causes chickenpox in the live attenuated form was licensed and added to the routine immunization program in children
• *1996:* A combined diphtheria, tetanus, and pertussis vaccine that contained only few pertussis antigens was approved
• *2003:* First nasal influenza vaccine was approved in the United States

(continued)

Table 25.1 (continued)

• *2006:* Human papillomavirus (HPV) vaccine, which is a protein subunit vaccine, became available for cervical cancer and prevention of other cancers and infections associated with high-risk types of HPV, like 16 and 18
• *2012:* First vaccine for hepatitis E was approved in China that provided long-term protection against the hepatitis E virus
• *2019:* First recombinant vaccine consisting of a gene encoding for the EBOV glycoprotein for people aged 18 years or older
• *2020:* First RNA vaccine for SARS-CoV-2 was approved for emergency use by US FDA for prevention of Covid-19 infection. The vaccine (tozinameran), code-named BNT162b2 has been developed jointly by Pfizer and BioNTech. The vaccine contains a modified RNA that encodes the spike protein of SARS-CoV-2 coronavirus (Sahin and Muik 2020)

25.3 Types of Vaccines

Figure 25.1 depicts the progress in the vaccine platforms with major licensed vaccines and vaccines in pipeline. Due to advancements in major technologies like genomics, recombinant DNA technology, synthetic biology approaches, and use of adjuvants have led to the licensure of 22 vaccines since 1980 (Baker et al. 2018).

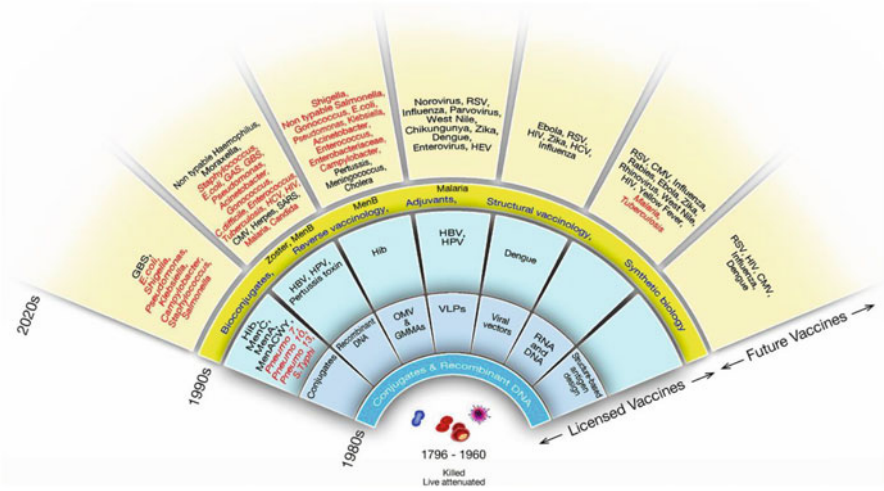

Fig. 25.1 History of development of new vaccine platforms with licensed and future vaccines. *CMV* cytomegalovirus, *GAS* group A *Streptococcus*, *GBS* group B *Streptococcus, HBV* hepatitis B virus, *HCV* hepatitis C virus, *HEV* hepatitis E virus, *Hib* type B Haemophilus influenzae, *HIV* human immunodeficiency virus, *HPV* human papillomavirus, *MenA* meningococcus A, *MenACWY* meningococcus ACWY, *MenB* meningococcus B, *MenC* meningococcus C, *Pneumo* pneumococcus, *RSV* respiratory syncytial virus, *SARS* severe acute respiratory syndrome, *VLP* virus-like particles Image Courtsey: Technologies to address antimicrobial resistance. Stephen J et al., Proceedings of the National Academy of Sciences, 2018

25.3.1 Live-Attenuated Vaccines

The live-attenuated vaccines represent the traditional forms of vaccine that are prepared from living microorganisms weakened under laboratory conditions. These "attenuated" organisms lose their pathogenic properties but are capable of replication in a vaccinated individual and produce immune response with a mild or no disease. This attenuation is usually achieved via suboptimal culturing or repeated passaging of pathogens for multiple generations in tissue cultures, embryonated eggs, or live animals. The concept of live attenuation is based on the principle introduced by Jenner, that is, using a related virus isolated from a different animal (cowpox to prevent smallpox). The process of attenuation allows the microorganism to adapt to another cell type and temperature that is nonexistent in human bodies. Basically, the process of attenuation causes accumulation of mutations, resulting in transformation of the microorganism, incapable of causing disease in its natural host. Carrying out repeated passages for multiple generations ensures stable integration of these mutations so that the microorganism does not revert to its pathogenic form. Upon injection of small doses of the attenuated strains in the human host, these attenuated forms of the microorganisms multiply and provide sustained immune response.

One of the most common examples of a live-attenuated vaccine is the oral polio vaccine (OPV) which is a mixture of three live-attenuated polio virus strains representing different serotypes (I, II, III) and passaged in monkey kidney cells (Bandyopadhyay et al. 2015). The polio virus targets intestinal mucosa via the fecal-oral route. The OPV protects mucosa and prevents its spread to the nervous system. Upon administration, active replication of OPV occurs in epithelial cells to activate the mucosal immune response which protects against wild-type infection and transmission. In OPV, the virus is alive and infects intestinal cells and produces viral proteins that are expressed on host MHC-I, eliciting effective CD8+ T cell responses that eliminates the modified virus and in this process create memory T cells that mount an exaggerated immune response against the wild-type poliovirus on a subsequent exposure. Usually one or two doses of these vaccines are capable of eliciting strong and long-lasting immune responses. The process of creating the live-attenuated vaccines is fairly easy for certain viruses; however, in the case of bacteria and parasites, it is quite challenging.

Advances in tissue culture techniques have enabled the development of live-attenuated vaccines that could be administered in combination (Minor 2015). For example, the MMR vaccine for three viruses, measles, mumps, and rubella, could be administered as a combined vaccine. Other examples of currently available live-attenuated vaccines against viral infections include cowpox, yellow fever, and influenza. Live-attenuated bacterial vaccines include BCG and oral typhoid vaccine. In the case of recently emerging infections, certain modified forms of live-attenuated vaccines have been generated. Chimeric vaccines are one such example in which few of the genes are substituted for similar genes from a less pathogenic related organism. A live-attenuated chimeric vaccine against Zika virus has been developed using the type 4 backbone of dengue virus that expresses Zika virus surface proteins and is

undergoing early-stage testing in humans (Li et al. 2018). Administration of a pathogenic or partially attenuated virus by an unnatural route is another means to reduce the virulence of a virus. Newly recruited military personnel were used to be immunized through this principle in which the vaccine against adenoviruses 4 and 7 were administered through oral routes and hence it prevented the acute respiratory distress syndrome in them (Collins et al. 2020).

There are certain drawbacks associated with live-attenuated vaccines. These vaccines cannot be administered to individuals with immunocompromised state (pregnancy, HIV infection, on immunosuppressive therapy). To maintain potency, live-attenuated vaccines require maintenance of cold chain and protection from light during storage and supplies for immunization in communities. The live-attenuated vaccines carry a risk of reversion to their pathogenic forms or emergence of new mutations that could also make them pathogenic or drug resistant. Therefore, the manufacturers have to maintain and subculture the attenuated strains by employing stringent quality checks.

25.3.2 Inactivated Vaccines

Since the live-attenuated vaccines carry a risk of reversion and knowing the fact that the immune system elicits responses against microbial components, vaccinologists had started preparing killed or inactivated forms of pathogens centuries ago (Stauffer et al. 2006). The process of killing or inactivation of microorganisms by treatment with heat, chemicals (formaldehyde, β-propiolactane), or radiations ensures that the vaccine is safe and carries no risk of infectivity or reversion. However, the process should ensure that the antigenicity of the major epitopes is retained, with minimal loss of immunogenicity in comparison to the live pathogen, and the vaccine preparation should be able to generate strong and long-lasting immuno-protective responses.

Currently there are a number of whole inactivated vaccines available, including those against viral infections, such as rabies, polio (IPV), hepatitis A, and influenza. Whole-inactivated bacterial vaccines include typhoid, cholera, pertussis, and plague. Besides being safer than the live-attenuated vaccines, the inactivated vaccines have less stringent culture requirements, as they can be abundantly grown in vitro. Once they are inactivated, these vaccines often do not require refrigerated conditions and can be stored in lyophilized forms, making them easier for storage, transport, and administration. However, treatment in any form alters the native antigenic structure of the microorganisms; therefore, the inactivated vaccines are usually not able to generate strong immune responses. Due to the lack of replication, there is absence of cellular production of antigens, due to which these vaccines have an impaired ability to induce T cell responses. These vaccines are not produced in the host by replication of pathogen, and therefore, inactivated vaccines must contain much more antigen than the live vaccines to be effective. Sometimes, excess of antigen or chemical treatment may cause allergic reactions in the recipients. Inactivated vaccines induce

immunity that generally wanes off with time and hence may require administration of additional doses either alone or with an adjuvant.

25.3.3 Subunit Vaccines

With the initial success of inactivated vaccines, it became clear that immune responses are elicited against the key antigens of the pathogens. Earlier, the preparations of inactivated vaccines are comprised of purified extracts of the cultured viruses or bacteria. Later, with advancements in protein purification techniques, it became possible to purify antigens from culture preparations. Further advances in biotechnology made it possible to identify the key epitopes of the pathogens. Hence, instead of whole microbe, the use of antigens or peptides (subunit) that could elicit protective immunogenic responses became prevalent for use as a vaccine (Tsoras and Champion 2019).

Since only the specific antigenic determinants or immunogenic regions of the antigens are used for making a subunit vaccine, there is minimal risk of adverse effects and no chance of reversal of virulence. With increase in purification steps required for their preparation, this often leads to decrease in immunogenicity of subunit vaccines; therefore, they are often coupled with adjuvants. In order to elicit broad spectrum immune responses, subunit vaccines can incorporate more than one (up to 20) antigens. However, identification of key epitopes or antigens capable of eliciting immuno-protective responses is a major prerequisite for development of subunit vaccines. Availability of bioinformatics tools, in silico algorithms, and immune epitope databases have facilitated the development of subunit vaccines carrying multiple peptides. Vaccines for influenza virus *Haemophilus influenzae* A and B (HiA & HiB) and hepatitis B antigen (HBsAg) are typical examples of purified subunit vaccines (Keshavarz and Mirzaei 2019).

25.3.4 Recombinant Subunit Vaccines

Advancements in recombinant DNA technology and protein purification techniques have provided a major boost in the development of subunit vaccines, now called as "recombinant subunit vaccines." Typically, the process involves cloning of a microbial antigen or epitope gene in a vector that is expressed in a bacterial, yeast, insect, or mammalian protein expression system. Once the desired protein is expressed in abundant quantities, it can be purified and formulated for use as a subunit vaccine (Burnette 1991). The current hepatitis B vaccines are produced by inserting a segment of the hepatitis B virus gene, encoding its surface antigen, into the genome of a yeast cell. Once the recombinant yeast cells produce hepatitis B surface antigen, it is purified and used for vaccine preparation. Similarly, the vaccines against human papilloma virus (HPV) are produced by recombinant DNA technology, and two such vaccines are available in the market. The first one is a bivalent vaccine in which HPV types 16 and 18 proteins are made to be expressed in insect cell lines and purified

from them. Another one is a tetravalent vaccine containing four viral proteins-HPV types 6, 11, 16, and 18, expressed and purified from yeast cells. Both vaccines are administered with adjuvants. These vaccines are also safe for use in humans because they do not contain any live viral or genetic material (Barnard 2010).

25.3.5 Toxoid Vaccines

Some pathogenic bacteria, like *Clostridium tetani* (causes tetanus), *Corynebacterium* (causes diphtheria), produce disease by secreting exotoxins (Yen and Thwaites 2019; Weinberger 2017). These exotoxins by themselves can cause serious illness and require to be neutralized. The production of toxoid vaccines involves purification of these toxins and treatments with chemicals (e.g., formaldehyde) or with heat, to inactivate them, and at the same time retain their immunogenicity to produce toxoids. These toxoids or their preparations are used as vaccines to produce antibodies against these toxins in the recipients. On subsequent exposure to these toxins, the recipients are able to quickly secrete neutralizing antibodies (mainly opsonizing antibodies) against these toxins. Vaccines against tetanus and diphtheria are the common examples of toxoid vaccines.

25.3.6 Conjugate Vaccines

Conjugate vaccines involve combination of a poorly immunogenic antigen along with a potent antigen that serves as a carrier for the weak antigen leading to a stable and effective vaccine formulation. The host mounts a strong immune response to the weaker antigen as well. This strategy has been developed in the case of polysaccharides (obtained from pathogenic bacterium like *Streptococcus pneumoniae*) by combining them with protein antigens, although peptide-protein and protein-protein vaccine conjugates have also been developed. Bacteria can synthesize various types of chemically and immunologically different polysaccharides. Some of these polysaccharides are able to generate antibodies in adults with varying efficacy. However, most of these polysaccharides by themselves do not generate immune response, as they cannot be presented on MHC-II molecules of the antigen presenting cells. Also, it has been observed in children that they are not able to mount immune responses against some bacterial antigens as they are masked by the polysaccharide layer. By linking these polysaccharides covalently to protein antigens as carriers, they become immunogenic. This observation was first reported in 1929 when the streptococcal type 3 polysaccharide antigen was linked with a protein carrier to generate immune responses to *Streptococcus pneumoniae* (Avery and Goebel 1929). However, it took another 60 years when the first conjugate vaccine against *Haemophilus influenzae* type b (Hib) became available. The Hib conjugate vaccine is made by combining with one of the several readily available carrier proteins, like the diphtheria toxoid (DT) or the tetanus toxoid (TT) (Ahmad and Chapnick 1999). This was followed by availability of conjugate vaccines against

pneumococcus and several serogroups of meningococcus, leading to a substantial decrease in the number of bacterial meningitis infections. For other bacterial diseases caused by group B streptococcus, *Salmonella* sp. and *Shigella* sp., conjugate vaccines have now become available.

Further, *Escherichia coli* strains have been metabolically engineered to synthesize polysaccharides that could be linked with carrier proteins in their periplasmic space, these are also known as bioconjugates and have currently undergone safe clinical trials (Ihssen et al. 2010). Although several conjugate vaccines have been developed, the mechanisms by which the immune responses, particularly T cell responses, are generated against the polysaccharide antigens are not completely understood. The immunogenicity of such conjugate vaccines, particularly the T cell responses, can be enhanced several fold by the formation of covalent bond between the polysaccharide and the proteins (Rappuoli et al. 2019). Recently, it has been shown that the structure of the bacterial polysaccharide has a great impact on potency of adaptive response against these glycoconjugate vaccines (Sun et al. 2019).

25.4 Recent Strategies in Vaccine Development

25.4.1 Nucleic Acid Vaccines

25.4.1.1 DNA Vaccines

Besides using microbial components or proteins, another approach in the development of vaccines involves introduction of genes encoding a particular antigen of interest. The host cells then use this newly introduced DNA to produce the protein antigens naturally, which is considered as foreign by the cells of host immune system to mount the immune response against that antigen. The DNA also carries adjuvant like properties that stimulate the expression of co-stimulatory molecules on the antigen-presenting cells along with production of other cytokines, which potentiate the generation of immune response against the desired antigen(s). This approach provides broad long-term immune responses that are stable and long-lasting with minimal side effects, and the large-scale manufacture of such vaccines is relatively easy. The DNA vaccines carry an advantage over the subunit or vector-based vaccines being devoid of other pathogen or virus-derived antigens. Majority of these vaccines are either in the research process or have entered phase I clinical trials. Most of these DNA vaccines being developed use plasmid (also termed as DNA plasmid vaccines) that carries the gene or gene encoding proteins of the microbial pathogen (Ghaffarifar 2018). The process of manufacturing DNA plasmid vaccines is now well established, enabling quick development of vaccines for newly emerging pathogens like COVID-19 or reemerging infectious diseases, such as influenza.

DNA vaccines for many viral diseases have been developed relatively at a faster pace to manage certain viral outbreaks. These include the SARS coronavirus (SARS-CoV) in 2003, H5N1 avian influenza in 2005, H1N1 pandemic influenza

in 2009, and Zika virus in 2016. Currently, efforts are on to develop DNA vaccines against the SARS-CoV-2 as well by various research groups across the world. The relative ease in the process of DNA vaccination (or with nucleic acid vaccination) development has led to a shorter turnaround time for the production of vaccines. As compared to SARS-CoV whose production in 2003 took around 20 months, the vaccine for Zika virus was produced in much shorter time within 3 months in the year 2016.

25.4.1.2 RNA Vaccines

Vaccines based on messenger RNA (mRNA) are also being developed. The process involves direct delivery of synthetic RNA molecules into immune cells. After internalization, the RNA vaccine functions like mRNA, making the cells to synthesize the foreign antigen as it would be synthesized by a usual pathogen or a tumor cell. These expressed antigens or proteins stimulate the host's adaptive immune responses on how to recognize and eliminate that particular pathogen or antigen expressing tumor cell. The first successful attempt of RNA vaccine was made in mouse by intramuscular injection of an in vitro transcribed mRNA, paving the way for development of RNA vaccines (Wolff et al. 1990). A fully functional mRNA vaccine requires the concerted action of all the structural components of its mRNA, and the preparation should be pure whereby impurities could be removed by either precipitation or extraction methods and other RNAs could be separated from the target mRNA chromatographically (Pardi et al. 2013; Pardi et al. 2018; Karikó et al. 2011).

Like DNA, the RNA vaccines also have a self-adjuvant property that ensures adequate immune response against the expressed protein. RNA vaccines carry an advantage over DNA vaccines, since mRNA directly encodes for protein and needs no prior transcription inside the host cell and thus no requirement to enter the cell nucleus. Further, the sequences of RNA can be altered to express the desired proteins. The in vivo expression of the RNA also ensures that it does not integrate into the host genome and interfere with the normal functioning of host cell.

Recent innovations in RNA vaccine technology platforms have dealt with problems associated with mRNA instability and the challenges of transfecting RNA (like use of cationic lipids, protamine, polymers) into host cells. Recently, encouraging results have been observed with some mRNA vaccines (Xu et al. 2020). As of now more than 20 RNA-based drugs have entered clinical trials, many of which are vaccine candidates. The mRNA vaccines are excellent candidates in the case of rapidly emerging infectious diseases, like COVID-19. There are many manufacturers like Pfizer-BioNTech and Moderna, whose RNA vaccines have been successful in preventing severe form of COVID-19 following their launch in early 2021 and have been granted emergency use authorization (EUA) in the United States and many other countries. The Pfizer-BioNTech collaboration developed the BNT162b1 vaccine, which is a modified mRNA vaccine encoding trimerized SARS-CoV-2 S protein receptor-binding domain formulated by lipid nanoparticles. The Moderna vaccine (mRNA-1273) encodes the spike (S) protein of SARS-CoV-2 in a prefusion confirmation.

In case of other deadly viruses as well, the RNA vaccines have shown considerable success. An experimental mRNA vaccine against Zika virus infection has been shown to protect mice and monkeys after a single dose (NIAID-supported research) that later on led to the development of a modified mRNA encapsulated in lipid nanoparticle vaccine against zika virus in the year 2017 (Pardi et al. 2017; Richner et al. 2017). Likewise, RNA vaccines against rabies (Alberer et al. 2017), Venezuelan equine encephalitis virus (Samsa et al. 2019), streptococci (Maruggi et al. 2017), and various cancers (Kreiter et al. 2012) are currently being developed or are in clinical trials (Xu et al. 2020).

25.4.1.3 Recombinant Vector Vaccines

The recombinant vector vaccines (also referred to as recombinant live vector vaccines) are similar to DNA vaccines except that the DNA encoding the antigen from the pathogen is cloned in an attenuated virus or bacterium (vector), which can directly be used for injection as a vaccine. These vaccines are quite safe as the DNA is delivered through a nonpathogenic organism as vector that introduces the foreign genetic material into the cells of the host.

Upon injection in the recipient, the vector (like adenovirus, modified vaccinia virus, vesicular stomatitis virus) directly introduces the microbial DNA to the host cells to elicit immune response. The recombinant viral vector vaccines can be developed either as replication competent (e.g., vesicular stomatitis virus (VSV) for various infectious diseases like yellow fever (YF) 17D virus for flaviviruses, such as Japanese encephalitis, Dengue West Nile) or replication defective (e.g., modified vaccinia virus Ankara for smallpox and other infectious viruses), modified simian adenoviruses for viruses belonging to the family of Ebola viruses, MERS-CoV, Zika virus, and SARS-CoV2. The replication competent viral vector vaccines elicit potent humoral and T cell responses. These can be simply produced via viral cultures. However, in some cases, the non-virulent virus may again become virulent, or there is reduced expression or loss of heterologous antigens that leads to ineffectiveness of some vaccines. With the requirement of a few additional manufacturing steps, the replication defective viral vector vaccines on the other hand is safe (Afrough et al. 2019). Nowadays, numerous vectors have been engineered to create novel recombinant vectors that allow incorporation of multiple genes from same or different pathogens along with their promoters for enhanced expression. Efforts are being made to develop recombinant vector vaccines against HIV, recently emerged pathogens like, Zika virus and Ebola virus. When attenuated or nonpathogenic bacterium is used as a vector, the inserted microbial gene is expressed and displayed as an antigen on its surface. This organism behaves similarly to the pathogen in provoking a protective immune response; however it does not pose any safety issues to the host and hence can be used without any harm.

The recombinant vector vaccines are capable of producing broad spectrum and long-lasting immune responses that are highly specific. The expressed antigens are broken down in the proteasomes of the transduced cells and presented as externalized peptides by the MHC-I molecules to activate CD8+ T cells. Additionally, the antigen-presenting cells can phagocytose the apoptotic or necrotic

transduced cells and present antigenic peptides in association with MHC-II molecules to the CD4+ helper T cells, by a process termed as cross-presentation.

25.4.1.4 Heterologous Prime-Boost Strategy

The prime-boost technology was typically developed using the same vaccine, which means using the same formulation expressing the same antigen/antigens for primary and booster doses. However, recent studies have demonstrated that different vaccine preparations that express the same antigen can be used to perform the prime-boost strategy with encouraging results (Fig. 25.2). This type of heterologous prime-boost strategy is now being investigated in many diseases caused by many viral pathogens, including HIV, HCV, pseudorabies virus and herpesvirus. Recently against Tembusu (TMUV) virus, a flavivirus that causes infection in ducks, chickens, geese, and pigeon, that poses threat to human beings also. Vaccination against this virus has been developed using heterologous prime-boost strategy. For this, priming is done with attenuated salmonella vaccine, and boosting is done with a recombinant adenovirus vaccine that expresses prM-E or the E gene of TMUV and has been shown to be more efficacious than the traditional homologous prime-boost strategy (Pan et al. 2020).

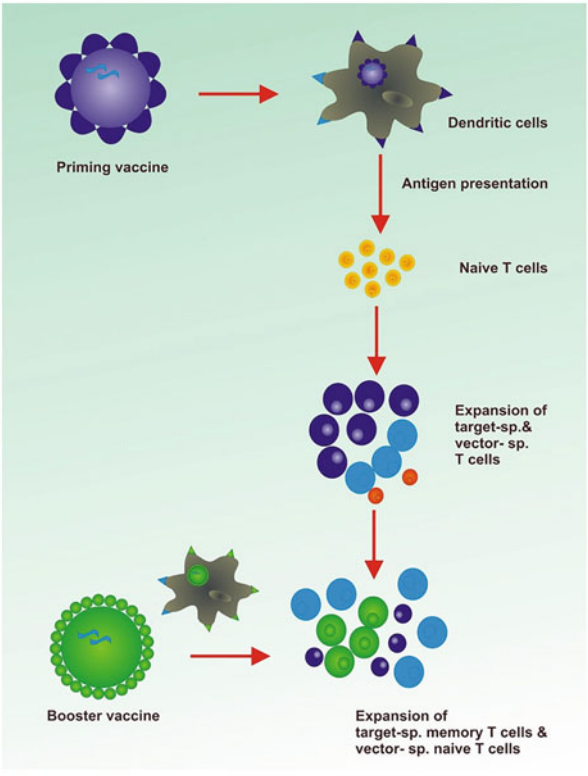

Fig. 25.2 Amplification of T cell immune responses by heterologous prime-boost strategy: The first vaccine activates the dendritic cells (antigen presenting cells) which then presents the processed target antigens to naïve T cells, resulting in their activation and expansion. Subsequent booster vaccine results in representation of target antigens and antigens specific to second vector. This booster vaccine results in the expansion of target-specific memory T cells and the vector-specific naïve T cells (second vector) leading to a synergistic expansion of T cells specific for the target antigen and selection of T cells having high avidity for the antigen

25.5 SARS-CoV-2 and Vaccine Development

While there are several uncertainties in the disease pathology of COVID-19, the development of vaccines against SARS-CoV-2 has occurred at a very brisk pace (within months), unlike the standard process of vaccine development which usually takes several years. Currently, there are more than 200 vaccine candidates that have been either released for public use or are in preclinical or phase 1, 2, or 3 stage.

Coronaviruses are enveloped viruses containing a positive-sense single-stranded RNA (~ 30 kb) capable of infecting a variety of mammals and birds. They have four main structural proteins: the spike (S) protein which facilitates entry of the virus in the host cells, the envelope (E) and membrane (M) proteins required for the production and assembly of coronavirus like particles, and the nucleocapsid (N) protein (Schoeman and Fielding 2019; Fehr and Perlman 2015). It must be noted that the vaccines against veterinary coronavirus diseases that include the avian infectious bronchitis virus [IBV], feline coronaviruses, bovine coronaviruses, and canine coronaviruses have provided important lessons in developing vaccines against SARS-CoV-2 (Tizard 2020).

25.5.1 Important Considerations in SARS-CoV-2 Vaccine Development

25.5.1.1 Selection of Antigen

The S or the spike protein forms the outermost component of the virus. It attaches to the host cell receptors through its region called the receptor-binding domain (RBD), which is located in the S1 subunit. Another subunit of S protein, the S2 subunit then fuses the viral envelope with the host cell membrane, to deliver the viral genome into the host cellular cytoplasm. It has been well demonstrated that the antibodies formed against the S protein can neutralize the virus, making the S protein a first choice as a primary antigen candidate for the SARS-Cov-2 vaccine.

In the context of appearance of mutated forms of the SARS-CoV-2 strains from different regions of the world, there is now growing interest on the effectiveness of the SARS-CoV-2 vaccines, particularly those using the S protein for vaccine development, since most of the mutations have been reported in this protein (Shi et al. 2020; Korber et al. 2020). It appears that the selection of these mutant variants is not driven by host immunity, as still there is enough population that is unexposed to the virus. Secondly, since the immune responses of the vaccine are directed against the whole protein, it is expected that few isolated mutations (which may increase the infectivity or replicative fitness of the virus) should not compromise the immunoprotection conferred by the vaccines, unless there is accumulation of large number of mutations thet may lead to immune escape of the virus.

The N protein is a conserved protein; however since it is not displayed on the surface of the virus, it is less likely to produce high titers of neutralizing antibodies. However, few studies have shown that N protein-based vaccines induce immunoprotection against SARS-CoV-2 and MERS that is CD4+ T cell-dependent,

suggesting that the N protein-based T cell inducing vaccines also present a viable option (Zhao et al. 2016). Data from animal studies has shown that the M protein-based vaccines can also induce high titers of antibodies (He et al. 2005). However, whether the M protein-based vaccines can induce protective immunity in preclinical models is yet to be determined. Similarly, immunization studies on the E protein-based vaccines have also been performed, but none of them demonstrated protective immunity (Buchholz et al. 2004).

25.5.1.2 Vaccine Platform

The vaccine for SARS-CoV-2 can include any of the established platforms, including the live attenuated virus, recombinant viral-vectored vaccines (bioengineered to express SARS-CoV-2 antigens in vivo), inactivated or killed virus, protein subunit vaccines, virus-like particles (VLPs), and nucleic acid-based (DNA or mRNA) vaccines.

Typically, a successful vaccine requires two main components: antigens from the target pathogen and a signal indicating infection (via the pathogen-associated molecular patterns or damage-associated molecular patterns) which activates the host immune system. Naturally, the live-attenuated vaccines provide both of the components; however, long periods of time are required to monitor the safety of such vaccines. The nonviral vaccine platforms do provide the antigens but generally require adjuvants and multiple doses. Similarly, the killed virus vaccines also sometimes require adjuvant and repeated boosters for maximum efficacy. The live vaccines, on the other hand, have the ability to provide single dose immunity (Rauch et al. 2018).

In terms of the abundance, various types of candidate vaccines under development or in trials or approved under EUA are mentioned (with examples) as follows (Jeyanathan et al. 2020) (World Health Organization. Draft landscape of COVID-19 candidate vaccines):

1. Protein subunit
   - SARS-CoV-2 rS/Matrix-M1 Adjuvant NVX-CoV2373 by NovaVax,
   - Recombinant new coronavirus vaccine (CHO cell) adjuvanted recombinant RBD-Dimer by Anhui Zhifei Longcom Biopharmaceutical/Institute of Microbiology, Chinese Academy of Sciences.
   - SARS-CoV-2 Sclamp vaccine (Molecular clamp stabilized S protein with MF59 adjuvant University of Queensland/CSL/Seqirus).
2. Recombinant viral vector
   - AZD1222 (ChAdOx1-S) by University of Oxford/AstraZeneca,
   - Ad5-nCoV (Adenovirus Type 5) by CanSino Biological Inc./Beijing Institute of Biotechnology.
   - Gam-COVID-Vac (Adeno-based (rAd26-S + rAd5-S) by Gamaleya Research Institute.
3. DNA
   - INO-4800 (DNA plasmid with electroporation) by Inovio Pharmaceuticals/International Vaccine Institute.

- AG0301 (COVID19 Adjuvanted DNA plasmid) by Osaka University/AnGes/Takara Bio.
- ZyCov- D Vaccine (DNA plasmid) Zydus Cadila Healthcare Limited.

4. RNA
   - mRNA-1273 (Moderna/NIAID),
   - BNT162b1 and BNT162b2 (LNP-mRNAs) by BioNTech/Fosun Pharma/Pfizer.
5. Inactivated, virus-like particles
   - Adsorbed COVID-19 (inactivated) vaccine by SinoVac.
   - Inactivated SARSCoV-2 vaccine (Vero cell) by Wuhan Institute of Biological Products/Sinopharm.
   - BBV152A, BBV152B, BBV152C inactivated by Bharat Biotech.
6. Live attenuated
   - COVI-VAC (intranasal, live attenuated vaccine against SARS-CoV-2) by Codagenix, Inc.
   - AttenuBlock (live attenuated synthetic SARS-CoV-2 vaccine on RSV platform) by Meissa Vaccines, Inc.

25.5.1.3 Route of Administration and Dosage

The route of administration and the regimen play an important role in the success of any vaccine. The respiratory tract forms the initial site of infection of SARS-CoV-2 (Sungnak et al. 2020). Upon entry in the respiratory tract, SARS-CoV-2 interacts with the angiotensin-converting enzyme-2 (ACE2) receptor on the bronchial and alveolar epithelial cells via the receptor-binding domain (RBD) of the spike (S) protein, which is subsequently primed by a specific cellular serine protease, the transmembrane protease serine 2 (TMPRSS2), facilitating entry in the host cells (Hoffmann et al. 2020). Therefore, it can be inferred that the vaccine must be able to elicit a strong respiratory mucosal immunity at first to prevent the infection. Parenteral vaccination as observed in the case of intramuscular measles or influenza vaccines has been able to show protective mucosal IgG responses. However, these vaccines do not induce desired levels of mucosal IgA antibodies or resident memory T cells in the lungs (Belyakov and Ahlers 2009). In contrast, the respiratory mucosal route of vaccination is suitable in inducing antibodies, resident memory T cells in the respiratory mucosa, and the macrophage-mediated trained immunity (Szabo and Miron 2019).

Inactivated virus, protein subunit, and nucleic acid vaccines are not suitable candidates for administration by the respiratory mucosal route since they require immune adjuvants and repeated dosage. The recombinant viral vector vaccines, like the human serotype 5 adenovirus (Ad5) or chimpanzee-derived adenovirus (ChAd) in particular, are considered safe and highly effective for respiratory mucosal vaccination (Afkhami et al. 2016). In fact, the ChAdOx1 nCoV-19 (also known as AZD-1222), developed by Oxford University UK, and AstraZeneca are one of the most successful COVID-19 vaccines that are now in clinical application following the EUA approval. Preclinical studies demonstrated that the ChAdOx1 nCoV-19

reduced viral load in lungs and prevented pneumonia in rhesus macaques. Humans have a very low seroprevalence for ChAd, making it suitable for inducing robust immunogenicity and utility for heterologous prime-boost vaccination (Mercado et al. 2020; Folegatti et al. 2020).

25.6 Conclusions and Futuristic Strategies

The field of vaccinology is continuously evolving. While there is a growing threat of reemergence of existing pathogens or appearance of novel infectious pathogens in the environment, great strides have been made in the understanding the pathogenesis of infectious pathogens. Further advancements in cell biology, genetic engineering, proteomics, and bioinformatics have enabled development of new platforms or modification of existing platforms for novel vaccines tailored against specific pathogens. Besides, advances in novel delivery systems, like the liposomes, virosomes, exosomes, microspheres, and proteosomes have enabled these vaccines to be more targeted in generating potent immune responses with enhanced memory. Of particular interest are the nanoparticle-based vaccines or nanovaccines that are derived from natural polysaccharides like chitosan and synthetic biocompatible polymers such as poly lactic-co-glycolic acid (PLGA), 1,8-bis (p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG), and calcium phosphate nanoparticles. These polymeric nanoparticles can be employed as carriers for antigens and as adjuvants that will further improve the efficacy and stability of the vaccine formulation against many infectious diseases (Maina et al. 2020). Nanovaccines offer the flexibility of administration through various routes, and because of their morphology, they are capable of crossing the blood-brain barrier. Unlike traditional vaccines, these formulations exhibit extended thermal stability and are capable of maintaining antigenic structure and function for long periods of time (McGill et al. 2018; Wagner-Muñiz et al. 2018). Nanovaccines also offer flexibility in designing, so that they imitate the disease-causing pathogen in size and shape, for easy engulfment and processing by various immune cells of the host. Since most of the pathogens first encounter host mucosal surfaces, strategies of sensitizing the mucosal immune system are also being developed. Newer mucosal vaccinations could be formulated as injectable liquids, nasal sprays, nebulizers, or lyophilized formulations (Narasimhan et al. 2016).

Overall, it can be said that there are several platforms now available for vaccine development and formulations. The current technologies are capable of developing efficacious vaccines at a rapid pace than ever before. The past decade has witnessed emergence of few infectious viruses from nowhere. The current global threat of SARS-CoV-2 has reemphasized the fact that vaccines are the most important available tools to prevent and reduce the incidence of infectious diseases, and further advancements in the field of vaccinology must continue.

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Preclinical Models of Intimal Hyperplasia and Restenosis to Predict Clinical Events and Develop Novel Therapies

26

Finosh G. Thankam, Victoria E. D. Wilson, and Devendra K. Agrawal

Abstract

Restenosis involves re-narrowing of the arterial lumen and leads to the restriction of blood flow. As a common occurrence following interventional procedures, including balloon angioplasty and intravascular stenting, to treat atherosclerotic lesions, restenosis can pose a serious problem. Animal models have significantly advanced our knowledge to better understand the underlying molecular pathophysiology of restenosis and helped in the development of novel and effective therapeutic strategies to improve the quality of human cardiovascular health. Though experimental models may not perfectly mimic clinical restenosis, they provide a better understanding of the pathophysiology; efficiency of therapeutics for potential clinical application, testing, and performance of novel devices; and investigation of the compatibility status of the novel interventional strategies. The information in this chapter is focused on the discussion regarding the contribution of various animal models, including mice, rats, rabbits, and porcine, as well as in advancing the current understanding of the cellular and molecular mechanisms of the pathophysiology of neointimal hyperplasia (NIH) and restenosis. A critical evaluation is made on the validity and translation of these findings in animal models to human disease and their application to develop novel translational strategies in developing better management of the disease process.

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Angiocardiopathy · Animal models · Atherosclerosis · Neointimal hyperplasia · Restenosis · Vascular interventions

Cardiovascular diseases (CVD) represent the pathologies associated with atherosclerosis that significantly affect the heart and blood vessels. The development and deposition of atherosclerotic plaques in the major arteries, including the coronary and carotids, limit blood flow and consequently result in complete occlusion. As an inflammatory disorder, atherosclerosis results in morphologically distinct stenotic and non-stenotic lesions. Generally, the stenotic lesions are clinically characterized with angina pectoris where the revascularization procedures including percutaneous coronary intervention (PCI), percutaneous transluminal coronary angioplasty (PTCA), and coronary artery bypass grafting (CABG) have been widely employed as corrective measures (Varela et al. 2020). In contrast, the non-stenotic lesions remain clinically asymptomatic for several years, and lifestyle modifications followed by pharmacological interventions (depending on the severity) support patient care. Despite the massive recovery of patients following the stenotic interventions, the increased rate of restenosis often hurdles the long-term success, which remains the serious challenge in current cardiology. Classically, restenosis refers to the narrowing of the vascular diameter to >50% to that of a reference blood vessel. Intimal hyperplasia, the invasion of vascular smooth muscle cells (SMCs) due to uncontrolled proliferation and migration from the medial layer to the intimal layer, reinitiates the luminal occlusion and results in stenosis. The emergence of stenting functions as another breakthrough in interventional cardiology; nevertheless, restenosis due to intimal hyperplasia of SMCs significantly decreases the rate of success (Varela et al. 2020; Forte et al. 2014).

Restenosis commonly occurs following interventional procedures, including angioplasty, to treat ischemic/atherosclerotic lesions. Failures are encountered in the early phase, in part due to technical incompatibilities and thrombosis, whereas failures in later stages (>18 months) are mainly due to chronic inflammation, remodeling of the extracellular matrix (ECM), scarring, and ongoing atherosclerosis. Typically, atherosclerosis requires decades to develop; however, restenosis occurs within months to years, which suggests the need for careful monitoring and effective strategies to prevent restenosis in the first place (Kenagy 2011). Moreover, the decreased endothelialization of stented vessels results in in-stent restenosis and proves to be a serious challenge in angiocardiopathy. The reendothelialization process post-angioplasty is vital in the prevention of restenosis, an avenue of study that has yet to be explored in sufficient detail (Chang et al. 2018). Extensive research has made strides to address these issues, and medical science anticipates next-generation strategies with better performance for improved patient care. Importantly, a multitude of animal models increase our understanding of the molecular pathology of restenosis in angiocardiopathy, the application of translationally relevant approaches, the development of novel and effective therapeutic strategies for better

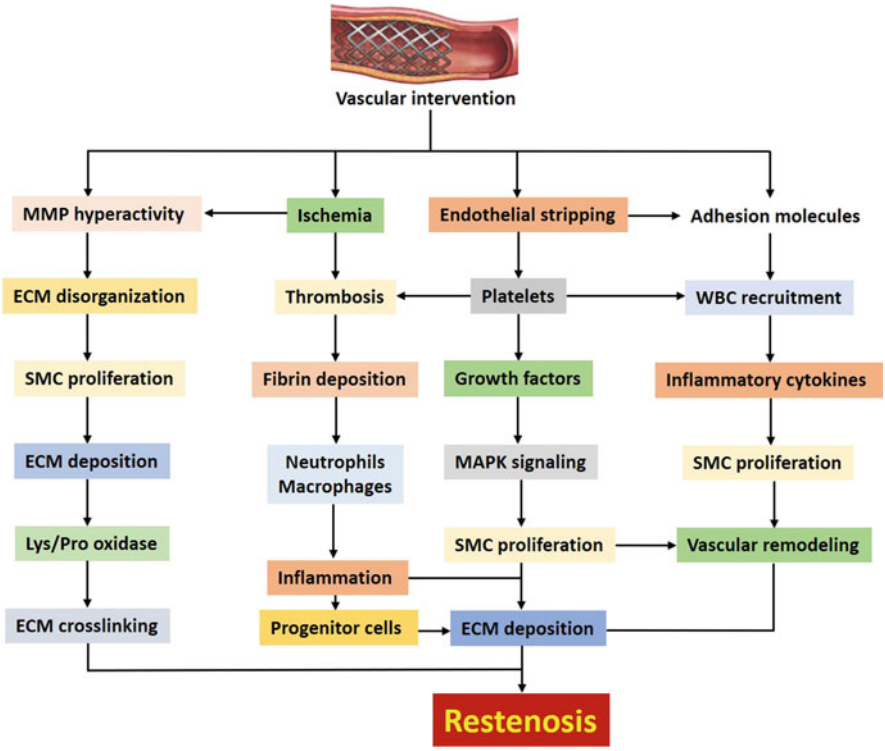

Fig. 26.1 The key biological events associated with restenosis. The vascular intervention and associated endothelial damage trigger the proliferation of SMCs via multiple signaling including MMP hyperactivity, platelet aggregation, inflammation, ECM damage and vascular remodeling ultimately leading to aggravated NIH and subsequent restenosis

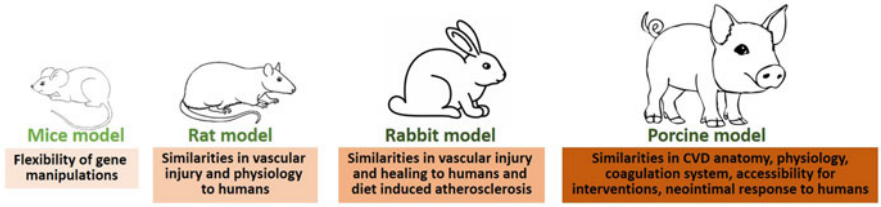

Fig. 26.2 The major animal models and their advantageous features used for endovascular interventions and restenosis research

management of disease processes, and extend human life. This chapter discusses the contribution of diverse animal models in the advancement of the current understanding surrounding vascular lesions, especially in the pathology of NIH and restenosis (Figs. 26.1 and 26.2).

26.1 Pathobiology of Restenosis

Restenosis involves multiple risk factors categorized according to patient-related factors and comorbidities, such as diabetic mellitus, lesion types, and procedural defects. For instance, the performance of drug-eluting stents (DES) in diabetic patients proves less satisfactory compared with nondiabetic subjects. In addition, the hyperactivation of matrix metalloproteinases (MMPs), along with the inflammatory episodes and subsequent ECM disorganization, facilitates the proliferation and migration of SMCs (Omeh and Shlofmitz 2020). Additionally, hematological indices such as the respective ratios of neutrophils and platelets to lymphocytes, RBC distribution width, and platelet volume and distribution width have been the predictive factors for restenosis following stenting. Furthermore, lesion size and vessel diameter are critical in the successful performance of vascular stents. For example, a stent length >35 mm doubles the failure risk when compared with the 20 mm stents (Omeh and Shlofmitz 2020; Piraino et al. 2017). Moreover, stent under-expansion due to inappropriate size, low-pressure deployment, vessel preparation techniques, and/or extensive calcification adversely affect the patency of stents and pave multiple paths to restenosis. Other key risk factors for restenosis following stenting include stent fractures, geographic miss, local hypersensitivity, and drug resistance in DES (Omeh and Shlofmitz 2020).

Generally, restenosis displays diverse clinical features delineating various levels of ischemia followed by arterial interventions that depend on lesion characteristics, anatomical location, and genetics. The pathological events associated with restenosis can be attributed to the extensive biological responses to vascular injury sustained from the intervention. The elastic recoil, platelet plug formation, and excessive thrombosis are the initial events that occur within minutes to hours post-intervention (Kim and Dean 2011). Subsequent to these initial responses, the immune system launches complex inflammatory and reparative events that persist for months to years. The acute inflammatory phase is characterized by the deposition of fibrin and platelets in conjunction with the recruitment and adhesion of neutrophils and monocytes at the site of injury (Fig. 26.1). The switch to chronic inflammation usually occurs over a few weeks and is marked by the presence of macrophages and giant cells, which in turn pave the way for the two principal biological mechanism of stenosis: NIH and neoatherosclerosis (Kim and Dean 2011).

The early phase of restenosis is marked by endothelial stripping, reendothelialization, and the formation of neo-endothelialization, all of which contribute to platelet activation, inflammation, and the upregulation of cytokines and growth factors. The intermediate phase features the proliferation and migration of SMCs from the medial layer to the intima. Normally, the SMCs exist in a quiescent nonproliferative G0 phase, which is the contractile phenotype (Mitra and Agrawal 2006). In the late/remodeling phase, the contractile phenotype of SMCs switches to a highly active synthetic phenotype characterized by increased ECM deposition in the intimal region. The upregulation of diverse growth factor signaling including FGF-2, EGF, PDGF, and IGF results in SMC proliferation mediated via MAPK signaling (Varela et al. 2020). Moreover, newly synthesized ECM sequesters inflammatory

infiltrates, while the proliferating SMCs secrete proteoglycans and hyaluronic acid to stabilize the fibrin-rich provisional ECM. Such vascular remodeling is also accelerated by extravascular cells, especially endothelial progenitor cells, dendritic cells, and stem cells (Varela et al. 2020; Mitra and Agrawal 2006).

The damage to the endothelium caused throughout interventions exposes tissue factors to blood, resulting in platelet aggregation and subsequent activation of the coagulation cascade and subsequent thrombotic occlusion. Integrins mediate platelet aggregation and result in the secretion of thromboxane A2, serotonin, and MMPs to continue the process of aggregation (Smyth et al. 2009). The growth factors and chemotactic mediators released by the platelets attract the SMCs from the medial layer where the thrombus acts as a scaffold for SMC migration, attachment, and proliferation. Moreover, the ADP released by the activated platelets aggravates the NIH via the P2Y12 receptor (Evans et al. 2009). Thus, antiplatelet therapies are prescribed to prevent restenosis, which prolongs the appearance of clinical symptoms. In addition, interventional procedures impart tension and stretch to the arteries, which proves a potent trigger for cell proliferation and leads to an increase in tissue mass, while tension in the cell-ECM interface facilitates tissue remodeling (Korshunov et al. 2007). In addition, the accelerated remodeling occurs via nitric oxide (NO) signaling and the upregulation of cytoskeletal components, especially vimentin (Kenagy 2011). Moreover, the adventitial fibrosis and collagen deposition result in negative remodeling (inward or maladaptive), as the SMCs secrete collagen fibrils based upon the relevant fibronectin template, α2β1 integrin, and RhoA signaling (Li et al. 2003). Furthermore, increased ECM cross-linking via the upregulation of collagen enzymes, including lysyl oxidase and prolyl oxidase, contributes significantly to negative remodeling and aggravated restenosis (Brasselet et al. 2005).

The increased cell density and ECM deposition denote NIH, which is initiated by endothelial injury and results in the partial or complete removal of the quiescent endothelium (Gulati et al. 2005; Clowes et al. 1988). Meanwhile, the medial layer SMCs de-differentiate to express proteins associated with cell proliferation, migration, and ECM synthesis. Interestingly, the proliferative rate of SMCs has been reported to be increased by 10–40% in the arteries of experimental models during the first 2–4 days of injury and declined to normal after 4–8 weeks. However, the SMCs at the intimal region and luminal surface continued to proliferate at a slower pace (Clowes et al. 1983; Kenagy 2011). On the other hand, human arteries possibly require approximately 2 years for intimal growth following interventions (Kang et al. 2010; Kimura et al. 2002). The differences in the time frame between human and experimental animals could be attributed to differences in thrombotic response, recovery rate of endothelial tissue, and genetics (Kenagy 2011). Adventitial proliferation displays a similar trend (Couffinhal et al. 2001). Unfortunately, the precise role and underlying mechanism of SMC migration remain largely unknown, and current technologies are insufficient to quantify NIH in human vessels. Moreover, the impact of preexisting intimal SMCs in the migration of medial SMCs requires further investigation.

The origin of the intimal SMCs in NIH/restenosis remains a topic of some debate. Interestingly, the studies in animal models suggest a population of intimal SMCs of bone marrow origin (Orlandi and Bennett 2010). Other studies suggest that adventitial cells may be a possible origin of intimal SMCs (Sartore et al. 2001). Another proposal posited the de-differentiation of endothelial cells to SMCs (Frid et al. 2002). Inflammation plays a crucial role in the initiation of NIH via the mediators secreted by recruited leukocytes and adhered platelets, as well as cell adhesion molecules, such as ICAM-1 and VCAM-1, released by damaged endothelium (Smyth et al. 2001; Tanaka et al. 1993). In addition, injured, surviving, and recruited cells release critical growth factors, including PDGF, FGF2, TGFα/β, VEGF, and M-CSF, as well as a group of pro- and anti-inflammatory cytokines, such as IL-1, IL-4, IL-6, IL-8, IL-18, and TNF-α that exacerbate inflammation and vascular remodeling (Fig. 26.1). These mediators exhibit intimate association with NIH and restenosis (Mitra et al. 2005).

The contribution of ECM warrants further consideration, as ~80% of the neointima is composed of ECM components, whereas ~20% has been constituted by slowly proliferating SMCs (Clowes et al. 1983). This suggests that alterations in ECM biology and matrisome composition following interventions act as key factors in the development of restenosis, which is supported by the inhibition of collagen and hyaluronic acid expression by rapamycin (Koyama and Reidy 1998; Gouëffic et al. 2007). Biglycan and hyaluronan act as mitogen for SMC proliferation, whereas decorin inhibits ECM deposition following an injury. Interestingly, the restenotic tissues displayed increased levels of collagen I, elastin, hyaluronic acid, and proteoglycans, especially versican, biglycan, and perlecan with a concomitant reduction in decorin (Kenagy 2011). In addition, the hyperactivation of multiple MMPs contributes to vascular remodeling and subsequent NIH. For instance, MMP9 upregulation is closely associated with inflammation and the increased density of CD34+ cells following coronary stenting, which in turn is the major predictor for NIH and restenosis (Heissig et al. 2002). Furthermore, reports indicate the collagen receptor discoidin domain receptor to be essential for SMC migration and MMP secretion (Hou et al. 2002). Additionally, CD44 facilitates the proliferation and migration of SMCs via binding to hyaluronic acid and collagen moieties (Cuff et al. 2001). Moreover, endogenous mediators, including versatile danger-associated molecular patterns (DAMPs), significantly accelerate NIH via the signaling through TLR2/4 receptors and warrant further translational investigations (Schaefer et al. 2005).

Our knowledge on the underlying molecular pathogenesis of restenosis remains limited, and the literature regarding the underlying molecular mechanisms proves scarce. Encouragingly, the growing wealth of experimental evidence reflect advancements in the pathology of restenosis and angiocardiopathies (Fig. 26.1). The current understanding regarding the biology/pathology of NIH has been supported and improved by versatile animal models of vascular injury simulating human pathology. A handful of established animal models utilize clinically relevant interventional strategies, such as angioplasty with balloon catheter or wire injury and stenting. While animal models do not perfectly mimic human disease, they provide a

fundamental understanding and reveal the sequence of biochemical episodes following injury. The subsequent sections discuss the contributions of various animal models, challenges and limitations, and the success stories in the study of restenosis following vascular interventions.

26.2 Animal Models in CVD

Experimental animal models play an indispensable role in the discernment of the etiology and molecular pathology of human CVDs; furthermore, animal models assist in the design and development of novel disease management strategies. Both past and current cardiology research successfully established small and large animal models to closely recreate/simulate human CVD pathology (Fig. 26.2). The replication of exact complexities associated with human CVD pathologies in animal models is challenging; however, these experimental models have significantly advanced our knowledge, especially with regard to molecular mechanisms, diagnostic avenues, and prevention and management strategies. Small animal models (rodents) are widely utilized due to their cost-effectiveness, smaller size and ease of handling, large number of litters, physiological similarities, and ease of genetic modifications (Chorro et al. 2009; Tsang et al. 2016). However, their relatively smaller size impedes intravascular interventions and post-sacrifice examinations (Vilahur et al. 2011). In contrast, larger animal models exhibit exceptionally similar pathophysiology to humans and are ideal to provide the mechanistic insights regarding molecular pathology. However, appropriation/correlation with age- and sex-related variables can prove difficult to obtain in larger animal models (Walters et al. 2012). For instance, neutrophils form the predominant cell population in porcine blood leukocytes, as well as in humans, whereas mouse leukocytes demonstrate a higher proportion of lymphocytes (Meurens et al. 2012; Doeing et al. 2003). While large animal models elicit increased maintenance costs, they share a similar anatomy, physiology, biomechanics, and size to their human counterparts. Moreover, they facilitate the effective implementation of human instruments and procedures, better options for intravascular imaging, which engenders promising translational relevance. In addition, the application of larger animal models can easily illuminate the biological pathways and regulatory mechanisms underlying CVD pathology. However, it is important to note that the lack of specific antibodies and assays, scarcity of transgenic models, and lack of information regarding the inherent repair/regenerative mechanisms offer challenges to the use of larger animal models (Tsang et al. 2016).

The CVD animal models largely focus on the replication of human atherosclerosis; however, the studies using the experimental models on NIH and restenosis following interventional procedures in the arteries are limited. Notably, multiple aspects of restenotic pathology have been successfully developed in smaller and larger animal models and are used in preclinical studies (Rodriguez-Menocal et al. 2010). Moreover, restenosis models provided a significant contribution to the ability to predict potential adverse clinical outcomes, as suboptimal animal models result in

unsuccessful human trials. This section critically reviews the contributions of the well-established small and large animal models in unveiling the molecular mysteries of NIH and restenosis.

26.3 Mouse Models

The extensive and well-established use of mouse models assists in investigations into the pathological, molecular, mechanistic, and translational aspects of restenosis. In light of the smaller vasculature in mice, the traditional arterial injury approaches, including angioplasty, prove impractical; instead, wire injury or electrical injuries are preferred and routinely employed. Encouragingly, a variable range of neointimal thickness with minimal thrombosis can be achieved by these methods. The thrombus resorption following inflammatory cell infiltration in mouse models creates a hurdle in the precise replication of clinical vascular healing (Schwartz et al. 2004). Importantly, the ease and flexibility of gene knockout and the development of transgenic strains in mouse models have been instrumental in order to unveil the cellular and molecular mechanisms underlying NIH and atherosclerosis. For instance, low-density lipoprotein receptor (LDLR) knockout and ApoE-deficient mice demonstrated the metabolic alterations associated with atheroma formation (Zaragoza et al. 2011). Nevertheless, mouse models introduce unique obstacles for endovascular treatments that require systemic administration. Moreover, the vasomotor functions and viscoelastic properties of murine arteries are disparate to human arteries, thus exhibiting poor translational significance (Leigh Perkins 2010). In spite of the procedural difficulties, mouse models became an inevitable source to advance our understanding of the underlying pathophysiology of restenosis.

26.4 Key Updates from the Mouse Model

The mouse models of balloon angioplasty and stenting that replicate human PCI provide novel insights into the molecular pathology underlying stent injury and restenosis (Ali et al. 2007). The androgen receptor plays a potentially preventative role in NIH, as reported in the androgen receptor knockout (ARKO) mouse model of vascular injury, as evidenced from the increased NIH in the ARKO mice when compared with the normal control (Wilhelmson et al. 2016). In addition, studies in p27-deficient mice demonstrated that the mechanism of the antiproliferative drug, rapamycin, may prevent NIH independent of p27 signaling (Roqué et al. 2001). Recently, a genetic model of NIH has been developed by selectively silencing the elastin gene expression in the vascular SMCs to create a discontinuous internal elastic membrane (IEM) of the aorta resulting in NIH (Fig. 26.2). The impaired IEM, which acts as a barrier for the migration of SMCs, accelerates NIH, and this model possesses the advantage of the absence of surgical procedures (Lin et al. 2019). An earlier study reported a rapid procedure for stent implantation in mouse carotid artery to study the effects of various drug coatings on arterial remodeling (Simsekyilmaz

et al. 2013). This procedure possesses minimal risk to nerves and other structures and is suitable for normal and knockout mice. Similarly, a simple and inexpensive model of in-stent restenosis was developed by implanting a bare-metal stent in Apo-E null mouse abdominal aorta (Rodriguez-Menocal et al. 2010). Admittedly, the literature regarding mice models for restenosis is limited; however, the growing body of evidence provides the translational significance of mice models in vascular injury.

26.5 Rat Models

Since the 1960s rat models have been employed extensively for CVD research despite similar size-related challenges, as witnessed in mouse models. Interestingly, similar physiology and vascular injury responses to human patients have been demonstrated by rat models of carotid and aortic vasculopathies (Leigh Perkins 2010). Moreover, increased availability of antibodies to target intracellular and extracellular proteins and the flexibility of creating transgenic strains have advanced the field to select rat models for CVD research, especially in endovascular studies. Critically, the response of several anti-restenotic compounds in rat arterial injury models contradicts the findings in human patients and clinical trials, suggesting the possibilities of skepticism when dealing with clinically relevant studies in rat models (Leigh Perkins 2010). In addition, impaired thrombosis, increased fibrin deposition, sparse vascular remodeling, and minimal inflammation need to be considered while exploring the restenotic responses in rat models (Leigh Perkins 2010). Nonetheless, rat models were initially considered to be the gold standard to investigate NIH following the endothelial husking and provided immense translational opportunities to unveil underlying molecular pathology (Schwartz et al. 2004).

26.6 Key Updates from the Rat Model

Lowe et al. demonstrated a simple and cost-effective rat model for in-stent restenosis (ISR) in the left common carotid and implemented the BiodivYsio human coronary stent following the standard surgical procedures without fluoroscopy assistance (Lowe et al. 2005). Feng and colleagues effectively utilized the rat carotid artery injury model to elucidate the active role of tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein β (YWHAB or 14–3-3β) in accelerating the SMC migration and subsequent NIH formation. The study highlights YWHAB inhibition as an ideal strategy to prevent restenosis following the interventional therapy, especially in diabetic patients (Feng et al. 2018). Similarly, Langevield and co-investigators presented a rat model of ISR in the abdominal aorta for high-throughput stent examinations. The stent deployment resulted in comparable responses including thrombus formation, inflammation, and NIH formation, as displayed in human patients and other animal models. Interestingly, this model exhibited anti-restenotic responses while employing anti-restenotic stents (Langeveld et al. 2004). Recently, in another study, homozygous $apoE^{-/-}$ rats

were utilized to develop a reliable abdominal aorta stenting model by simulating a western diet. This model reflects the atherogenic phenotype combined with restenosis following the application of human-sized coronary stents (Cornelissen et al. 2019). In addition, heme oxygenase 1, an oxidoreductase enzyme, in a rat ISR model was reported to be a limiting factor and a potential target for ISR (Hyvelin et al. 2010). Furthermore, the advantage of the combined therapy of rapamycin and α-cyanoacrylate was reported in preventing NIH in the rat carotid injury model (Tianshu-Chu et al. 2019). Marshall et al. utilized a similar model of carotid balloon catheter injury to demonstrate the dual mode of medial artery calcification based on the expression status of BMP-2. The study laid a strong foundation for future approaches to improve therapeutic outcomes to address restenosis (Marshall et al. 2019). A similar approach was also employed to study the anti-NIH effects of the saponin isolated from the roots and rhizomes of *Panax notoginseng*, which effectively prevented the proliferation of SMCs via the inhibition of pERK/p38 MAPK signaling following carotid balloon injury (Yang et al. 2020). The research on NIH and restenosis using various rat models is rapidly progressing to open novel translational avenues and to advance the understanding of underlying molecular pathology.

26.7 Rabbit Model

The small size, ease of handling, and inexpensive maintenance reward rabbit models a prominent place within CVD research (Leigh Perkins 2010). The vital arteries, including the carotid, aorta, and iliofemoral, are the common anatomical sites for restenotic injury and are comparable to human coronary arteries (Leigh Perkins 2010). The rabbit ear crush injury has also been used for localized restenosis studies (Banai et al. 1991). Of note is the fact that the vascular injury and healing responses in rabbits are akin to larger animal models. For instance, stent-induced injury in a rabbit iliac artery closely parallels with the porcine coronary injury model (Virmani et al. 2003). However, the difficulties in cardiac access and the elastic nature of rabbit vascular injury when compared with muscular coronary arteries present major concerns when employing rabbit models in translational research. These challenges affect the extent of NIH, thrombosis, and inflammation. Due to delayed post-injury reendothelialization, rabbit models could be useful for endoluminal investigations, as opposed to studies with a focus on endothelial healing and function (Leigh Perkins 2010). Importantly, rabbits prove highly susceptible to diet-induced hypercholesterolemia, which is advantageous in order to simulate the clinical pathology of restenosis. Generally, the rabbit model for restenosis is combined with cholesterol-supplemented diet and balloon injury where the atheromatic lipid composition and histomorphometry are comparable to human lesions.

26.8 Key Updates from the Rabbit Model

More than two decades ago, the iliac injury model of NIH in rabbits was reported to reveal the mechanical infiltration of mononuclear cells to the injury site independent of lipid metabolites. In addition, heparin treatment significantly prevented cell density and subsequent NIH (Rogers Campbell et al. 1996). Interestingly, Meng et al. reported a vein graft rabbit model to establish the adventitial route of delivery of nonviral vector formulations to achieve transfection of endothelial cells and macrophages throughout the vessel wall (Meng et al. 2013). This novel approach exhibited significant benefits in the prevention of NIH and subsequent restenosis. Similarly, a model for carotid venous bypass grafting in rabbits was developed to study the therapeutic efficiency of a spectrum of anti-NIH drugs suggesting the potential benefits of rabbit models in high-throughput drug screening (Yucel et al. 2009). In addition, angioplasty-driven myointimal hyperplasia in the iliac artery of rabbit models was recently reported to study the anti-NIH effect of natural compounds (Rolim et al. 2016). Another inexpensive and highly reproducible model of NIH was demonstrated utilizing the common carotid arteries of NZ white rabbits and underscored potential advantages of assessment with the use of color Doppler ultrasonography (Mehrad et al. 2018). In addition, the protective effect of hyperbaric oxygen was reported against NIH following arteriovenous fistula (AVF) ameliorating the AVF failure in rabbit models where the AVF was created between the left common carotid artery and left common jugular vein (Li et al. 2017). In another study, the performance of an atorvastatin-eluting stent was not satisfactory in suppressing NIH in the rabbit iliac artery overstretching model (Lim et al. 2013). Importantly, Zhang et al. studied the effect of systemic methotrexate in combination with sirolimus-eluting stents in an in-stent neoatherosclerosis rabbit model (Zhang et al. 2016). This model demonstrated the advantage of employing optical coherence tomography (OCT) to reveal the status of restenosis and intimal thickening. Findings from a different study, which utilized a rabbit balloon angioplasty with stent implantation model, revealed that a moderate level of local heating favors healing responses. These results revealed immense translational potential owing to study simplicity, feasibility, and relevance (Brasselet et al. 2008) (Fig. 26.2).

26.9 Porcine Model

The porcine model has been the gold standard for preclinical CVD research largely due to the similarities in the cardiovascular anatomy, size, morphology, collateral vessels, well-defined vasa vasorum, physiology, and coagulation system that effectively simulate the human cardiovascular system in health and disease (Gross 1997; Lowe et al. 2003) (Fig. 26.2). In addition, the relatively larger dimensions of the vessels are ideal for assessing and accessing the performance of stents, catheters, guide wires, and intravascular imaging systems that are used in clinical cases. Moreover, the local flow dynamics of porcine vasculature, especially the coronary

arteries, intimately parallel that of humans, which improves confidence in pharmacokinetics studies. Importantly, the neointimal response and histology are similar to human restenotic vasculature (Leigh Perkins 2010). The disadvantage of the maintenance costs and economic viability are overcome by the possibility of multiple stent implantations including single or overlapping stents. In addition, the rapid growth rate and increased weight gain (>400 kg domestic farm pigs) combined with the associated difficulties in logistics, including the challenges in handling, instrumentation, and transportation, are challenging and require substantive resources. Hence, the miniature swine breeds, including Yucatan, Sinclair, Hansford, and Gottingen, are widely used, as they maintain modest weight during adulthood and provide appreciable scientific outcomes. More importantly, the swine are highly susceptible to diet-induced atherosclerosis and familial hypercholesterolemia, and the feasibility of endovascular interventions/treatments draw potential insights into the clinical setting of vascular pathologies (Leigh Perkins 2010). However, the increased penchant for inflammation with significant involvement of eosinophils following the stenting and/or endovascular interventions offers complex challenges to the clinical simulation of human restenotic response and warrants careful interpretations of relevant findings (Leigh Perkins 2010). Nonetheless, pig models are translationally the most relevant preclinical experimental models for investigating the molecular pathology underlying restenosis, developing gene therapy, and stem cell therapy, as well as other novel therapeutic modalities.

26.10 Key Updates from the Swine Model

We have successfully developed a coronary interventional model of restenosis in atherosclerotic Yucatan microswine to investigate the protective effects of vitamin D (Chen et al. 2016; Rai and Agrawal 2017; Gunasekar et al. 2018; Satish and Agrawal 2019). The findings revealed the underlying molecular mechanisms of atherosclerotic processes in the swine model and demonstrated a considerable reduction in the degree of NIH and restenosis in pigs supplemented with vitamin D compared with vitamin D-deficient groups (Yin et al. 2015; Satish et al. 2020). Studies illustrated a concomitant reduction in the localized expression of pro-inflammatory cytokines. These findings support the use of vitamin D as a potent adjuvant therapy in the prevention of coronary artery NIH and restenosis following coronary interventions (Gupta et al. 2016). Continued investigations using the same model revealed the proatherogenic cytokines induced downregulation of the vitamin D receptor in the proliferating SMCs to be the major underlying mechanism associated with restenosis, which was prevented by supplementation of vitamin D (Gupta et al. 2012). In addition, the swine model has been found to be effective for gene therapy and stem therapy to prevent NIH and restenosis (Satish and Agrawal 2020; Hall and Agrawal 2018). Moreover, the possibilities of DAMPs and the subsequent activation of sterile inflammatory episodes can be linked to NIH and restenosis. Recently, we reported the association between hypoxic insults and mitochondrial DAMPs in the pathology of restenosis associated with CABG vein graft failure in the Yucatan microswine

model (Thankam et al. 2020). Similarly, Bilder et al. revealed the potency of oral doses of TKI963, a selective inhibitor of platelet-derived growth factor-receptor tyrosine kinase, in the prevention of restenosis following coronary stent placement in the atherosclerotic swine model. This study demonstrated the activation of PDGFR to be the major determinant of stent-induced restenotic response, which indicates immense potential for translational significance (Bilder et al. 2003). Interestingly, the first in vivo application of a resonant-heating stent toward wireless hyperthermia treatment of in-stent restenosis was reported in swine models where the radiofrequency heating expanded the stent devices to different diameters; even in live blood streams, this approach attained a rise in temperature in a reproducible manner. This study indicates the possible application of hyperthermia treatment in the management of restenosis (Yi et al. 2020). Another seminal study by Shimamura et al. revealed that infection with swinepox virus significantly reduced restenosis owing to the appreciable immune suppression in the pig models of angioplasty injury (Shimamura 2012). Additionally, Suzuki et al. reviewed the clinically relevant findings revealed from multiple swine models to address various pathological challenges in the cardiovascular system (Suzuki et al. 2010).

26.11 Other Animal Models

In addition to the previously discussed models, several animal models including canine, sheep, and primates are currently in use to investigate restenosis pathology and endovascular interventions. Dogs are widely used due to their manageable size, ease of handling, economic viability, and easy deployment of stents and vascular interventions in coronary and peripheral arteries. However, the resistance to NIH following balloon angioplasty and/or coronary stenting, higher fibrinolytic activity, low acute stent thrombosis, resistance to atherosclerosis, and the increased onset of sudden myocardial events demonstrate the array of challenging elements in the simulation of clinical restenosis in canine models (Leigh Perkins 2010). On the other hand, the sheep models possess similar coagulation and fibrinolytic system to that of humans, as well as favorably translational coronary anatomy. Despite the minimal number of reports focused on the stent-induced injury in ovine coronary arteries, the ovine model parallels the swine restenosis models. Notably, the larger bifurcations of coronary vasculature allow the sheep models to effectively investigate the bifurcation devices (Leigh Perkins 2010). The phylogenic, dietary, metabolic, pathologic, and physiological similarities support the use of nonhuman primate models for CVD research. However, the risk of zoonoses, ethical considerations, and maintenance costs present major roadblocks in the use of primate models in preclinical studies (Leigh Perkins 2010).

26.12 Summary and Perspective

The establishment of various large and small animal models has significantly improved our knowledge of the underlying cellular and molecular mechanisms and led to the development of superior therapeutic approaches to increase the life expectancy of patients living with CVD. The advancement in knowledge regarding the underlying molecular pathology of neoatherosclerosis following vascular interventions paved multiple paths to refine patient care, which owes a great debt to diverse and versatile animal models. The comprehensive review on the contributions granted by the experimental models to understand the biology of restenosis and NIH requires several volumes to present. However, this chapter discussed the molecular pathology and briefly pointed the advantages, disadvantages, challenges, and key findings revealed by the widely employed animal models including mice, rats, rabbits, and swine to investigate the underlying pathophysiology of NIH and restenosis. The growing body of evidence suggests drastic advancements in the basic science and applied technology where animal models provide a trustworthy platform for deciphering molecular pathology, testing, and innovation. Even though experimental models prove imperfect in simulating exact clinical restenosis, they provide a thorough understanding regarding the pathology, the efficiency of therapeutics for clinical applications, and performance of devices to be tested and/or reveal the compatibility status of the novel intervention strategies. In addition, the emerging research in the field of cardiovascular tissue engineering and regenerative medicine and the allied biomedical research, including 3D bioprinting, in vitro modeling, artificial intelligence, robotics, and bioreactor systems, would increase the multifaceted nature of next-generation CVD interventions. Without a doubt, the research on experimental animal models provides a strong foundation for the current understanding of restenosis pathology, which continues to improve our knowledge and understanding in order to address existing potential challenges and thereby opens novel, clinically worthwhile translational avenues to improve the patient care.

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Sphingolipids: A Roadmap from Biomarker Identification to Clinical Application

27

Priyanka Bhadwal and Navneet Agnihotri

Abstract

Sphingolipids are a class of bioactive lipids that have recently gained paramount attention in the field of cancer biology. Sphingolipid metabolism and its underlying regulation have been implicated to play critical role in cancer development, proliferation, progression, metastasis, and chemoresistance in various cancer types. Accumulating evidence has shifted the focus of the clinical research toward the use of these bioactive lipids as potential biomarkers. However, there has been a large gap in the translation of biomarkers from basic research to clinical application due to the complexities associated with their identification and validation. The current advent of metabolomics has made a significant contribution in the unravelling of perturbed regulatory pathways and screening of effective noninvasive biomarkers. Metabolomics is a viable approach that holds a promising future in clinical research by enabling the identification of metabolites that not only may serve as important therapeutic targets but also aid in the diagnosis and prognosis of the disease. However, the large volume and complexity of data generated by metabolomics poses a data analysis challenge making it difficult to identify biomarkers important to the outcome. Integration of metabolomics to highly efficient statistical measures could bridge this gap by extracting the valuable information and providing much deeper insights to the current clinical needs. The present chapter highlights the role of sphingolipids in cancer and the recent trends in metabolomics that can be employed to decipher their significance as biomarker. Further we have discussed the statistical concerns in the interpretation of the metabolomic data that may add to the robustness, reproducibility, and high performance of the selected biomarkers.

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Keywords

Sphingolipids · Biomarker · Metabolomics · Statistical analysis

27.1 Background

Rapid expansion of novel technologies has provided a platform to accelerate the process of biomarker discovery in cancer. A cancer biomarker is a unique metabolic signature or identifiable characteristic that provides useful information in multiple clinical settings including diagnosis, prognosis, prediction, and monitoring (Henry and Hayes 2012). Besides playing an increasingly important role in the clinical management of the patients, cancer biomarkers have also been linked to metabolic pathway dysregulations. Current translational studies have provided an unprecedented opportunity to study the association between sphingolipid metabolism and clinical outcomes. The clinical significance of sphingolipid-related biomarkers has acquired a forefront in cancer diagnosis and prognosis. The circulating levels of bioactive sphingolipids were found to corelate with the patient survival and treatment response in various tumor types (Sedić et al. 2019). More recently, the role of sphingolipid species in cancer progression and metastasis suggested their use as potential biomarkers in advanced ovarian cancer (Knapp et al. 2017). A comprehensive data analysis also highlighted the significance of sphingolipid metabolites as potential biomarkers in colorectal cancer (Shen et al. 2017a). These discoveries have initiated an upsurge of interest in the role of sphingolipid metabolites as biomarkers in cancer processes. Despite vast advancements in the cancer biomarker development, there is a very low (0.1%) success rate for the clinical translation (Poste 2011). More than 90% of the drugs that enter clinical development fail to reach the market approval which suggests that a large void still exists between initial discovery of a biomarker and their clinical application (Paul et al. 2010). A potential biomarker must surpass a number of obstacles prior to incorporation into routine clinical care. The most common reasons of failure in developing a robust cancer biomarker involve the methods of identification along with the successful validation (Shen et al. 2017a; Schully et al. 2015; Parkinson et al. 2014). The present chapter focuses on the rational exploitation of the sphingolipid metabolic pathways as well as the techniques that can be utilized for the identification and quantitation of sphingolipids. We have also discussed recent progress in our understanding of the appropriate statistical methods that could provide a wealth of evidence toward the use of sphingolipids in biomarker studies.

27.2 Sphingolipid Metabolism

Sphingolipids represent the largest class of bioactive lipids recently recognised as crucial regulators in cancer biology. Sphingolipids along with the cholesterol constitute components of membrane specialized domain named lipid rafts (Bieberich

2018). A large number of proteins are embedded in the lipid rafts that control various aspects of cellular activities such as cell growth, proliferation, migration, and drug resistance (Hryniewicz-Jankowska et al. 2014; Gomà et al. 2014). The basic structure of sphingolipids comprises of an18-carbon sphingoid base attached to fatty acyl group via an amide linkage. Sphingolipids are synthesized in the endoplasmic reticulum (ER) and Golgi apparatus while they travel across the organelles via transport vesicles. The de novo synthesis of sphingolipids commences inside the endoplasmic reticulum with the condensation of serine and palmitoyl CoA to 3-keto-dihydrosphingosine catalyzed by the enzyme serine palmitoyl transferase. The 3-keto-dihydrosphingosine thus formed is converted to dihydroceramide which is further converted to ceramide by desaturation (Knapp et al. 2019). Ceramide is transported via ceramide transfer protein (CERT) to Golgi apparatus where it is transformed to complex sphingolipids by the addition of various head groups (Hanada et al. 2009; Rao and Acharya 2008). Ceramide holds a central position in the sphingolipid metabolism and is considered as a metabolic hub (Hannun and Obeid 2008; Di Pardo et al. 2017). It can be synthesized either by de novo biosynthetic pathway or breakdown of complex sphingolipids (salvage pathway) (Gault et al. 2010). The salvage pathway takes place in the acidic compartments, late endosomes, or lysosomes and involves constitutive degradation of glycosphingolipids to sphingosine (Mashima et al. 2019). Sphingosine thus produced is salvaged through reacylation and is converted back to ceramide by the ceramide synthase (Kitatani et al. 2008). The salvage pathway entails the participation of large number of enzymes such as sphingomyelinases, cerebrosidases, and ceramidases. In addition to de novo and salvage pathways, ceramide can also be generated through the catabolism of sphingomyelins by acid or neutral sphingomyelinases (Marchesini and Hannun 2004).

Ceramide and sphingosine have gained significant attention in cancer research by orchestrating a number of signaling pathways. They act as potent tumor suppressors and have traditionally been associated with various aspects of cell growth, survival, and apoptosis in cancer (Che et al. 2017; Ponnusamy et al. 2010). Ceramide has earlier been reported to exhibit antiproliferative effects through apoptosis by inhibiting Akt and NFκB signaling in lung cancer H1299 cells (Lin et al. 2014). Apoptosis is identified as the major pathway adopted by the cytotoxic agents to induce cell death (Modrak et al. 2006). Most of the anticancer drugs such as, daunorubicin (Bose et al. 1995), etoposide (Perry et al. 2000), and cannabinoids (Gómez del Pulgar et al. 2002) are known to exert their effects through increased de novo ceramide synthesis. Ceramides are known to induce apoptosis by forming channels in mitochondrial membrane and causing the release of cytochrome *c (*Siskind et al. 2006*)*. Any aberration in ceramide synthesis or metabolism may thus lead to enhanced cell survival and resistance to chemotherapy in tumor cells (Morad and Cabot 2012).

Ceramide can either be converted to its phosphorylated derivative ceramide-1-phosphate (C1P) or may act as a substrate for sphingosine and sphingosine-1-phosphate (S1P). S1P is a potent pleiotropic lipid mediator involved in many physiological processes including cell survival, differentiation, and tumor

progression (Nakajima et al. 2017). It is generated intracellularly by the phosphorylation of sphingosine by the enzyme sphingosine kinase (SK)—a key regulator of the ceramide/sphingosine 1-phosphate rheostat. Most of the biological effects of S1P in carcinogenesis are attributed to the "inside-out" signaling of the molecule. S1P is produced inside the cells and is exported out of the cell via ATP-binding cassette transporters such as ABCA1, ABCC1, ABCG2, and spinster (Takabe and Spiegel 2014). Extracellular S1P binds to a family of five G-protein coupled receptors ($S1P_1$-$S1P_5$) in an autocrine or paracrine fashion and initiates a downstream cascade of signaling pathways (Fig. 27.1).

A growing body of evidence has documented the role of "sphingolipid rheostat" model in cell fate determination as well as modulation of drug sensitivity in cancer cells (Newton et al. 2015). The model was proposed in 1996 to elucidate the differential regulation of cell growth and survival by ceramide and S1P. The two bioactive metabolites are known to exert opposing effects, and the cell fate is determined by the balance between pro-apoptotic ceramide and pro-survival S1P (Newton et al. 2015; Cuvillier et al. 1996). A current study has suggested that modulation of sphingolipid rheostat can protect the human prostate cancer cell line PC3-PR against paclitaxel drug (Aoyama et al. 2017).

The metabolites generated by sphingolipid metabolism play key cellular roles both as structural components of membranes as well as signaling molecules. These compounds are anticipated to participate in tumor cell growth, differentiation, apoptosis, resistance to chemotherapy, and various other cellular aspects (Voelkel-Johnson et al. 2018; Hannun and Obeid 2018). The above findings suggest that a deep understanding of the sphingolipid metabolism and associated pathways can provide rare insights in identifying key players in cancer etiology.

27.3 Metabolomic Approaches for the Sphingolipid Analysis

Metabolomics is a recently emerged branch of 'omics' that can be employed to study the metabolic aberrations in sphingolipids. It refers to the comprehensive analysis of the metabolic products (metabolome) in a biological specimen (cell, biofluids, tissues, organs, or organisms) (Clish 2015). A number of studies have predicted the role of sphingolipid metabolism in cancer employing this technique (Knapp et al. 2019; Sullards et al. 2003; Shen et al. 2017b). Metabolomics not only allows the identification of metabolites but also helps in recognizing the classifiers between normal and diseased states (Dougan et al. 2018). The technique also extends opportunities to discover novel predictive biomarkers and therapeutic targets by providing the insights into deregulated tumor metabolism (Kaushik and DeBerardinis 2018). Metabolomic approach was employed to discover the role of sphingolipid metabolism in cell proliferation and screen novel biomarker for epithelial ovarian cancer detection (Kozar et al. 2018). The significance of sphingolipids as potential sensitive and specific biomarkers for lung cancer diagnosis and prognosis was also established using serum metabolomic studies (Chen et al. 2015).

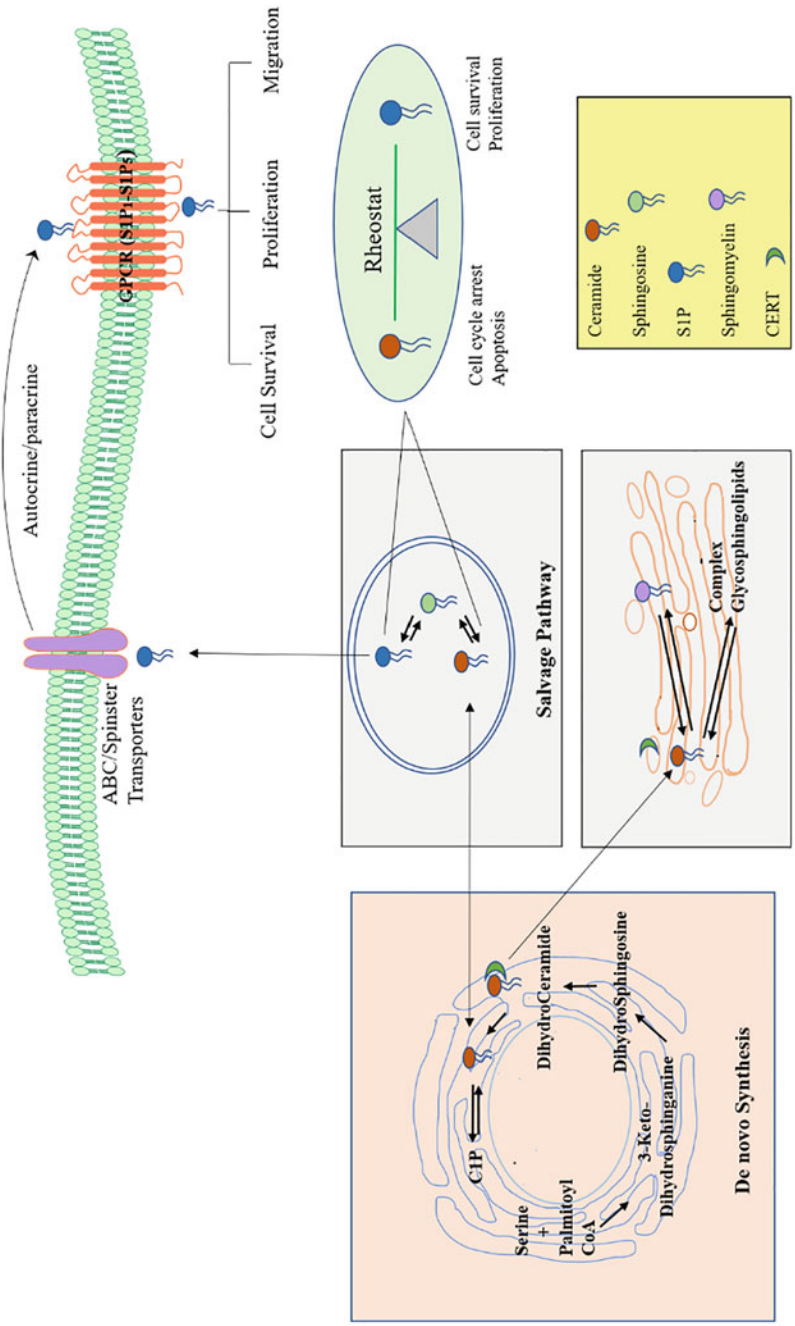

Fig. 27.1 Representation of the sphingolipid metabolism and its significance in cancer pathways

The two main techniques that can be employed for the sphingolipid metabolic profiling include nuclear magnetic resonance (NMR) and mass spectrometry (MS) (Fuhrer and Zamboni 2015). A number of studies have demonstrated the significance of NMR and MS in metabolic pathway disruption studies in various cancers (Cheng et al. 2005; Chan et al. 2009). The choice of metabolomic platform however, depends primarily on objective of the study, nature of sample, and availability of resources (Emwas 2015).

27.3.1 NMR

NMR is vital analytical technique that can be employed to identify and quantify molecules in complex mixtures by measuring the interaction of nuclear spins in a magnetic field (Martin-Pastor 2014). Though recent advances in analytical techniques and instrumentation have shifted the focus of metabolomic studies toward the use of MS methods (covering around 80% of the published studies), the NMR technique is still of considerable interest. It offers several advantages over MS as it is noninvasive, highly selective and requires little or no sample preparation and chromatographic separation. The method is highly reproducible and can easily characterize sugars, alcohols, organic acids, polyols, and other highly polar compounds that are less amenable to MS detection (Emwas et al. 2019). Further, nondestructive nature of NMR makes it an ideal approach for the real-time metabolite profiling of live cells, which cannot be accomplished with MS methods due to their destructive nature. One negative aspect of NMR over MS, however, is that it has limited specificity which makes it difficult to determine the less abundant metabolites (Veenstra 2012). A plethora of studies have utilized NMR to determine the metabolic profiles of tumor tissues, biological fluids, and cell extracts (Kaplan et al. 1997; Li and Deng 2016; Ramachandran et al. 2016). A recent study has used NMR-based metabolomic studies to monitor real-time dynamic changes in serum metabolome of head and neck squamous cell carcinoma (HNSCC) patients during radio/chemotherapy (Boguszewicz et al. 2019). Another report described the metabolic changes in the serum of colorectal polyps and colorectal cancer patients using the NMR-based metabolomics (Gu et al. 2019). NMR metabolomics was also used as a diagnostic tool to discriminate preinvasive colorectal neoplasia (CRN) and colorectal cancer (CRC) patients from healthy individuals (Kim et al. 2019). The significance of SK1 axis in metabolic reprogramming of A270 ovarian cancer cells was also highlighted using NMR-based metabolic profiling (Bernacchioni et al. 2017). The technique is being widely utilized to screen novel biomarkers with promising ability in disease diagnosis, prediction, and treatment (Zikuan et al. 2019). A recent study has utilized urine-based NMR profiling as a cost-effective approach for the early detection and risk stratification of prostate cancer (Yang et al. 2017a). NMR-based metabolomic profiling was also used to identify 32 potential biomarkers involved in several metabolic processes including sphingolipid metabolism from serum samples of hepatocellular carcinoma and liver cirrhosis patients (Liu et al. 2014).

27.3.2 Mass Spectrometry

Mass spectrometry is a high-throughput analytical technique used for the structure elucidation and qualitative as well as quantitative identification of the biomolecules (Mittal 2015; Che et al. 2015). It ionizes the samples according to their mass-to-charge ratio, and the resulting mass spectrum is used to calculate exact molecular weight of the compounds. MS is characterized by very high sensitivity and thus has specific advantage of being more useful than NMR in identifying low concentration molecules (Mal et al. 2012). High degree of sensitivity and reliability makes MS-based metabolomics a widely utilized technique in clinical research (Kennedy et al. 2018). However, few disadvantages also exist for MS including sample preparation which may lead to some degree of metabolite loss and high cost (Wang et al. 2010). Moreover, the method is destructive to the samples and hence cannot be utilized for live cell studies.

MS is integrated to a separation step based on some chromatographic technique such as gas chromatography (GC-MS) or liquid chromatography (LC-MS) that extensively improves the accuracy, speed, and sensitivity of the technique (Silva et al. 2019). GC-MS is mainly employed for volatile and less polar compounds such as fatty acids, alcohol, terpenes, and organic acids. The method is highly favored for its robustness and reproducibility and thus has become a valuable tool in metabolomics (Kennedy et al. 2018; Ramautar 2016). However, GC-MS has a limited specificity in the identification of compounds since it has a restricted mass range and it is often necessary to derivatize the samples. On the other hand, LC-MS is well suited for the polar, nonvolatile, and thermolabile compounds such as proteins, polymers, and peptides (Goodacre et al. 2004). It is the most popular and accepted technique for the analysis of sphingolipids and other high molecular weight compounds (Ogiso et al. 2014; Wu et al. 2014). The method is highly versatile enabling the identification of wide variety of molecules and thus is predominantly used in metabolomic studies (Wang et al. 2010). Further, unlike GC-MS sample dativization is not required for LC-MS, hence avoiding the problems associated with high heat conditions (Perez et al. 2016).

A mass spectrometer mainly consists of an ion source, mass analyzer, and a detector. The ion source is used to convert the molecules to gas phase ions which are moved through and manipulated by electromagnetic fields. The commonly used ion sources in MS include electron impact ionization (EI), atmospheric pressure chemical ionization (APCI), electrospray ionization (ESI), and matrix-assisted laser desorption/ionization (MALDI) (Lee et al. 2010). Mass analyzers resolve the ions into their characteristic masses and then separate the ionized analytes on the basis of their mass-to-charge ratio. The selected ions (precursor ions) can further be fragmented to product ions by a second mass analyzer—a technique known as tandem MS or MS/MS. There are many types of mass analyzers employed in metabolomic including quadrupole, ion trap, time of flight (TOF), Fourier transform (FT) MS, and Orbitraps (Wang et al. 2010). The detection system measures the separated ions and their relative abundance thus leading to the formation of mass

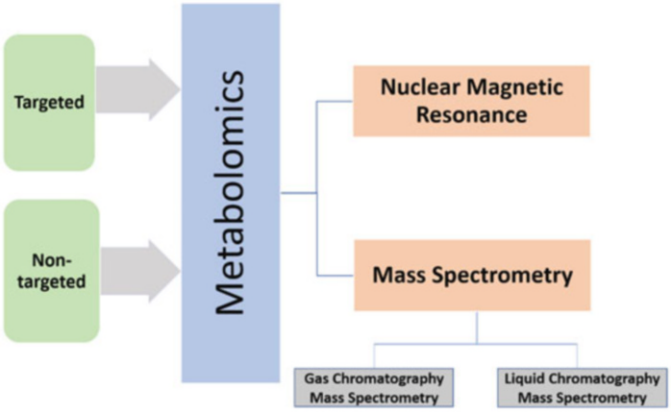

Fig. 27.2 Types of analytical approaches employed for metabolic profiling

spectrum. A mass spectrum is a two-dimensional plot representing the ion abundance versus mass-to-charge ratio.

MS has evolved as an attractive metabolomic tool for identifying biomolecular changes linked to disease progression, diagnosis, and prognosis (Zhang et al. 2020). It poses a substantial impact in cancer biology by covering various spheres of cancer research. In a previous study, we reported an elevation in the levels of sphingolipid metabolites in breast cancer patients employing LC-MS-based metabolomics (Bhadwal et al. 2020). A similar approach was also utilized to identify differential metabolic biomarkers of metastatic colorectal cancer (Martín-Blázquez et al. 2019). Alteration in sphingolipid levels between human ovarian cancer cell line A2780 and its Taxol-resistant strain A2780T (Huang et al. 2016) as well as human lung adenocarcinoma cell line A549 and its Taxol-resistant strain A549T (Huang et al. 2018) were determined using LC-MS-based metabolomics. The method was also used to delineate the association of high plasma concentration of S1P and total ceramide with increased future risk of lung cancer (Alberg et al. 2013). The sphingomyelins and the enzymes involved in sphingolipid metabolism were identified as potential prognostic marker and therapeutic targets in triple negative breast cancer patients employing LC-MS lipidomics profiling (Purwaha et al. 2018).

Perturbations in the sphingolipid metabolic pathways of an organism can be determined using two primary analysis platforms—non-targeted metabolomics and targeted metabolomics (Fig. 27.2). While the non-targeted approach involves broad metabolomic coverage and helps in the global metabolome profiling, targeted approach is more useful to investigate specific metabolic pathways or to validate classifier metabolites already determined using non-targeted approach (Zhang et al. 2016).

Non-Targeted Approach

Non-targeted metabolomics is a comprehensive metabolome profiling that helps to capture as many metabolites as possible in a single analysis. It involves full scanning

of the metabolome and allows the identification of novel biomarkers (Ribbenstedt et al. 2018). The method can be used for the relative quantification of the sphingolipid metabolites. The technique was employed to identify >900 metabolite ions in breast cell lines under both under both normoxic and hypoxic conditions (Dubuis et al. 2017). In a previous study, we have also used a non-targeted approach to identify novel sphingolipid biomarkers in breast cancer patient samples (Bhadwal et al. 2020). Metabolic alterations were also evaluated in lung cancer patients using both non-targeted and targeted metabolomic approach, and a total of 25 metabolites differentially regulated between lung cancer patients and healthy controls were identified (Zhang et al. 2016).

Targeted Approach

Targeted metabolomics involves multiplexed analysis and validation of the identified metabolites. The method can be owned for the absolute quantification of the sphingolipids using a standard of known concentration. However, one main drawback of the targeted approach is that it covers limited metabolome which may lead the researchers to overlook the metabolite of interest (Ribbenstedt et al. 2018). The method was exploited to create a model for lung cancer screening using serum amino acids and acylcarnitines (Ni et al. 2019). Targeted metabolomics was employed to investigate the tissue metabolome associated with low- and high-grade serous epithelial ovarian cancer (Garg et al. 2018). The association between metabolic deregulation and pancreatic cancer in postmenopausal women was established using targeted serum metabolomics (Jiao et al. 2019). The method also revealed potential serum biomarkers by determining the difference in metabolic concentrations of ovarian cancer and benign ovarian tumor in comparison to healthy controls (Plewa et al. 2019).

Metabolomics is a rapidly maturing technique that can provide rare insights to many facets of sphingolipid metabolism in cancer research. However, generation of immensely high information content by such techniques poses a data analysis challenge. Therefore, integrating the approach of metabolomics with pertinent statistical approaches is mandatory for the felicitous interpretation of data.

27.4 Statistical Approaches Employed for the Metabolomics Data

The selection of appropriate statistical measures is very crucial in the analysis of data and need to be elected suitably as this may affect the integrity of the data. The validation of a biomarker seeks remarkable perception of the statistical concerns in order to assure the reproducibility of the studies (Ensor 2014). The use of sound statistical principles may help in minimizing bias and maximizing the precision in biomarker validation studies (Gosho et al. 2012). The current chapter summarizes the choice of suitable statistical approach that can be employed to translate the high dimensional sphingolipid metabolomics data and extract the valuable information needed for biomarker exploration. Further, suitable efforts have been made to

incorporate the wide range of data analysis methods covering from simple univariate analysis to complex multivariate analysis methods.

27.4.1 Univariate Analysis

Univariate analysis is the simplest statistical method employed in metabolomic study for the evaluation of a single variable (Canova et al. 2017). It is generally used to infer a relationship between a dependent and an independent variable. The statistical methods commonly employed for univariate analysis in metabolomics include student's t-test, Mann-Whitney U test, analysis of variance (ANOVA), and receiver operating characteristics (ROC) analysis (Nagana Gowda et al. 2017; Bartel et al. 2013) (Fig. 27.3). The student's t-test and Mann-Whitney U test both are used to predict the probability of a particular biomarker to discriminate between normal and the diseased groups. Of the two, the student's t-test is widely used test and is based on the assumption that the data is normally distributed among the two groups. However, when the above assumption is in doubt, which is mostly the case with the metabolomics data, Mann-Whitney U test is used as an alternative for student's t-test.

ANOVA is an analysis tool used in statistics to study the difference between two or more means. It is simple, popular, and most frequently used statistical method to test the experiments involving two or more groups (Smalheiser and Smalheiser 2017). There are generally two main types of ANOVA—one-way ANOVA and two-way ANOVA. The one-way ANOVA is used to compare the means of two or more samples based on one independent variable. The two-way ANOVA is an extension of one-way ANOVA that determines the influence of two independent variables on one dependent variable. It compares the effect of multiple levels of two independent factors. ANOVA was used to establish the correlation between prognosis of gastric adenocarcinoma patients and tumor diameter, regional lymphatic metastasis, vascular invasion, and pTNM stages (Liu et al. 2017). The method was employed to study the effects of dietary behavior on the risk and incidence of breast cancer (West 2018). ANOVA was used to determine alterations in sphingolipid signaling in the immune cells of breast cancer patients and identify target pathways

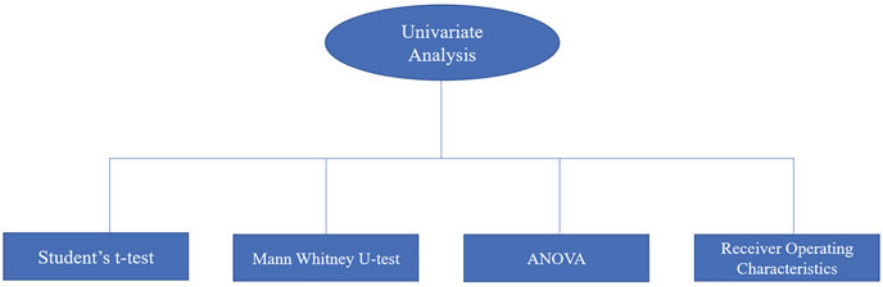

Fig. 27.3 Various statistical approaches employed for univariate analysis

for breast cancer therapeutics (Maia et al. 2017). The methods also helped to discover the variations in specific sphingolipid metabolites during staurosporine-induced apoptosis in colon cancer cells (del Solar et al. 2015).

Receiver operating characteristic (ROC) curve is a performance measurement to quantify how accurately a classifier biomarker can discriminate between the two patient states. The performance evaluation of a biomarker however, requires the assessment of sensitivity and specificity. The sensitivity is considered as the probability of identifying a true positive when the outcome is actually positive, and the specificity identifies a true negative when the outcome is actual negative. ROC curve is a plot of the sensitivity and specificity which is often summarized into a single metric known as area under curve (AUC). The AUC value is a measure of the extent of separability of the biomarker and has a value ranging from 0 to 1. Generally, an AUC score of 0 indicates that biomarker has a no separation ability, while the score of 1 suggests that biomarker is the best measure of separability among the two groups (Xia et al. 2013).

The ROC analysis is being widely employed in clinical epidemiology for the prediction of a biomarkers and its diagnostic potential. The ROC analysis has been used to determine the diagnostic accuracy of serum and urine cytokeratin fragment-19 (CYFRA 21-1) in bladder cancer diagnosis (Fu et al. 2018). A recent study has also used the ROC analysis to determine the clinical relevance of cholesterol homeostasis genes in colorectal cancer patients (Sharma et al. 2019). Another report has suggested the use of serum sphingolipids as cholesterol-independent biomarkers in coronary artery disease classification by evaluating their ROC scores (Poss et al. 2020). ROC curve analysis was employed to identify sphingosine as potential serum biomarkers which could distinguish lung cancer patients from the normal subjects (Chen et al. 2015). Previous report by us also suggested the diagnostic potential of sphingosine-1-phosphates and ceramide-1-phosphates in breast cancer patients using individual and cumulative ROC analysis (Bhadwal et al. 2020). Individual and cumulative ROC analysis has also earlier been used to study the interaction between meiotic recombination 11 homolog A (MRE-11) and breast cancer recurrence status (Yang et al. 2017b). A cumulative ROC also resulted in a significant improvement in the ability of serum carcinoembryonic antigen (CEA) plus microRNA-17-3p to discriminate colon cancer patients from healthy controls (Zhu et al. 2015). The cumulative ROC extends over individual ROC in its ability to use two or more variables as discriminants on a shared basis (deCastro 2019). Cumulative ROC was employed to analyze the interaction between tissue visfatin and clinicopathological characteristics in breast cancer patients (Moi et al. 2018).

A number of metabolomic studies, particularly in cancer, are designed with the objective of identifying classifiers capable of discriminating between normal and diseased states. Univariate analysis was used to assess the characteristics variations among the breast cancer patients who had survived or not survived the disease (Momenyan et al. 2018). Another study in breast cancer has also utilized the univariate analysis to study the effect of clinicopathological characteristics on survival time analysis (Baghestani et al. 2015). Univariate analysis has also been

employed to establish the association of high Ki67 score with reduced cancer-specific survival in endometrial cancer (Kitson et al. 2017).

Although univariate analysis models had a good predictive efficiency in all the above studies, multivariate analysis proved be a more appropriate method. This can be due to the fact that outcome in patient studies is affected by various factors such age, stage, and other clinicopathological characteristics, and the compared studies of univariate and multivariate analysis with these confounding factors may end up with different outcomes. Thus, univariate analysis could help with a lead in the selection of suitable biomarkers which further need to be validated by multivariate approach.

27.4.2 Multivariate Analysis

Multivariate approach involves the handling of complex data sets comprising of more than one variable (Hair 2011). The method is employed to discover comparison or association between a dependent and one or more independent variables. A recent study has suggested multivariate approach as more effective than univariate analysis in order to assign biological roles to the genes of unknown function (Gorfine et al. 2015). Most common statistical methods employed for the multivariate analysis include regression analysis, multivariate analysis of variance (MANOVA), correlation analysis, principal component analysis (PCA), partial least square (PLS) regression, and hierarchical cluster analysis (HCA) (Fig. 27.4).

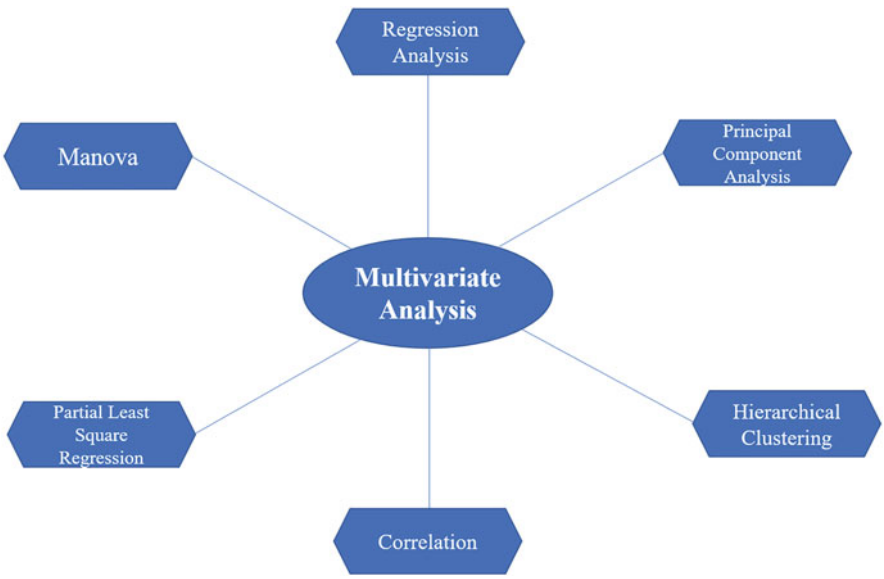

Fig. 27.4 Various statistical approaches employed for multivariate analysis

1. The regression analysis is used to assess the strength of association between a dependent and one or more independent variables. This statistical measure is a way of sorting out the biomarkers that matter the most, the biomarkers that can be ignored, and the impact two biomarkers have on each other. Although there are several different types of regression models, the two most common types employed in metabolomics include linear regressions and logistic regressions. The linear regression model is used to predict outcome for a continuous dependent variable using the independent variables. The linear regression is classified as simple regression analysis or multiple regression analysis depending upon the number of independent variables used for the prediction (Denis 2018). Logistic regression is mainly used for the classification problems where the probability between two classes is required, for example, whether or not, true or false. It is used for the prediction of categorical dependent variable with the help of independent variables. The logistic regression is very much similar to multiple linear regression except for the difference that response variable is binominal (Sperandei 2014). The regression model was employed to identify miRNA and their link to a set of target genes involved in CRC microsatellite instability (MSI) and chromosomal instability (CIN) signaling pathways (Wang et al. 2014a). Regression model was also adopted to predict the response to multidrug regimens in cancer patients using cell line experiments (Falgreen et al. 2015). Multivariate linear regression analysis was employed to identify the significant predictors and determine the relationships between food intake variables and human serum sphingolipid levels (Abnet et al. 2001). The logistic regression was used to establish the association of lung cancer risk with between plasma concentrations of ceramides and S1P (Alberg et al. 2013).
2. Multivariate analysis of variance (MANOVA) is simply an ANOVA with several dependent variables. It can measure several dependent variables in a single experiment and find the differences not revealed by ANOVA. While ANOVA is used to compare the difference between the means of two or more groups, MANOVA is used to study the difference between two or more vectors of means. The test has been used to analyze the multivariate data in a number of applications. The method is proposed for the gene set analysis in the studies involving two or more experimental conditions (Tsai and Chen 2009). The clinical factors that were most strongly associated with grouping of nonmalignant and tumor miRNA expression in lung cancer were determined using MANOVA (Vucic et al. 2014).
3. Correlation coefficient is also used in statistics to measure the strength and direction of relationship between two variables. The value of correlation coefficients lies in the range of -1 to $+1$. The closer the correlation coefficient approaches the value ± 1, the stronger the correlation among variables. A value of 0 indicates no correlation, while the $+1$ indicates strong positive, and -1 indicates strong negative association between the variables. The two main types of correlation coefficients include Pearson correlation coefficient and Spearman's rank correlation coefficient (Mukaka 2012). The Pearson coefficient is used to determine the correlation when both variables being studied are normally

distributed. However, if one or both variables do not follow normal distribution, Spearman's correlation is more appropriate to use. The multivariate extension of correlation is known as canonical correlation analysis (CCA). It helps to determine the linear combinations of variables that maximally correlate by exploring the association between two multivariate sets of variables (Thompson 2007). A number of clinical studies have employed the correlation coefficient to decipher the association among different parameters. A previous observation suggested that the expression of three cancer-related genes *PIK3C3*, *PIM3*, and *PTEN* was correlated in various cancers and could be used as an indicators of early cancer diagnosis (Ling et al. 2014). A significant positive association between plasma ceramide levels and cancer progression was observed in ovarian cancer patients (Knapp et al. 2017). Another study in ovarian cancer reported strong correlations between sphingolipid regulatory networks and tumor immune microenvironment (Meshcheryakova et al. 2019).

4. Principal component analysis (PCA) is a method that reduces the data variability and increases the interpretability while keeping the information loss to a minimal (Ringnér 2008). It minimized the number of predictive variables and look for the combinations that can abridge the data. The method converts a set of possibly correlated variables into a set of linearly uncorrelated variables known as principal components (PCs). The first principal component has the largest possible variation, and the variance decreases with each succeeding component. Generally, the first four components are considered valuable as the components of higher order are assumed to contain irrelevant information or high noise level (Butte 2002). PCA is a descriptive method that can be used to visualize the relatedness between populations and thus making the predictive models. Further, it can be applied to data of various types as it does not consider the distributional assumptions (Jolliffe and Cadima 2016). The method was employed to elucidate the metabolic differences between human rectal cancer biopsies and colorectal xenografts (Seierstad et al. 2008). PCA has also been reported to reduce multidimensionality of the data and obtain a clear separation between non-small cell lung cancer patients and healthy controls (Chen et al. 2018). The principal component scatter plots show the extent of correlations among two variables by arranging the variable points on X-Y axis (Salkind 2010). The better the correlation, the tighter the points will concentrate near a straight line. If no correlation exists between the variables, the points will fall apart and appear randomly scattered. The scatter plot diagram has been used earlier to reveal a positive association of cancer stage and grade with red cell distribution in renal cell carcinoma (Wang et al. 2014b). Scatter plots were also used to validate the role of miR-146a in melanoma migration, proliferation, and mitochondrial fitness within the STAT1/ IFNγ axis (Mastroianni et al. 2019). The differential expression of 52 sphingolipid biomarkers between A2780 and A2780T cell lines of ovarian cancer was identified employing scatter plots (Huang et al. 2016).
5. Partial least square (PLS) regression is a statistical method used to construct predictive models. The method combines the characteristics of PCA and multiple regression analysis. A general limitation of the PCA technique is that it does not

allow to determine the association between predictive or independent variable and the target or dependent variable. PLS helps to bridge this gap by finding the linkage between the two and thus can be utilized as an alternative to the PCA approach. PLS-based method has been reported earlier to produce better results in ovarian cancer classification problems than PCA-based methods (Boulesteix et al. 2008). PLS-DA was also found to produce better separation results between control group and CRC patient groups in comparison to the PCA (de Figueiredo Junior et al. 2018). Classification with PLS is termed as PLS-DA where DA is termed as discriminant analysis. PLS-DA is a classification model used to sharpen the variations between the different observation groups (Lee et al. 2018). The variable responsible for the separation among the groups is identified using variable importance in projection (VIP) score. The VIP score provides a quantitative estimation of the variable's discriminatory power. In general, VIP values of 0.7–0.8 have been considered acceptable for the variable selection. The predictor variable having VIP $>$ 1 is considered as the most influential to the group separation (Eriksson et al. 2013). The VIP score was used to identify T, N, and TNM stage as important factors for miRNA 106b~25 expression both in plasma and tumor tissues in gastric cancer (Li et al. 2019). A total of ten potential metabolic biomarkers including sphingosine were identified for lung cancer diagnosis using PLS-DA approach (Chen et al. 2015). PLS-DA-based VIP score $>$ 1.5 was used as a basis for the selection of 18 potential serum metabolic biomarkers that may be useful in the clinical diagnosis and treatment of ovarian cancer patients (Yang et al. 2018).

6. Hierarchical cluster analysis (HCA) is a method that sorts similar variables into groups in such a way that the variables within each cluster are broadly alike. The final results of each hierarchical clustering are represented as a tree, called as dendrogram. The HCA is mainly divided into two types: divisive or top-down clustering and agglomerative or bottom-up clustering. In divisive clustering, all observations start within single cluster and are split while moving down the hierarchy. In agglomerative method, each observation starts in a separate cluster, and the pair of clusters unites, while one moves up the hierarchy (Nielsen 2016). The agglomerative clustering is the more common type of the two and is more frequently employed in statistical analysis. Classification of the tumor-infiltrating immune cells using HCA helped identifying ovarian cancers with poor prognosis and high levels of COX expression (Liu et al. 2009). HCA method was used to identify the patients with more aggressive phenotype in metastatic colorectal cancer (Opinto et al. 2017). Agglomerative clustering has recently been used to identify the cancer subtypes form the omics data in a variety of cancers (Nidheesh et al. 2018). A common way to visualize the HCA data is the generation of heatmaps. A heatmap is a graphical representation of the data that uses a color coding system to depict the magnitude of a phenomenon. In heatmap, the values are displayed in different colors that vary in intensity over the different values. The variation in color intensity gives a visual cue about how the values are clustered. The data in heatmap is represented in grid where rows and columns delineate the phenomena and categories, respectively (Qu et al. 2019). If there are

large number of data points that need to be plotted, a heatmap can be a used as good substitution to the scatter plots in order to avoid over-density issues. They help to understand large volumes of complex data sets by providing a visual summary (Lee et al. 2017). Heatmaps were used as a way of representing multidimensional cancer omics data, where rows and columns were clustered according to the molecular or clinical features (Schroeder et al. 2013). A previous study has used the heatmap to discover the biomarkers in human breast cancer tissues by clustering the genes identified by gene expression profiling into various groups (Fu et al. 2014). Heatmap was also used to categorize the serum sphingolipids such as ceramides, sphingomyelins, and hexosylceramides from the CRC patients and normal controls into positively and negatively corelated clusters (Separovic et al. 2017).

27.4.3 Survival Analysis

Survival analysis is another branch of statistics involving both univariate and multivariate approach. It determines the occurrence and timings of an event in a particular population under study (Tolley et al. 2016). The time interval from the beginning of an observation period to occurrence of an event such as death, disease occurrence, relapse, cure, or equipment failure is called as survival time. The methods commonly employed in survival studies include Kaplan–Meier (KM) plots, log rank tests, and Cox proportional hazards model (Hoffman and JIE 2019). Kaplan–Meier plot is defined as a measure of the patient's survival over time. It computes the probability of occurrence of an event over certain point of time (Rodrigues et al. 2018). The log rank test is employed to compare the survival curves between two or more independent groups (Johnson 2018). Generally, the survival curves are prepared for each group separately using Kaplan–Meier plots, and then intergroup comparisons are done using log rank tests. Both the above tests are examples of univariate analysis and are used only when predictor variable is categorical. These methods do not qualify for quantitative predictor variables such as age and weight. Therefore, a method that covers both categorical variables and quantitative predictor variables is required. The best alternative method that can be applied to investigate the effect of several variables on a specified event occurrence is Cox proportional hazards regression model. It is the most commonly employed multivariate approach to review the survival time data in medical research (Bradburn et al. 2003). Cox proportional hazards regression model adjusts for both categorical and quantitative prognostic factors and thus provides an estimate for the treatment difference. The method has been used to evaluate the association between different treatments and survival time in breast cancer patients (Abadi et al. 2014). Kaplan–Meier method and Cox proportional regression analysis were used respectively to study the survival probability and hazards ratios (HR) in hypopharyngeal cancer patients (Krishnatreya et al. 2019). Log rank tests were used to determine the importance of ceramide-metabolizing enzymes in colorectal cancer cell proliferation and predict their role in patient survival (Tang et al. 2017). Kaplan–Meier analysis

revealed a positive association of S1P receptor S1P1 and a negative association of S1P2 with patient's survival time in glioblastoma multiforme (GBM) patients (Bien-Möller et al. 2016). Another study found that an increased accumulation of sphingoid base intermediates and a decreased ceramide production are linked to better survival outcomes in triple negative breast cancer patients (Purwaha et al. 2018).

Each multivariate analysis technique mentioned above has its own merits and shortcomings and is suited for a specific scientific quest. Therefore, it becomes imperative to have a better understanding of these statistical methods before making a choice regarding the interpretation of the data and selection of a biomarker.

27.5 Conclusion

Biomarker discovery has recently become a major thrust area in the field of cancer treatment. Cancer biomarkers have revolutionized the treatment of various cancer types by playing an increasingly important role in risk assessment, diagnosis, and prognosis of the disease. The clinical utility of cancer biomarkers however is limited, and only 5% of the oncological drugs entering the clinical trial have been successfully commercialized. The principal reason underlying the restricted use of a cancer biomarker is the lack of coherent and comprehensive biomarker discovery methods. Following discovery phase, another major factor that influences the outcome of a biomarker study is the precision and reproducibility of the reported findings. Numerous studies have highlighted the importance of technical robustness of the statistical procedures to enable a fair identification and evaluation of the reported biomarkers. Current progress in lipidomics has expanded the knowledge on the role of sphingolipids in cancer studies. Sphingolipids are a diverse class of lipids whose metabolic products not only participate in cancer signaling pathways but also may serve as potential diagnostic as well as prognostic cancer biomarkers. However, limited information is available on the methods of choice for the identification of sphingolipids. Existing literature also reflects the need to understand suitable data analysis methods in order to establish the role of sphingolipids in biomarker studies. Acknowledging the abovementioned challenges may help to streamline the process of biomarker study. The present chapter illuminate the role of sphingolipid metabolites in cancer as well as the metabolomic approaches that can be employed for the sphingolipid identification. We have also made an attempt to address the fundamental statistical concepts that must be discerned in order to address the critical flaws in the data analysis methods and establish the association of identified sphingolipid biomarkers with clinical characteristics.

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Future of Gastrointestinal Endoscopy

28

Surinder Singh Rana

Abstract

The field of medical gastroenterology has come a long way from the era of x-rays and empirical diagnosis to an era of therapeutic gastrointestinal endoscopy (GIE) and precision medicine with accurate diagnosis. GIE is an important component of gastroenterology clinical practice with optical innovations like high-definition endoscopes, magnification endoscopy and optical imaging adjuncts such as narrow band imaging and confocal laser endomicroscopy having transformed an endoscope into a miracle tool. The advent of endoscopic ultrasound, an amalgamation of endoscope and ultrasound technology, has further revolutionised GIE. Development of wireless capsule endoscope has made possible to visualise the unexplored blind areas of small intestine, and lot of efforts are underway to develop a therapeutic capsule endoscope that may make possible remotely controlled endoscopic surgeries. The future of GIE appears to be like a fiction movie with advancements in three-dimensional imaging, computers, electronic platforms, artificial intelligence, biomechanical engineering and molecular imaging driving GIE to an altogether different unbelievable level. The field of third space endoscopy is another promising innovation in the field of endoscopic surgery that provides endoscopic access to the enteric nervous system and thus plethora of possibilities to treat various gastrointestinal motility disorders. Computer and robotically assisted endoscopic surgery platforms of future will further enhance the therapeutic capabilities of endoscopists. This brief review discusses the probable future of GIE and how it is going to transform the practice of gastroenterology.

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Endoscopy · Endosonography · Third space endoscopy · Surgery · Capsule endoscopy

28.1 Introduction

The field of medical gastroenterology has come a long way from the era of x-rays and empirical diagnosis to an era of therapeutic gastrointestinal endoscopy (GIE) and precision medicine with accurate diagnosis. Over last several decades the GIE has transformed from a cumbersome and difficult procedure for both the patient and doctor to a highly accurate and comfortable procedure. Because of these advancements the field of endoscopy has entered into the arena of therapeutics and has gradually replaced invasive surgical procedures with safe and minimally invasive endoscopic interventions. The GIE is now an important component of gastroenterology clinical practice with optical innovations like high-definition endoscopes, magnification endoscopy and optical imaging adjuncts such as narrow band imaging and confocal laser endomicroscopy having transformed an endoscope into a miracle tool. The advent of endoscopic ultrasound (EUS), an amalgamation of endoscope and ultrasound technology, has further revolutionised GIE taking it into an altogether different level where the organs not visible on endoscopy can be visualised by EUS. Thus, these innovations over last few decades have transformed GIE from simple diagnostic tool to interventional tool and subsequently to an advanced diagnostic tool that uses adjunctive image enhanced technology to establish diagnosis without need of biopsy. Development of capsule endoscope has made possible to visualise the unexplored blind areas of small intestine, and lot of efforts are underway to develop a therapeutic capsule endoscope that may make possible remotely controlled endoscopic surgeries. These wonderful innovations have made possible to see the gastrointestinal tract just like the skin. The future of GIE appears to be like a fiction movie with advancements in three-dimensional imaging, computers, electronic platforms, artificial intelligence, biomechanical engineering and molecular imaging driving GIE to an altogether different unbelievable level. This brief review discusses the probable future of GIE and how it is going to transform the practice of gastroenterology.

28.2 Historical

The GIE developed from an inherent human desire to see within the living human body in real time to diagnose as well as treat diseases. The early innovators and fathers of GIE had to face two difficult problems to achieve the goal of seeing inside the gastrointestinal tract in real time (Sivak Jr. 1994, 2006). These were:

1. The gastrointestinal tract is not a straight tube, and therefore one has to develop a device that can negotiate the curves and bends of the human gut.
2. There is no light inside the gut, and therefore to see inside one had to develop a system that can illuminate inside the gut.

The first gastroscopy was done *by Adolf Kussmaul and Julius Müller using reflections from mirrored candlelight to see the human oesophagus and stomach, and this was probably the starting point of development of GIE. This was followed in 1881 by two Austrian Surgeons J*ohann von Mikulicz-Radecki and Viktor von Hacker who used platinum loop as a direct light source at the tip of their instrument that glow with an external battery source. The development of endoscopes was accelerated by the invention of rubber and the incandescent lamp. The first semi-flexible endoscope was developed in 1932 by an instrument maker, Georg Wolf, and a physician, Schindler. The flexible part of endoscope consisted of a rubber-coated steel wire spiral, and inside it were 51 converging lenses (Schäfer and Sauerbruch 2004; Modlin and Farhadi 2000; Gordon and Kirsner 1979). This semi-flexible endoscope remained in practice for almost 25 years, and then a path-breaking discovery of image transmission using flexible quartz fibres was demonstrated. Along with this, availability of highly transparent optical quality glass made possible the development of fibre-optic endoscope in 1958 by Larry Curtiss, a graduate student in physics, and Basil Hirschowitz, a trainee in gastroenterology (Hirschowitz et al. 1958).

Over the next six decades, this fibre-optic endoscope has undergone phenomenal transformation that is unbelievable. Invention of charge-coupled device (CCD) that produced an electronic image in 1969 led to its incorporation of this path-breaking discovery into the flexible endoscope (Sivak Jr. 1994, 2006). In 1979, this led to transformation of flexible endoscope to video endoscope with images being transmitted onto television monitor. This was followed by a golden era of GIE where the images were digitalised and an interface was developed between the endoscope and computer leading on to unbelievable images of gastrointestinal tract. Image enhancement techniques like high-resolution images, chromoendoscopy, narrow band imaging, endocytoscopy and confocal laser endoscopy have revolutionised endoscopic imaging, and the early innovators, who were struggling to see the dark and tortuous gut, could have ever imagined that the technology would reach such a level that an individual living cell of the gut could be seen in real time one day. Development of these advanced endoscopes led on to development of various interventional procedures like endoscopic retrograde cholangiopancreatography, colonoscopy, cholangioscopy and pancreatoscopy. As evident from the above discussion, these innovations in GIE happened because of close collaborations between physicians and engineers with the incorporation of technology from other fields. The future technologies in GIE will also probably develop from close collaborations as well as borrowing from other scientific fields.

28.3 Major Techniques/Important Observations

After the development of video endoscopy, a number of pathbreaking innovations have happened in the field of GIE (Pasricha and Motamedi 2002; Braden et al. 2019; Riemann 2004; Pasricha 2013). These innovations are going to further improve in future leading on to better diagnostic and therapeutic tools. Although surgery has seen a great revolution following the advent of laparoscopic surgery, better endoscopes and accessories will propel the field of endoscopic surgery to newer heights, and the future seems to be an era of minimally invasive endoscopic surgery. EUS, another important innovation, has markedly improved over last one decade, and in the future better technologies like three-dimensional EUS, contrast EUS and elastography are going to further improve and expand the role of this technology. Capsule endoscopy is a technique that seems to be straight from a fiction book, and therapeutic capsule endoscope seems to be a future. Therapeutic endoscopy has also markedly improved with development of various ablative technologies and newer advanced therapeutic accessories. Along with these future prospects, the field of GIE also faces various challenges. The evolution and advancement in radiological imaging especially virtual imaging is going to challenge the field of diagnostic endoscopy. Development of magnetic resonance cholangiopancreatography (MRCP) is a classical example which led to obsolescence of diagnostic endoscopic retrograde cholangiopancreatography (ERCP).

28.3.1 Capsule Endoscopy and Wireless Endoscopy

Capsule endoscopy is an example of development of disruptive technology that has revolutionised the field of diagnostic endoscopy. It has considerable advantages over conventional fibre-optic video endoscopes including visualising the entire gut without any pain/discomfort or sedation and has very high patient compliance. There are already studies that are reporting that capsule colonoscopy is as good as video colonoscopy, and in the future, it may replace screening video colonoscopy (Hosoe et al. 2020; Fockens 2002). The important rate limiting issues of capsule endoscopy including powering the capsule for longer duration as well as development of technology that helps in steering/moving the capsule from outside the body are being resolved (Luo et al. 2019; Alsunaydih et al. 2017). The future capsule endoscope will not only visualise the gut but also monitor the various physiological, vascular, immune, metabolic and microbiological parameters of the gut (Byrne and Donnellan 2019; Gu et al. 2015; Qiao et al. 2016). This advanced smart capsule along with advanced endoscopic technologies like magnification and endocytoscopy and artificial intelligence will probably make video endoscopy obsolete. The future capsule endoscopy will be nothing short of a fiction movie with patient ingesting a smart capsule endoscope and advanced therapeutic procedures being done inside the gut by remote controlling the capsule from outside.

28.3.2 Endoscopic Surgery

Advent of endoscopic clipping laid the foundation of endoscopic surgery, a minimally invasive alternative to surgery (Lee and Tan 2015; Cho et al. 2011; Kim 2013). Improvement in endoscopic surgical techniques especially development of endoscopic cutting and apposition techniques has made endoscopic surgery safe and feasible. Endoscopic submucosal dissection and full thickness resection are endoscopic surgical techniques that are helpful in treatment of early gastrointestinal cancers. Natural orifice transluminal endoscopic surgery (NOTES) is a technique that enables the resection of abdominal organs without resorting to skin incision. In the future, this technique will get more refined with development of small-calibre flexible endoscopes that can be introduced into the peritoneal cavity and development of appropriate accessories (Pasricha 2013). The exciting field of endoscopic surgery is still in its infancy, and future development of multitasking endoscopic platforms, robotic interface, artificial intelligence and endoscopic surgical instruments will probably lead on to replacement of conventional surgery with flexible endoscopic surgery.

The field of third space endoscopy is another promising innovation in the field of endoscopic surgery. In this technique, the mucosa of gut is lifted from the layers beneath it by injection with fluid and then making an incision in the mucosa. Thereafter flexible endoscope is inserted in this so called 'third space', and a number of interventions can be done in this third space as demonstrated by peroral endoscopic myotomy (POEM) for achalasia. A number of other interventions like submucosal tunnelling and endoscopic resection for removal of intramural tumours as well as intramural drug delivery are some of the potential application of third space endoscopy (Manolakis et al. 2019). In the future this endoscopic technique is going to further expand, and it will be a boon for treatment of various gastrointestinal motility disorders by manipulating the enteric nervous system (Pasricha 2013).

28.3.3 Endoscopic Bariatric Surgery and Metabolic Interventions

Obesity and consequent associated metabolic diseases like cardiovascular diseases, diabetes mellitus, stroke, osteoarthritis, gastric oesophageal reflux disease and cancer are an increasing health problem worldwide. Laparoscopic bariatric surgery has evolved into an effective and durable treatment for morbid obesity. It has also beneficial effects on insulin resistance and diabetes and therefore can help in the control of metabolic disorders. Although effective, bariatric surgery is an invasive procedure with complication rate of up to 17% and the re-surgery rate of up to 7%.

Endoscopic interventions using flexible gastrointestinal endoscope offer an alternative non-surgical treatment approach of obesity. The advantage of this endoscopic approach is that it is safer and more cost-effective than surgery. Over last few years a number of endoscopic interventions have been developed for treatment of obesity (Coté and Edmundowicz 2009; Stimac and Majanović 2012; Lopez-Nava et al. 2020; Choi and Chun 2017; Štimac et al. 2016). These endoscopic interventions

include endoscopic gastroplasty, endoscopic placement of an intragastric balloon, endoluminal malabsorptive bariatric procedures that induce malabsorption in duodenum and gastric electrical stimulation (GES) for the modulation of gastric emptying. These endoscopic interventions especially endoluminal malabsorptive bariatric procedures have an immense potential for treatment of various metabolic diseases including diabetes.

28.3.4 Endoscopic Ultrasound

EUS is considered as one of the greatest innovations in the field of GIE as it opened an altogether different field in gastroenterology. All the previous innovations in the field of GIE were improvement in the optics and image acquisition of the flexible endoscope, but EUS enabled the endoscopist to see beyond the mucosa of the gut. Over last few years this field has seen phenomenal innovations that have considerably improved the quality of EUS images as well as added various enhancement technologies like colour doppler, elastography and contrast EUS (Rana et al. 2020a, b; Rana and Vilmann 2015; Rana and Rana 2019). Also, EUS has graduated from a diagnostic procedure to an interventional procedure, and a number of interventional non-surgical procedures now can be safely performed under EUS guidance (Sharma et al. 2015; Rana et al. 2017). In the future, the hardware and software of EUS are going to be further refined, and artificial intelligence is going to play a major role in improving diagnostic capability of EUS.

28.3.5 Future Endoscope

The future gastrointestinal endoscopes are going to become more slimmer and better designed ergonomically. Moreover, further refinements in the high-definition television technology (HDTV) compatible endoscopy systems will lead on to improved resolution of images. The fusion with artificial intelligence is going to improve the diagnostic capability of the endoscopes of the future. The future endoscope is probably going to be a 'bioendoscope' which along with morphological diagnosis via high-resolution images will also provide pathophysiological diagnosis using magnification endoscopy as well as functional diagnosis using thermal, biochemical and pressure sensors (Tajiri 2005). The concept of molecular imaging is going to revolutionize gastrointestinal endoscopy by making possible the identification of both structural and molecular changes in tissues. Moreover, the concept of target-specific therapy based on principles of molecular imaging offers a highly selective approach for treatment of gastrointestinal cancers without systemic side effects (Ahmed et al. 2019).

Computer and robotically assisted endoscopic surgeries are going to be developed to enhance the capabilities of endoscopists (Patel et al. 2015). A number of newly designed robotic endoscopes are in pipeline and will probably change the way the GIE is being done (Yeung et al. 2019). Endoscopes with 180 degree bend

backwards using robotic mechanisms are being developed to improve the diagnostic visualization. Also endoscopes with locomotion methods to reduce the force applied and consequent discomfort to the patients are being developed. Third eye retroscope and peerscope system are the newer endoscope designs that have improved the diagnostic capability of endoscopy. Robotic assisted multiple channel endoscopes have also been evaluated that allow bimanual grasping of tissues possible and therefore will make endoscopic surgery easier. EndoSamurai, Anubiscope, R scope, Incisionless Operating Platform and NeoGuide are some of the recently developed therapeutic endoscopy platforms for endoscopic surgery.

28.4 Concluding Remarks

GIE has come a long way since the inception of first semi-flexible endoscope in 1932, and the journey of last 88 years is a fascinating journey of innovations that have gone beyond the wildest of imaginations. The advances in the fields of lens, television, computer and bioengineering technology have revolutionised the field of endoscopy. Wireless capsule endoscopy, endoscopic surgery, third space endoscopy, metabolic endoscopy, molecular endoscopy and robotic endoscopy are some of the exciting fields in GIE that hold considerable promise in the future. The future innovations driven by curiosity of human mind and desire to solve clinical problems will propel the field of GIE to newer heights. We have just started achieving our dreams, and it is a long way to go!

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Beating Heart in a Box: The Future of Cardiac Transplant

29

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Abstract

More than 100,000 heart transplants have been carried out worldwide, following the first successful heart transplant in 1967. These procedures mostly rely on cold ischemic preservation of the donor's heart as it is inexpensive and relatively reliable. However, the well-known limitations of cold ischemic preservation impose significant challenges to heart transplantation and limit the number of donor's hearts that can be safely transplanted annually. The technology to transport donor hearts in this state has only been developed within the last decade, although the advantages of normothermic donor heart perfusion have been recognized for over a century. TransMedics Inc. designed and manufactured the Organ Care System (OCS) which is currently the only commercially available device with this capability.

Keywords

Ischemic preservation · Heart transplant · Organ Care System

29.1 Introduction

Despite 40 years of research into the management of end-stage heart failure spanning total artificial hearts, ventricular assist devices and more recently stem cell therapy, there remains no comparable alternative to human heart transplantation. This gold standard therapy remains unparalleled in improving survival and quality of life. Unfortunately, the Achilles heel of this excellent treatment is the severe shortage of

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donor organs. The number of heart transplants performed in the United Kingdom (UK) and many Western countries has dropped dramatically over the last decade. The traditional predictors of primary graft failure (PGF) remain constant even as the acceptance criteria for donor's hearts continue to expand. According to the International Society for Heart and Lung Transplantation (ISHLT) registry, the 30-day mortality after heart transplantation is 8% with the leading cause of death attributable to PGF. The length of the ischemic time and donor age are strong predictors of PGF. The ISHLT registry discloses that the risk of PGF begins to increase once the ischemic time exceeds 3 h (Stehlik et al. 2012).

Since the first successful human transplant in 1954, a lot has changed in medicine. But one part of the transplant process that hasn't changed much since then is that the organs have to be transported in a cold environment. New devices have made it possible to keep the donor organs in a functional state at body temperature during transportation. Organ's health is more closely monitored by these devices before it's transplanted. The technology used by these devices known as the ex vivo warm perfusion basically allows organs to stay outside a human body for long periods, such that they can be transported to waiting for recipients far away. The "cold ischemic time" is the time between cross-clamping of the donor's heart and reperfusion of the heart in the recipient. Irreversible damage to the harvested organ can ensue if the ischemia time is prolonged.

Greater than 6 h of cold ischemia time is associated with a greater incidence of cardiac allograft dysfunction and decreased transplant recipient survival, hence, the need for techniques of beating heart procurement, or "warm perfusion." Cold ischemic storage is known to be faulty as low levels of anaerobic metabolism continue in the background leading to depletion of adenosine triphosphate (ATP) stores and an increase in acidosis (Buckburg et al. 1997). Reduction of the cold ischemic time is the most apparent benefit of normothermic donor heart perfusion during transportation. Currently, nearly 5500 heart transplants are being performed annually across the entire world (Lund et al. 2017).

29.2 Historical

The idea of replacing a bad organ with a good one is an ancient one well documented in ancient mythology. Skin grafting was probably the first organ transplant that was performed in India around the second century B.C. Vladimir Demikhov is credited with the first heart transplant in an animal. In 1946 in Moscow, Vladimir switched the hearts between the two dogs. The dogs survived the surgery. However, it is Dr. Christiaan Barnard who is credited with the feat of performing the first heart transplant in human beings. The surgery was performed in South Africa in 1967 (Barnard 1967). However, the patient could survive postoperatively for only 18 days. Most of the research that led to successful heart transplantation was carried out at Stanford University, USA, under the able leadership of Dr. Norman Shumway. Once Stanford started reporting better results, other centers also followed suit. Nevertheless, for successful transplantation of the human heart, certain medicine

was required which could prevent the recipient from rejecting the donor's heart. This came about in 1983 when a drug called cyclosporine was approved by the Food and Drug Administration (FDA) (Reitz et al. 1981). Before the arrival of cyclosporine, the overall results of heart transplants were not very good.

Dr. Juro Wada in 1968 performed the first heart transplant in Asia at Sapporo Medical University in Japan (Krittayaphong and Ariyachaipanich 2015). Compared to the experience in the United States, the initial results were poor, until the introduction of cyclosporine in 1983. Dr. P. Venugopal and his team in 1994 carried out the first successful heart transplant in India at the All India Institute of Medical Sciences (AIIMS), New Delhi (Venugopal 1994). The patient survived for 14 years postoperatively.

Normothermic ex vivo heart perfusion allows continuous metabolic and functional assessment. It was almost 150 years ago that the idea of explanting and perfusing the heart was first established in frogs by the scientist Elias Cyon (Zimmer 1998). Oscar Langendorff is accredited with the idea of the first isolated perfused mammalian heart (Taegtmeyer 1995). It was in 1895 that he postulated the principle of cannulating the ascending aorta and delivering the perfusate retrogradely down the aorta. The aortic valve would close under a hydrostatic pressure head, and the perfusate would be directed anterogradely through the coronary arteries. This technique was modified by Katz in 1939 wherein he incorporated a pump to set the perfusion pressure allowing vasoactive properties of various drugs to be assessed (Broadley 1979). In 1914, Ernest Starling used this technique to investigate ventricular volumes, formulating the Frank-Starling laws of the heart (Starling and Visscher 1926). Robicsek modified Starling's heart-lung preparation in 1959 to investigate the methods of perfusing the donor heart before transplantation. Hearts survived for an average of 11 h using this ex vivo perfusion technique (Robicsek et al. 1963; Robicsek et al. 1967). Further attempts were made using the technique of autoperfusion, but the practice was abandoned due to significant bleeding and pulmonary edema (Ladowski et al. 1985).

29.3 Major Techniques

The foundation of the TransMedics (Andover, Massachusetts) Organ Care System (OCS) followed the development of a portable perfusion apparatus used as a research tool for investigating donor heart preservation (Hassanein et al. 1998). This was the first commercially available device to transport donor hearts in a normothermic perfused state. The perfusate is a proprietary priming solution with the addition of antibiotics, insulin, sodium bicarbonate, multivitamins, methyl prednisolone, and fresh donor blood. Integrated plate heater maintains normothermia, while the pulsatile flow is generated by a diaphragmatic pump. After systemic heparinization, around1.2–1.5 l of donor blood is collected just before aortic cross-clamping. The collected donor blood is passed through a leucocyte filter and added to the priming solution in the OCS organ perfusion module. The donor heart retrieval proceeds in the standard fashion, the only difference being that a lower volume of a

short-acting cardioplegic solution is used in the initial cold ischemic period for no more than 20–30 min. The transected donor aorta is connected to a specially designed aortic tip connector, and the pulmonary trunk is cannulated with a malleable cannula. This aortic tip connector is joined to the perfusion port of the OCS organ chamber that supports the heart on a sloping cradle.

As the donor's heart is reperfused, sinus rhythm is either restored spontaneously or with the aid of a direct current shock delivered through defibrillator pads placed inside the organ chamber. The superior vena cava and inferior vena cava are sutured closed once the heart begins to beat. Coronary sinus blood returning to the right atrium flows into the right ventricle which pumps it through a low resistance membrane oxygenator before it enters a blood reservoir. The oxygenated blood is then pumped into the donor aortic root by a pulsatile pump. A wireless monitor controls the perfusion rate of the OCS and displays a comprehensive panel of information including aortic pressure, coronary flowrate, hematocrit, temperature, and oxygen saturation. During transportation, the goal is to maintain the aortic pressure between 65 and 90 mmHg with a coronary flow of 650–850 ml/min. The operator can increase the rate of the vasodilatory maintenance solution or increase pump flow, in case the coronary flow is deemed inadequate. After reviewing the first 49 OCS clinical heart transplants, the most powerful predictor of outcome was the final lactate level at the end of OCS preservation (Hamed et al. 2009). Used as a univariate, lactate level of >4.96 mmol/l at the end had a sensitivity of 0.625 and specificity of 0.976 to predict a poor outcome. Rising lactate levels over 5 mmol/l have also been shown to be a sensitive tool predicting a poor outcome. Compared with the current method of hypothermic arrest and storage, OCS exhibits the ability to safely extend the preservation period of donor's hearts beyond the current 4–6-h limit. An increase in the pool of available donors may be achieved by the development of a clinical, portable perfusion device for donor organ preservation and transport, thus permitting distant procurement of organs. Prolonged safe preservation would help in reducing the risk of early graft rejection (DiSesa et al. 1994) and would also create the flexibility to perform the transplant procedure on an elective basis. This new technique may also reduce the incidence of cardiac allograft vasculopathy, the leading cause of late cardiac allograft failure (Ventura et al. 1995). Besides, hearts from non-heparinized donation after cardiac death (DCD) can be successfully resuscitated by applying the OCS in a setting that accurately simulates clinical conditions. However, the full potential benefits of ex vivo heart perfusion has yet to be realized. The ultimate aim of the technology would be to thoroughly characterize the biochemical, anatomical, metabolic, and mechanical function of the donor's heart before implantation. Utilizing the OCS for donor heart maintenance requires several resources including additional surgical and technical support personnel, appropriate transport, proprietary equipment, and the collection of donor blood to prime the perfusion module. Compared with cold static preservation techniques, this is inevitably costlier. However, this has to be against the value of potentially making more donor hearts available for trans plantation and the cost savings from a potential reduction in the incidence of PGF. During the early experience with the OCS, there were a few issues. Some of the donor's hearts

instrumented on the OCS became unfit for transplantation, either because of high levels of vasoconstrictors in the perfusate which were traced to the collected donor blood or the donor's heart became accidentally detached from the connector during transportation. The development of myocardial edema is another potential concern of continuous cardiac perfusion utilized in the OCS. When crystalloid solution was used to perfuse Langendorff hearts in the laboratory, it was recognized that a 10% decline in function was associated with myocardial edema (Sutherland and Hearse 2000). When blood was used as the perfusate to increase its oncotic pressure, edema was significantly reduced, and there seemed to be no deleterious effect on the function. To reduce the tendency for myocardial edema, TransMedics have developed a synchronization mode, replicating that of a balloon pump timed against the electrocardiogram to perfuse the donor heart during diastole. The OCS Heart PROCEED II (Ardehali et al. 2015) FDA pivotal trial demonstrated that the OCS is as safe and effective as the current standard of care (SOC) in preserving standard donor hearts for transplantation. These results were achieved despite the OCS arm having nearly double the total cross clamp time as compared to the SOC. In addition, the data from PROCEED II Trial demonstrated that circulating lactate level of the OCS heart perfusion could be a useful variable to evaluate donor hearts status ex vivo.

29.4 Summary

In conclusion, cold ischemic preservation for the donor heart has been universally embraced by clinicians across the globe over the last 45 years. However, the diminishing pool of ideal donors coupled with the drive to further improve heart transplant outcomes mandate a reevaluation in this field. Normothermic donor heart perfusion is the logical next step, and from the clinical experience to date, it definitely appears to hold promise.

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The Journey of Coronary Stent Technology 30

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Abstract

Coronary artery disease is one of the leading causes of morbidity and death worldwide. Significant stenosis (≥70% luminal reduction) of the coronary artery hampers the blood flow to the myocardial territory it supplies, leading to symptoms and other consequences related to reduced blood flow. Dilatation of the stenosis followed by stenting is the current standard of management. The technique, technology, and evidence behind this simple-looking procedure have been through a long journey of evolution. Coronary metallic stent scaffold was introduced in 1986, which would prevent the artery from recoiling and sealed the dissection/tear in the vessel wall resulting from the dilatation of the stenosis. Bare metal stents (BMS) were the first available scaffolds, however, with its inherent risk of stent restenosis due to neointimal hyperplasia. The next advancement came in the form of drug-eluting stents (DES) that contained antiproliferative agents to be released at the local site inhibiting the proliferation of inflammatory cells, thereby reducing the rate of restenosis. These DES have evolved tremendously so that the currently second-generation stents are available with bioabsorbable polymer or are polymer-free. These second-generation DES are the most commonly used coronary stents worldwide, with good clinical and angiographic outcomes. Here we briefly describe the historical aspect of the evolution of the stent technology, followed by a discussion on the current design, completely bioabsorbable stents, and the promise some of emerging technology holds.

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Keywords

Coronary heart disease · Stenosis · Bioabsorbable stents · Angiographic outcomes

30.1 Introduction

Coronary artery disease is one of the leading causes of morbidity and death worldwide. It accounts for 16.7% of all deaths globally, 10% of global years of life lost (the largest portion of all diseases), and 7% of all disability-adjusted life-years lost. The predominant form is where there is significant stenosis ($\geq$70% stenosis) which hampers the blood flow to the myocardial territory it supplies leading to symptoms of ischemia in the form of chest pain, breathlessness, and dysfunction of the myocardium. In a patient who comes to the health-care setting with symptoms of coronary artery disease, a coronary angiogram is done to detect and quantify the presence of coronary artery stenosis. In the present era, the next step is to typically dilate the stenosis with a balloon mounted on a catheter followed by stenting the lesion with a drug-eluting stent. The technique, technology, and evidence behind this simple-looking procedure have been through a long journey of evolution. Here we briefly describe the historical aspect of the evolution of the stent technology followed by a discussion on the current design, completely bioabsorbable stents, and the promise some of emerging technology holds.

30.2 History

Balloon angioplasty alone wherein the catheter mount balloon pushes the plaque against the vessel wall was the earliest approach used for opening the stenosed segment. It was introduced by Dr. Andreas Gruentzig back in 1977. Although there was a good acute increase in luminal diameter, it carried the risk of dissection of the vessel wall, arterial recoil within a short period resulting in reocclusion. Coronary metallic stent scaffold was introduced in 1986 which would prevent the artery from recoiling, and it also sealed the dissection/tear in the vessel wall resulting from the dilatation of the stenosis. These bare metallic stents (BMS) were shown to achieve a better patency rate. An ideal stent possesses the following features—good biocompatibility, flexibility to cross the coronary vessel, less thrombogenicity, strong radial force to keep the artery open, good radio-opacity to be seen under fluoroscopy, and low rates of stent thrombosis and neointimal hyperplasia in the long run. Stent thrombosis is the phenomenon where there is clot formation within the lumen of the stent that carries the risk of vessel occlusion. The presence of a metallic stent without endothelial lining carries the risk of stent thrombosis. The luminal surface is completely endothelized by 12 weeks on an average in BMS thereby posing less risk of stent thrombosis. However, it was noted that BMS still carries the risk of stent restenosis in 20–30% of cases due to neointimal hyperplasia (ingrowth causing

luminal compromise secondary to tissue proliferation as a response to vessel micro or macro injury due to the plaque rupture or angioplasty) long after stenting is done.

To overcome this, drug-eluting stents (DES) were designed that contained antiproliferative agents to be released at the local site inhibiting the proliferation of inflammatory cells thereby reducing the rate of restenosis.

30.3 Drug-Eluting Stents

A first-generation DES typically consists of three components: metallic scaffold, polymer coating, and an antiproliferation drug (Fig. 30.1). The polymer acts as a barrier that prevents blood from coming in contact with the metallic surface, and it also helps in the sustained and prolonged release of the drug in the milieu. Stainless steel was the metal used in these stents. Cypher and Taxus were the prototype first-generation DES introduced in 2003. The Cypher stent used parylene tie layer, poly (n-butyl methacrylate) (PMBA), and polyethylene-co-vinyl acetate (PEVA) polymer with sirolimus drug. Taxus stents had poly(styrene-b-isobutylene-b-styrene) (SIBS) polymer and used paclitaxel drug. Sirolimus and paclitaxel are antiproliferative agents used to prevent neointimal hyperplasia. These stents released the drugs over the first 30 days resulting in a decrease in the incidence of target vessel revascularization by 60–70% of what was seen in BMS and reduction of stent thrombosis (Stettler et al. 2007). Studies comparing DES and BMS showed a peculiar phenomenon with DES called late (1 month to 1 year) and very late stent thrombosis (>1 year) occurring in a significantly higher number of patients compared to BMS. The incidence of very late stent thrombosis was around 0.2–0.5% per year, which was higher than that of BMS (Slottow et al. 2008; Mauri et al. 2007). The retained polymers and antiproliferative drugs were implicated in delayed endothelial healing and neoatherosclerosis resulting in stent thrombosis (Higo et al. 2009).

To bring down the rate of late and very late stent thrombosis, second-generation stents were introduced that used more biocompatible polymer [(polylactic acid (PLA), poly(vinylidene fluoride-co-hexafluoropropylene) (PVDF-HFP), and polyvinylpyrrolidone (PVP)] coating with better antiproliferative agents

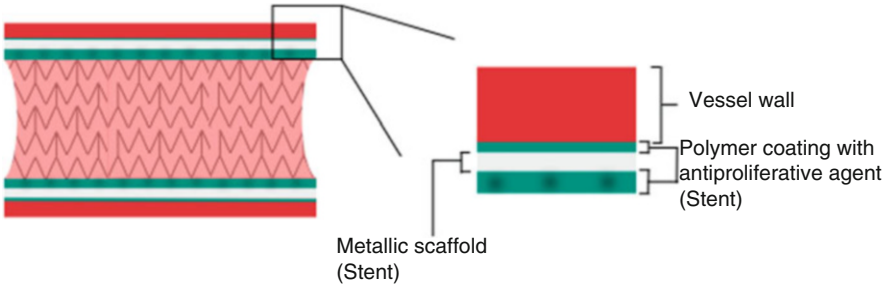

Fig. 30.1 Schematic diagram showing components of a typical drug-eluting coronary stent

(zotarolimus, everolimus, and novolimus) that accelerated the vessel healing. They also used new metal compounds (cobalt-chromium and platinum chromium alloy) resulting in a significant reduction in thickness of stent struts (70–90 μm). These metal compounds also allowed better radio-opacity under fluoroscopy, flexibility, and deliverability.

Currently, second-generation stents are the most commonly used coronary stents worldwide with good clinical and angiographic outcomes.

30.4 Bioabsorbable Polymer Stents

As polymers have been implicated in inflammation and thrombosis, polymer-free and bioresorbable polymers have been seen as a technological step forward (Van der Giessen et al. 1996; Tada et al. 2010). Endothelial healing improves as the polymer degrades; hence polymer-free or bioresorbable stent designs may theoretically reduce the complications of both BMS and DES. Conventionally dual antiplatelets (two platelet inhibiting drugs) are recommended for typically 1 year after stenting to reduce the risk of stent thrombosis that may arise because of non-endothelization. However, this prolonged administration of two antiplatelet drugs is associated with an increased risk of major bleeding episodes. Early healing with polymer-free stents may allow for a shorter duration of dual antiplatelet therapy safely (Urban et al. 2015).

SYNERGY was the first FDA-approved bioabsorbable polymer drug-eluting stent (BP-DES). It has a poly(lactide-co-glycolide) (PLGA) coating that eventually degraded into water and carbon dioxide which are excreted by the kidneys and lungs, respectively. Optical coherence tomography showed complete resorption of polymer with 4 months of implantation. The coating was done on the abluminal surface of the stent further improving endothelization. The EVOLVE and EVOLVE II trials showed no difference in the incidence of adverse events, stent thrombosis, or target lesion revascularization at 12 months with SYNERGY stent when compared to a second-generation drug-eluting stent (Meredith et al. 2012; Kereiakes et al. 2015).

Various other notable examples of BP-DES include Nobori and MiStent. A meta-analysis of 16 studies with 2 years mean follow-up did not show any difference in the incidence of adverse cardiac events, stent thrombosis, target lesion revascularization, or target vessel revascularization in comparison to the current-generation durable polymer DES. Six studies among them with follow-up of more than 12 months indicated a trend of lower late stent thrombosis with BP-DES (El-Hayek et al. 2017).

30.5 Polymer-Free Stents

Innovations were made in stent design to substitute for the property a polymer held as a carrier and controlled releaser of antiproliferative drugs by creating polymer-free stents. Initially, paclitaxel was directly loaded over stainless steel platform to look for the feasibility of polymer-free strategy; however this failed to show clinical

benefits over BMSs (Lansky et al. 2004). Then the drug was directly coated on a porous stent surface using chemical bonding. The Yukon stent is a popular example that has sirolimus coated on a microporous stainless steel surface. Most of the drug elution takes place in the first 7 days. This was shown to have better imaging outcome than sirolimus-eluting stents at 3 months follow-up with better endothelial coverage evident by optical coherence tomography intravascular imaging (Moore et al. 2009). A one-year outcome study comparison with a second-generation zotarolimus-eluting stent showed non-inferior results (ISAR-TEST 5 trial) (Massberg et al. 2011).

The BioFreedom stent uses a stainless steel platform with Biolimus A9 attached to the microstructure abluminal surface without using a polymer. A study in patients at high bleeding risk showed better efficacy and safety than BMSs when 1 month of dual antiplatelet therapy was used (Urban et al. 2015).

The VESTAsync stent uses a microporous hydroxyapatite surface on which sirolimus is coated. The hydroxyapatite gradually dissolves in a year. The OPTIMA stent has grooves over the abluminal polymer-free stent surface on which tacrolimus is coated as a reservoir.

30.6 Fully Bioresorbable Stents (BRS)

After the success of bioabsorbable polymer and polymer-free stents, the scientific community showed a large interest in the development of a fully bioabsorbable stent where both stent scaffold and polymer would be resorbed completely. Theoretically, it is expected to overcome the shortcomings of previous stents like impaired coronary vasomotion and reduced luminal size because of the retained obstructive scaffold, chronic inflammation secondary to the retained metallic foreign body, and very late stent thrombosis due to impaired endothelization.

ABSORB stent was the first fully bioresorbable stent (BRS) introduced. The scaffolding is made of poly-L-lactide (PLLA), which eventually degrades to carbon monoxide and lactate. The bioabsorbable scaffold provides lower radial strength necessitating thicker stent struts to maintain the required radial strength.

In porcine studies ABSORB stent showed favorable vascular remodeling compared to cobalt-chromium DES (Lane et al. 2014). Complete endothelization was demonstrated from as early as 1 month and complete resorption of stent scaffold by 3 years despite the thick struts (Nishio et al. 2012). ABSORB III trial comparing ABSORB with a second-generation DES showed that it was non-inferior with respect to a 1-year outcome following which it was FDA approved (Ellis et al. 2015). Subsequently, a 3-year result from the ABSORB II trial revealed inferior angiographic and clinical outcome from ABSORB stent compared to DES. It had a higher incidence of target vessel myocardial infarction primarily due to scaffold thrombosis (Serruys et al. 2016).

Few studies attributed the poor results to suboptimal pre-implant and post-implant techniques and improper vessel selection for which ABSORB IV was carried out to study the role of the bioabsorbable scaffold in the setting of optimized

coronary stenting. But the study reproduced the inferior outcomes (Stone et al. 2018).

Two pooled analyses of ABSORB trials and seven RCTs showed an increased risk of target vessel failure as well as device thrombosis between 1 and 3 years in the BRS group (Ali et al. 2018; Sorrentino et al. 2017). They also did not show any improvement in the anatomy of the stented segment. Due to safety concerns the product was pulled off the market in 2017. The mechanisms for early and late stent thrombosis in BRS are different. Studies so far have implicated increased stent strut thickness leading to turbulence as the most important contributing factor responsible for the increased incidence of acute stent thrombosis. Yamaji et al. showed that nonuniform absorption of vascular scaffold led to isolated malapposed scaffold stent struts that are out of plane with the rest of the scaffold which led to thrombosis. This selective discontinuity was seen in 42% of subjects making it the most common cause of very late scaffold thrombosis (Yamaji et al. 2017).

All these studies of the mechanism of stent thrombosis have provided insights into the pitfalls and potential areas of improvements for future BRS. The emerging generation of BRS stent if designed to have thinner stent struts, better apposition, and lower discontinuity, it is likely that they might be non-inferior or superior to the current second-generation DES.

30.7 Future

One of the possible innovations would be coating the metallic stent with special nanocomposite polymers like polyhedral oligomeric silsesquioxane polycarbonate urea (POSS-PCU) with the incorporation of endothelial progenitor cell (EPC) specific antibodies and nitric oxide (NO). NO maintains healthy endothelium and prevents thrombosis (Tan et al. 2012). These coatings can also incorporate multiple drugs. POSS-PCU is a nonbiodegradable coat that prevents blood from coming in contact with bare metal. POSS PCL (polyhedral oligomeric silsesquioxane polycaprolactone) is a biodegradable coating that can help in the controlled release of drugs.

Studies are underway to use the bio-nanodelivery system to promote re-endothelization and inhibit smooth muscle cell proliferation so that stent thrombosis and in-stent restenosis are reduced (Paul et al. 2010).

30.8 Summary

Stent technology has come a long way beginning with simple metallic support till the current designs with excellent biocompatibility, deliverability, flexibility, and clinical outcome. The second-generation drug-eluting stents have stood the test of time with excellent clinical results and safety profile. They are the most widely used stents to date. The second-generation DES with bioabsorbable polymer has good safety and at times better outcomes than the conventional DES. Although the completely

bioabsorbable scaffolds raised the expectations, initially they failed to meet the same. With the necessary improvement in stent structure, they may be the future of stent technology.

The active integration of biotechnology and nanotechnology is needed more than ever in the development of stent technology.

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Materiovigilance in Clinical Surgery

31

Ram Samujh and Nitin J. Peters

Abstract

Materiovigilance is the systematic reporting and analysis of unwanted events with the use of medical devices. The regulatory perspectives of these devices are also important from safety and a medicolegal point of view. There is increase in the use of health-care devices, which can vary from a humble bandage to a complex heart lung machine. The reporting of these events will go a long way in patient safety across the spectrum. The Indian materiovigilance program even though is in the early stages is becoming well established and robust system. This chapter looks at the issues of materiovigilance with special emphasis on clinical surgery.

Keywords

Materiovigilance · Reporting · Indian Pharmacopoeia Commission · Serious adverse events

31.1 Introduction

The last century has seen an exponential growth in the different types of medical devices being used in clinical surgery. The integration of technology and clinical practice has made several advances in the improvement of patient care (Fouretier and Bertram 2014). The latest being artificial intelligence (AI) and its use in clinical application.

There has been advancement in terms of various gadgets and other aspects of technologies in the betterment of patient care; however this comes with a downside.

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Medical devices can also carry various potential risks, which may range from simple malfunction to a life-threatening complication. There are several incidents where medical devices have been recalled either due to a defect or because of their causation of morbidity and in some cases even mortality in patients (Heneghan et al. 2011; McGee et al. 2012).

The international market for medical devices has increased from 260 billion US dollars (US$) to over US$ 380 billion over a decade (2006–2016) (Ted Fuhr et al. 2019). Currently there is a vast number of "medical devices" in the market which may range from a humble bandage to a complex device like an ECMO machine (Jefferys 2001).

The World Health Organization's definition of a medical device is any "instrument, apparatus, implement, machine, appliance, implant, reagent for in vitro use, software, material or other similar or related article, intended by the manufacturer to be used, alone or in combination, for human beings, for one or more of the specific medical purpose(s) of diagnosis, prevention, monitoring, treatment or alleviation of disease or injury." "This device may be used for investigation, replacement, modification, or support of anatomy or of a physiological process, supporting or sustaining life, control of conception, disinfection of medical devices providing information by means of in vitro examination of specimens derived from the human body".

As mentioned earlier the advantages of using medical devices in modern medicine have to be balanced by the adverse or deleterious effects these devices may incite. For example, the malfunctioning of a simple machine as weighing machine may miscalculate all the treatment parameters in a pediatric patient, or a wrong reading by a glucometer may alter the course of euglycemic management in a diabetic patient. A complex machine like a cardiopulmonary bypass may malfunction and cause significant morbidity or mortality in a patient undergoing surgery.

Thus, it is imperative to have a system in place for such events to be reported and noted by an agency so as to aid in identifying the risks associated with medical devices and to have a policy decision to withdraw these medical devices from the market. This would reduce the dangers of such adverse events occurring in future. This is the basic premise and principle of the concept of materiovigilance (Rani and Singh 2018).

Materiovigilance includes close monitoring of any adverse or undesirable events by collecting, reporting, and analyzing these events. The backbone of materiovigilance is the assurance of patient safety by decreasing such events (Radhadevi et al. 2012).

31.2 Surgical Instruments and Devices

There are more medical devices being used in surgical disciplines than any other branch of medicine. Even from the historical perspective, even a simple procedure will require one or another instrument for even starting the procedure. The medical devices in surgery with special reference to pediatric surgery include the following (Fig. 31.1).

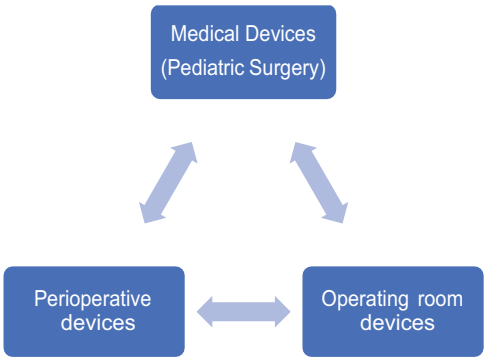

Fig. 31.1 Spectrum of medical devices in pediatric surgery

Operating room devices can be further classified as general surgical equipment or special surgical devices. General surgical devices include:

- suture materials,
- energy devices,
- surgical instruments,
- OT tables and lights.
- anesthesia-related equipment,
- autoclaves and sterilization devices,
- miscellaneous devices,

Special surgical devices (pediatric surgery) include:

- VP shunts.
- DJ stents.
- testicular,
- Nuss procedure metal bands.
- fiberoptic instruments like bronchoscopes cystoscopes.

laparoscopic instruments (3 mm and 5 mm).

31.3 Medical Devices Rule in India

The Medical Devices Rule 2017 was commissioned on January 1, 2018, under the Drug and Cosmetic Act 1940. Earlier to this rule, medical devices were regulated as drugs (pharmaceutical products) in India. There was a dire need of differentiating between medical devices and pharmaceutical products. The Central Drugs Standard Control Organization (CDSCO) categorized devices from time to time and displayed them on their official website. The classification list provided by Medical Devices Rule 2017 is mentioned in Table 31.1.

Table 31.1 Device risk classification as per Medical Device rule (Gupta 2016)

Device risk class	Type of risk	Examples
Class A	Low risk	Bolster suture, alcohol swabs, nasopharyngeal catheter
Class B	Low to moderate	Disinfectants, intravenous catheter, rectal catheter
Class C	Moderate to high	Biliary stents, bone cement, imaging catheter
Class D	High risk	Coronary stent, heart valve, copper-T

31.4 Materiovigilance Program of India (MvPI)

31.4.1 Origin and Inception

A well-organized vigilance system is the core of a reliable regulatory framework, which ensures the quality check and in turn promotes the safe use of medical devices. The regulation of medical devices, however, is a complex and evolving area that is often complicated by legal technicalities. For example, legal terminologies are sometimes nonuniform even within the same regulatory system (Shukla et al. 2020).

CDSCO (under the aegis of Ministry of Health and Family Welfare, Government of India) is the regulatory body which is responsible for controlling medical devices. The Medical Devices Regulatory Authority of India (MDRA) was established with the scope and mandate of regulation and legal aspects of medical devices in India. The Medical Devices Regulation Bill (MDRB) was introduced in 2001 by the Ministry of Science and Technology and is aimed at creating a system and environment for the regulation of safety, quality, and accessibility of the Indian Medical devices. (Radhadevi et al. 2012)

The Drugs and Cosmetics (Amendment) Bill 2013 was brought out to establish regulatory measures which included regulatory limitations for import, export, sale, manufacture, and distribution of medical devices in India. It also lays down guidelines and technical support for obtaining import license and registration. (Rani and Singh 2018)

31.4.2 Objectives of MvPI

The program was initiated with the objectives to protect the health and ensure the safety of device users and others by reducing the recurrences of adverse events and malfunctions.

- To create a nationwide system for patient safety monitoring.
- To analyze the risk–benefit ratio of medical devices uses.
- To generate evidence-based data on the safety of medical devices and to support CDSCO in the decision-making process on the use of medical devices.
- To communicate the safety information on the use of medical devices to various stakeholders to minimize the risk.

- To emerge as a national center of excellence for materiovigilance activities.
- To collaborate with other health-care organizations and international agencies for the exchange of information and data management.

The mandate is to be inclusive with all the relevant stakeholders and improve the outcome for patients by encouraging that more and more adverse events are reported. They may be very simple events, but the culture of reporting needs to be inculcated. The adverse events may be serious, nonserious, frequent, or rare. Along with these events the malfunction or deterioration of a medical device or errors in labelling instructions for user manuals should also be reported and encouraged.

A document has been prepared by the MvPI which captures all the relevant details regarding the patient, adverse event, device, regulator, and reporter. This form is freely available on the official website of IPC (www.ipc. gov.in). The duly signed form can be sent to the nearest medical device monitoring center (MDMC) or can be directly sent to the National Collaborating Centre (NCC). It can also be scanned and mailed @sctismt.ac.in and copy to mvpi.ipcindia@gmail.com. A help line number can be reached to report the adverse events: 1800-180-3024. All different stakeholders should be involved in the documentation and reporting, and this is possible only if a positive interdepartmental relationship is maintained (Kalaiselvan et al. 2014).

The various stakeholder in the plan include:

1. Professional staff at IPC, SCTIMST, NHSRC, and all such institutions that would serve as stakeholders of the program.
2. Representatives of Medical Device Monitoring Centre.
3. Staff and consultants in CDSCO.
4. Policy makers at all levels of health care, particularly those concerned with Medical Device policy.
5. Under MvPI clinicians, biomedical engineers, clinical engineers, hospital technology managers, pharmacists, nurses, and technicians can report medical device adverse events. Medical device manufacturers/CDSCO-notified medical device manufacturers/ medical devices' importer-traders can also report adverse events specific to their product to the National Coordinating Centre.
6. Medical technologists and innovators.

31.4.3 Organizational Structure

Materiovigilance Program of India (MvPI) was initiated by the Drugs Controller General of India launched at Indian Pharmacopoeia Commission (IPC), Ghaziabad on July 6, 2015. The primary aim of this program is to monitor medical device-associated adverse events (MDAE), create awareness among health-care professionals about the importance of MDAE reporting, and generate credible safety data of medical devices, which is without bias. The feedback to all relevant stakeholders is an important aspect of the program. Sree Chitra Tirunal Institute

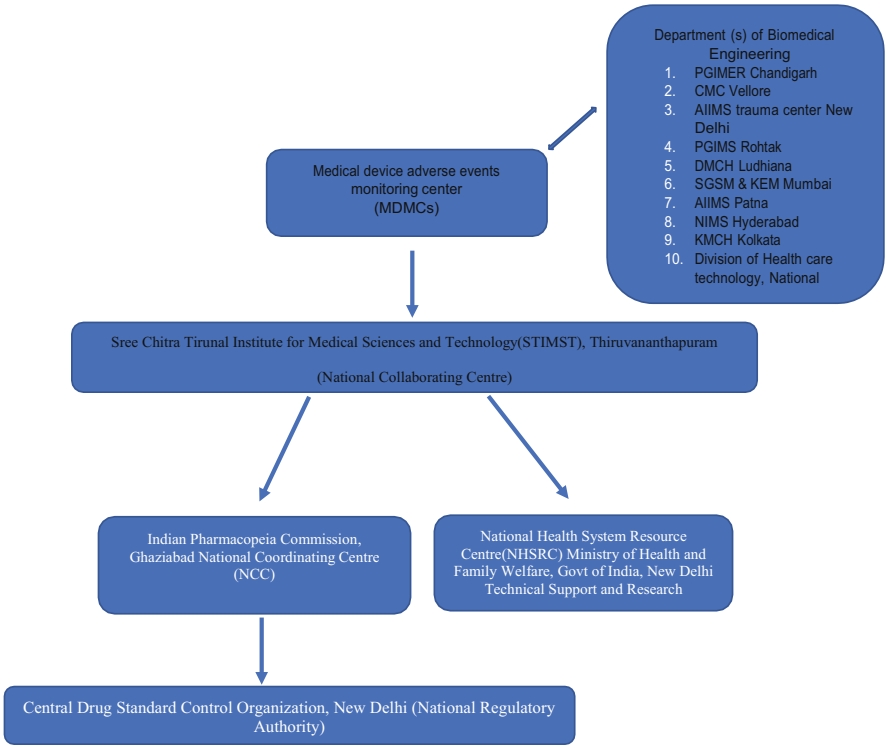

Fig. 31.2 Organizational structure of MvPI

for Medical Sciences & Technology (SCTIMST), Thiruvananthapuram, is the National Collaborating Centre; National Health Systems Resource Centre (NHSRC), New Delhi, is the technical support partner; and Central Drugs Standard Control Organization (CDSCO), New Delhi, functions as regulator (Fig. 31.2).

31.4.4 Responsibilities of Different Units in the MvPI

MDMC are tasked with the collection and review of MDAE, analyze failures effect, assess causality as per the standard operating procedures (SOP), and send the monthly consolidated report to National Collaborating Centre. As per a proposed list, there are ten medical colleges which have been identified to send in data pertaining to materiovigilance (shown in Fig. 31.2).

National Collaborating Centre receives the adverse event reports from MDMC and collates, analyzes and performs signal detection, and communicates the outcome to National Coordinating Center (NCC). It is also involved in conducting awareness program, training, and the workshop on materiovigilance periodically at various zones of the country.

The Indian Pharmacopoeia Commission is responsible for coordinating the program among all stakeholders. It also needs to identify and recruit new MDMCs across the country. It also prepares and disseminates SOP, guidance documents, training manual, and newsletter. It formulates the data received from SCTIMST and recommends to the CDSCO for appropriate action.

DGCI-CDSCO formulates the regulatory decisions and communicates to the different stakeholders. As regulator, the CDSCO should join the International Medical Device Regulators Forum (IMDRF) and other international forums for exchange of post-marketing safety information. National Health Systems Resource Centre Ministry of Health and Family Welfare, Government of India, New Delhi, functions as TSRC. It provides technical support to NCC and National Coordination Centre for the preparation of SOP, guidance documents, newsletters, and training manuals. It also helps in identifying new MDMC.

31.4.5 Process of Materiovigilance

Currently the MvPI has 26 centers across the country. In India the reporting is voluntary. The existing structural organization of the pharmacovigilance program is also being harnessed in terms of over 270 adverse drug reaction monitoring centers are also enrolling adverse events of medical devices.

As and when a medical college or fresh center is recruited, the designated research associate (RA) of that particular center starts collating and sending the MDAE data to the commission. The RA is the liaison between the commission, the patient or user, and the manufacturer.

A time lag of 15 calendar days is permitted to report any serious adverse event and the relevant action taken to be reported to the CDSCO. The commission is open to receive reports of nonserious incidents from any person at a voluntary basis. At the commission, each report received is segregated into initial, follow-up, or final and allotted a unique reference number. The commission is mandated to follow up the event diligently from the reporting person/agency or the patient till a valid conclusion is achieved. Trained personnel at the commission then assess these reports for quality and completeness of data, and if found valid, they are further evaluated by a group of external subject experts and sent to the core technical committee to prepare any necessary recommendations.

The recommendations of the core technical committee are forwarded to the Central Drugs Standard Control Organization for further discussion and regulatory action, if any. If the data are incomplete or invalid, reports are relayed back to the relevant monitoring center or reporter with the query or necessary comments, so that the report can be corrected or completed and returned to the commission for evaluation. (Shukla et al. 2020)

31.5 Surgical Materiovigilance

As mentioned earlier in this report, the gamut of appliance in clinical surgery is overwhelming. A procedure even as simple as an incision and drainage of an abscess relies on various devices like the basic infrastructure of the operating room and its paraphernalia. There are system and informal SOPs available in most busy surgical centers regarding troubleshooting and reporting of device malfunctions built over the years with inputs from experiences surgeons, anesthesiologists, nursing officers, and technical staff. Most logical responses involve:

(a) identifying the cause of the malfunction and the correlation with the adverse event,
(b) removing the device from the patient care (if possible),
(c) reporting to the biomedical engineering department,
(d) getting replacement from the manufacturer till the problem is rectified.

There is usually no system in place where a causality is identified and the feedback is given to the manufacturer specially if there is a one-off episode of adverse events. However, the lack of a robust system may cause serious adverse events and even mortality of patients. One such example which was highly publicized by the media was the metal on metal articular surface replacement (ASR) device which was manufactured by Johnson and Johnson subsidiary De Puy Orthopedics. The device was recalled in 2010 by the manufacturer in view of high failure rate, beside other adverse effects like metal deposits in the body which even led to the death of one patient. (The Times of India 2014)

In a 4-year period since its inception, the Materiovigilance Program and the Indian Pharmacopoeia Commission have received and analyzed more than 1931 medical device adverse events. Approximately 60% of these were serious adverse events. Report of device-associated adverse events in India increased markedly after 2017, when the Medical Devices Rules came into effect and after the development of various user-friendly reporting procedures.

The most commonly reported events were in the medium-/high-risk category of devices. Adverse events associated with cardiac stents were the most commonly reported (926 events; 47.95%), followed by intrauterine contraceptive devices and orthopedic implants. Most of the events (1439; 74.5%) were received from marketing authorization holders.

It is essential that resource-intensive and user-friendly methods are utilized for surgical centers to increase the materiovigilance and causality identification. Each MDAE reported will go a step further in ascertaining patient safety and improving quality of care in patient care in clinical surgery. More and more surgical centers must be included and involved in the process of reporting MDAEs.

31.6 Conclusions

Health-care workers use several medical devices to aid in patient care and save lives across the globe. The devices are in no way supposed to do any harm to the patients. A doctor always treats his patient with good intention, but if any adverse event occurs, the medicine prescribed or the device used in the treatment may be at fault. Hence, to minimize these adverse events and to analyze them, we require robust programs which include vigilance across the spectrum. These may include pharmacovigilance, hemovigilance, and more recently materiovigilance. The common goal of all these programs is to avoid the errors for recurring and in turn make medical treatment safe for all.

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