R.C. Sobti
Naranjan S. Dhalla *Editors*

Biomedical Translational Research

Drug Design and Discovery

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Editors
R.C. Sobti
Department of Biotechnology
Panjab University
Chandigarh, Chandigarh, India

Naranjan S. Dhalla
St. Boniface Hospital Albrechtsen Research Centre
Institute of Cardiovascular Sciences
Winnipeg, Manitoba, Canada

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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. Moreover, organismal physiology has been a most significant challenge ahead in basic and clinical research. Attempts are on to understand the integrated function of organs and organisms. Recently, researchers have facilitated to understand the disease-causing pathological and phytophysiological mechanisms. 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 of OMICS technologies in combination with computational and imaging technologies have provided new language to the understanding of occurrence, mechanism, and prevention of disease. Now molecular mechanisms of many acquired and inheritable diseases have been delineated. The 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 in understanding 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 and develop new strategies to prevent, diagnose, and treat them. Its 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 now also termed "translational" researchers. They are trained to be knowledgeable in the primary and clinical biomedical sciences and proficient in patient care.

The book *Biomedical Translational Research* is a platform for clinical researchers, basic scientists, biomedical engineers, and computational biologists from different countries to express their experiences and futuristic thoughts in the form of chapters.

"Biomedical Translational Research" has been compiled in three volumes, i.e., volume I summarizes 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 book, which is the third volume of biomedical translational research, focuses on the fundamental role of biomedical research in the development of new medicinal products. It emphasizes on the importance of understanding biological and pathophysiological mechanisms underlying the disease for the discovery and early development of new biological agents. The book comprehensively reviews different genomic and microfluidic and computational approaches that employ to guide drug repositioning. It also summarizes the major challenges in drug development and the rational design of the next generation of more effective but less toxic therapeutic agents.

The editors are thankful to all the authors who have contributed chapters to this volume. R.C. Sobti acknowledges the support given by his wife Dr. Vipin Sobti, daughters Er. Aditi and Dr. Aastha (their spouses Er. Vinit and Er. Ankit), and grand daughter Irene in preparation of the manuscript of this volume. The platform to prepare the volume was given by the Indian National Science Academy under their Senior Scientist Program.

Chandigarh, Chandigarh, India — R. C. Sobti
Winnipeg, Manitoba, Canada — Naranjan S. Dhalla

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About the Editors

R. C. Sobti is an Emeritus Professor at Panjab University. He is also Senior Scientist of Indian National Science Academy, New Delhi. He is a former Vice Chancellor of 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-cellularization protocol. He has published more than 300 research articles in 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 of Life Time Achievement Awards of the Punjab Academy of Sciences, Zoological Society of India, and the Environment Academy of India. He was awarded the third highest award Padmashri by the Government of India in 2009 for his contributions to science and education.

Naranjan S. Dhalla is a principal investigator, Experimental Cardiology, Institute of Cardiovascular Sciences; Distinguished Professor, Max Rady College of Medicine; Professor of Physiology and Pathophysiology, University of Manitoba; and Senior Fellow, Centre for the Advancement of Medicine. Dr. Naranjan S. Dhalla has received more than 170 honours and awards from all over the world. These include the Order of Canada, Order of Manitoba, Order of the Buffalo Hunt from the Province of Manitoba, Fellowship in the Royal Society of Canada, Medal of Honour of the Canadian Medical Association, Research Achievement Award of the Canadian Cardiovascular Society, Chair in Cardiovascular Research, and Honorary Professorship at different universities.

Dr. Dhalla was elected 2nd Greatest Manitoban of All Time and was featured in "Greatest Manitobans", a book published by the Winnipeg Free Press. Dr. Dhalla has published a large number of research papers and book chapters. He has travelled widely and has addressed various national and international conferences.

Part I

Basic Aspects

1 Emerging Technologies: Gateway to Understand Molecular Insight of Diseases, Newer Drugs, Their Design, and Targeting

R. C. Sobti, Mamtesh Kumari, Mandakini Singhla, and Ranjana Bhandari

Abstract

In the present time, our understanding of disease pathogenesis has changed significantly due to the advent of newer technology and recent scientific breakthroughs. The network models consisting of the genomic regions are being prepared by combining the developed molecular phenotyping profiling with deep clinical phenotyping, which can influence the levels of transcripts, proteins, and metabolites and can be exploited in various ways in diagnosing diseases and personalized drug development. Digital biomarkers (BM) can support in disease diagnosis in multiple ways, including patient identification to treatment recommendation. The use of "omics" technology and large sample sizes has resulted in vast data sets, providing a wealth of knowledge about different illnesses and their links to intrinsic biology. The analysis of such extensive data requires sophisticated computational and statistical methods. New data can be converted into usable knowledge to allow for faster diagnosis and treatment choices using these advanced technologies, such as artificial intelligence, machine learning algorithms, computational biology, and digital BMs. As a result, it is expected that such advancements would aid in the fight

R. C. Sobti
Department of Biotechnology, Panjab University, Chandigarh, India
e-mail: rcsobti@pu.ac.in

M. Kumari (✉)
Department of Zoology, Radhe Hari Post Graduate College, Kashipur, Uttarakhand, India
e-mail: Mamteshkumari2016@gmail.com

M. Singhla
Department of Zoology, Panjab University, Chandigarh, India

R. Bhandari
University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh, India

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against infectious disorders, epidemics, and pandemics. Hence, in this article, we would explore the importance of various AI tools that can be utilized for drug discovery and precision medicine.

Keywords

Molecular phenotype · Clinical phenotype · Personalized drugs · Digital biomarkers · Omics · Artificial intelligence · Machine learning algorithm · Computational biology

1.1 Introduction

In determining the causes of different illnesses, we are in a higher situation than at any other time in history. Our understanding of disease pathogenesis has changed significantly due to the advent of newer technology and recent scientific breakthroughs. Scientists today have a much more refined view of living molecules, and science is advancing toward understanding the pathophysiology of disease at the molecular level of living beings ranging from humans to plants, such as dementia, cancer, heart disease, and diabetes, which are developed during a person's life span. New and innovative diagnostic techniques for genetic disorders are now being developed. Epigenetic modifications, in tandem with genomics and genetics, are helping to explain and control several diseases. The construction of causal network models consisting of the genomic regions has become possible by combining the developed molecular phenotype profiling with deep clinical phenotyping. These network models can influence the levels of transcripts, proteins, and metabolites and can be exploited in various ways in diagnosing diseases and personalized drug development. Digital biomarkers (BM) may help with disease diagnosis in multiple ways, including patient identification to treatment recommendation. Individualized healthcare programs, custom-specific nutrition, living practices, and better therapies will benefit from this kind of treatment. The use of "omics" technology and large sample sizes has resulted in vast data sets, providing a wealth of knowledge about different illnesses and their links to intrinsic biology. The analysis of such extensive data requires sophisticated computational and statistical methods. Insignificant data processing, artificial intelligence (AI), and deep machine learning algorithms are beneficial. New data can be converted into usable knowledge to allow for faster diagnosis and treatment choices using these advanced technologies such as artificial intelligence, machine learning algorithms, computational biology, and digital BMs (Seyhan and Carini 2019). As a result, it is expected that such advancements would aid in the fight against infectious disorders, epidemics, and pandemics. Hence, in this article, we would explore the importance of various AI tools that can be utilized for drug discovery and precision medicine.

1.2 Advanced Technologies for Control of Infectious Diseases

Novel diseases have arisen in different parts of the world over the past few years. The most recent example is the COVID-19 virus, which has spread across the globe and becomes a pandemic. Infectious diseases may be caused by a new pathogen or reemerge in a population or geographic region, spreading locally or globally depending on the mode of transmission and pandemic potential. Outbreaks of various viral infections around the world emphasize the significance of surveillance networks for enormous infectious diseases. This includes the SARS epidemic in 2003, the H1N1 influenza pandemic in 2009, the reemergence of *Chikungunya* virus, the outbreaks of Middle East respiratory syndrome coronavirus (MERS-CoV) and Ebola virus in West Africa, and the latest outbreak and dissemination of COVID-19 virus, which has caused widespread disruption across the world.

On the other hand, these novel diseases can be linked to not only human factors, such as population density, transport, and migration from one location to another, but also environmental factors, such as agricultural practices and climate change. In the healthcare industry, newer and more sophisticated technologies are being developed rapidly, and as a result, these technologies are becoming more widely available and accessible. These may be effective in monitoring and stopping the transmission of infectious diseases.

Highly advanced molecular technology can not only assist in the accelerated identification of pathogens at the molecular level but can also be used to more accurately track the activities of infectious diseases. Public health institutions employ software focused on Web-based surveillance and epidemic intelligence approaches to enable risk management and predict outbreaks in time during situations such as epidemics or pandemics (Allam and Jones 2020; Yu et al. 2020; Michelozzi et al. 2020; Chu et al. 2020; Ricoca Peixoto et al. 2020). Event-based surveillance systems (e.g., GPHIN, ProMed-mail, HealthMap, EpiSPIDER, BioCaster) are among the applications used to track outbreaks and emerging health risks, such as SARS. Real-time tracking of epidemic incidence is done using Web-based real-time surveillance (e.g., Google Trends, Google Flu Trends); Early Warning, Alert, and Response Networks (such as GOARN) are used to identify interinstitutional contact, public health risks, and the execution of preventive and control measures (i.e., WHO Global Alert and Response). Infectious disease modeling, such as agent-based model and metapopulation models (GLEAM, FRED, gravity model), is used for epidemic simulation assessment of disease spread determinants. Such social media benefits in reporting and advising people about the state of a given infectious disease and hence plays a vital role in participatory epidemiology, for example, illnesses of food, seasonal flu activity, etc. New technology, such as genome-wide sequencing, microarrays, and bioinformatics, aids in the discovery of pathogens and viruses, wildlife sampling and surveillance, predictive modeling, and drug discovery by numerous approaches, such as detecting existing therapeutic agents, early detection of COVID-19, discovering the genetic sequence of COVID-19, and its categorization as well as an exploration of COVID-related antiviral and potential biomarkers (protein targets) (Yu et al. 2020; Christaki

2015; Tahir Ul Qamar et al. 2020; Cleemput et al. 2020; Elfiky 2020; Ahmed et al. 2020; Wang 2020; Kim et al. 2020; Yue et al. 2020).

It is critical to managing an epidemic of infectious diseases that threaten public health. Currently, the COVID-19 pandemic has emerged as a significant threat to the world, and its management is of immense importance for every nation to save humanity. In this respect, information technology will play a critical role, as its use in COVID-19 emergency management in terms of prevention/mitigation, preparedness, response, and recovery is vital. A variety of IT-based systems may be helpful in the direction of outbreaks, especially during the response phase. Surveillance technologies, artificial intelligence, computational methods, remote sensing sensors, Internet services, and geographic information systems (GIS) are among them (Asadzadeh et al. 2020). Many other viral diseases, such as H1N1, SARS, and MERS, have benefited from information technology (Cai et al. 2005; Xie et al. 2005; De Groot et al. 2013; Bogoch et al. 2016; Lan et al. 2016; Sandhu et al. 2016; Francis et al. 2017; Rovetta and Bhagavathula 2020; Song et al. 2020).

1.3 Role of Artificial Intelligence (AI) in Epidemiology

The propagation of infection can be detected using artificial intelligence. In the case of the ongoing COVID-19, a health monitoring AI platform, "BlueDot," located in Toronto, used big data analytics to map and forecast the virus's transmission from Wuhan to Tokyo after its first arrival (The Medical Futurist 2020). The use of deep learning algorithms, which assist in resolving complex problems and improving the reliability of performance, is the concept on which AI operates. Consequently, AI assists in the accelerated detection of positive cases and the control and prevention of COVID-19 outbreaks (Yu et al. 2020; Hu et al. 2020; Xu et al. 2020; Xie et al. 2020; Srinivasa Rao and Vazquez 2020; McCall 2020; Vaishya et al. 2020; Ghoshal and Tucker 2020; Zhang et al. 2020; Bherwani et al. 2020).

Because of its numerous strengths, AI has been seen to be effective in protecting healthcare personnel by supplying them with reliable knowledge and guidance (McCall 2020). Deep learning has been used in several studies, including lung infection quantification, tracing, improving diagnosis, patient management, fast screening, and drug discovery (Asadzadeh et al. 2020; Shan et al. 2020).

1.4 Drug Discovery-Associated Technologies

In "omics" techniques, such as genomics, epigenomics, transcriptomics, proteomics, and metabolomics, significant advances have been made. These are also known as system-based methods, and they can profile and monitor molecular markers, such as biomarkers (BMs), for a variety of diseases by combining clinical, physiological, and pathobiological anomalies. This helped clinicians and scientists develop a learning data set that allowed them to obtain a deeper understanding of disease pathogenesis at the molecular level.

Various genetic or epigenetic changes cause autoimmune disorders, infectious diseases, transplantation, and even cancer by DNA methylation and altered miRNA expression. Mutated epigenetic regulators, such as histone acetylation and methylation, are the most impaired epigenetic pathways in cancer. These abnormal epigenetic modifications in cancer have been revealed by sequencing chromatin modifications with deep sequencing technologies. Scientists are focusing on developing personalized drugs using "omics technologies." Appropriate biomarkers are needed to carry out specific therapies with each patient. Scientists are highly optimistic about "omics-based" healthcare interventions because knowledge in the fields of genomics and transcriptomics as well as understanding of the potentials of modified proteomes has grown significantly. Some of the essential biomarkers, such as altered gene expression signatures, germline or somatic gene variations (i.e., polymorphisms, mutations), chromosomal defects, and chosen protein biomarkers, functional disorders with a genetic etiology, are used to select therapies for patients; which are linked to pharmacogenomic knowledge available in medication labeling (Chow 2017). New advancements in proteomics have the potential not only to improve health outcomes but also to reduce the expense of therapies (Matthews et al. 2016; Aravanis et al. 2017; Quezada et al. 2017). One such technique is liquid biopsy, which involves collecting and analyzing molecules from body fluid, such as urine, sweat, whole blood, serum, and plasma. A large number of biomarkers, such as circulating tumor cells (CTC); circulating tumor proteins; cell-free DNA (cf-DNA); cell-free RNA (cf-RNA), including microRNAs (miRNAs); and extracellular vesicles, especially exosomes, have been identified as circulating molecules. These biomarkers effectively recognize the very early stages of cancer, preneoplastic disorders, etc., demonstrating their practical necessity for patient survival (Moutinho-Ribeiro et al. 2019) (Fig. 1.1).

1.5 Speedy Drug Discovery with Artificial Intelligence (AI) and Machine Learning

Artificial intelligence is widely used in the drug discovery process due to its many capabilities. AI will gather and interpret biomedicine knowledge quite effectively to adopt patient-driven biology and accumulate data for deriving more predictive hypotheses. It helps in the development of novel patient-specific drugs by specifically identifying and validating drug targets. It may also be used to repurpose medications, thus increasing research and development quality (R&D). Artificial intelligence is being used to track down drug targets and therapies for disorders such as Parkinson's disease and diabetes. It can solve both simple and complicated problems by learning from its past solutions and personified experience. As a result, advances in AI technology, along with dramatically increased computational resources, are revolutionizing the drug discovery process (Fleming 2018; Mak and Pichika 2019).

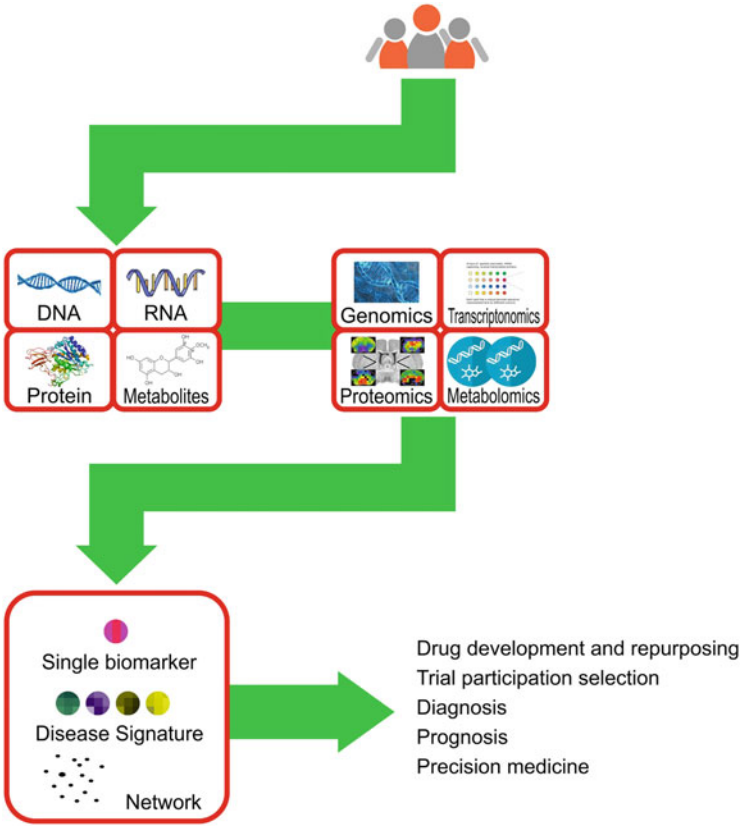

Fig. 1.1 Precision medicines can be benefitted by "omics technologies" by identifying new disease biomarkers, for diagnostics, prognosis, patient stratification, and drug development as well as repurposing

1.6 How Do AI Platforms Work?

BenevolentBio, a London-based start-up, has developed its own AI platform. The platform receives data from various sources (clinical trials, patient records, patents, research papers, etc.). A representation of billions of known and drawn-out relationships between biological entities, such as genes, proteins, species, tissues, symptoms, diseases, and candidate drugs, is designed using the cloud. Such information can be used to learn more about how a gene is linked to a specific medical condition and which compounds can be used to treat it. As a result, AI will bring all of this evidence into perspective and provide the essential information to drug-development scientists (Fleming 2018). In silico development of new compounds with desired activity has been made possible due to artificial intelligence. When AI is

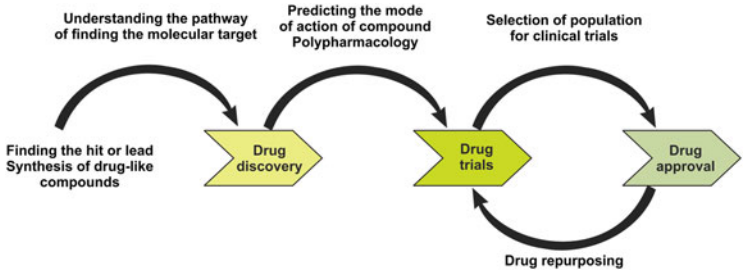

Fig. 1.2 Artificial intelligence in drug discovery

used in combination with computational de novo technology, it assists in extracting data from previously defined compounds, allowing for the development of chemically accurate and biologically active compound structures (Oskooei et al. 2018) (Fig. 1.2).

- One of the advanced technologies is the recurrent neural networks (RNNs). RNN produces the new character strings corresponding to molecules within the chemical space. In this way, many more drug-like molecules can be synthesized, matched with the drug target information to be placed in a particular region of chemical space of drugs (Eureka 2019a).
- An open-source software, the "CellProfiler" can quantitatively measure the phenotypes from thousands of images by automatically recognizing the cells and measuring their properties in the image (Steensberg and Simons 2015). The phenotype of cells is then recorded using "CellProfiler Analyst." For example, a healthy or a diseased cell can be taken to compare their morphology from a patient. Their profile difference can be used as a diagnostic tool (Eureka 2019b). Other open-source applications, such as PaccMann, INtERAcT, and PIMKL from IBM Research, Zurich (Switzerland), are also available these days (Manica and Cadow 2019). PaccMann's sensitivity of cancer cells is predicted by incorporating transcriptomics, cellular protein interactions, and compound molecular structure (Oskooei et al. 2018). Likewise, INtERAcT uses unsupervised machine learning to scrutinize cancer research publications and draw interactions, such as protein-protein interactions. Similarly, a machine-learning algorithm PIMKL is used to infer phenotype from multi-omics data.
- Pharmacovigilance (drug safety science) is the science of collecting, detecting, assessing, monitoring, and preventing adverse drug reactions (ADRs). Since there is now such a massive amount of data available, AI and machine learning will enhance the above processes. Due to the expanded compilation of electronic health records (EHRs) and access to freely accessible resources, the use of AI approaches for pharmacovigilance is growing day by day. Machine learning (ML) and deep learning (DL) techniques are now being used to replace conventional strategies, such as quantitative structure-activity relationships (QSAR) for determining preclinical safety (Kantarjian et al. 2012).

A high-performance screen (HTS) is handy to researchers as it helps to classify a good selection of active compounds that can influence a particular metabolic pathway of interest and, consequently, can move to an essential pipeline for discovery and validation. As a result, it's been commonly used in the early phases of drug production for a long time. HTS robotics is used in two situations: first, where the drug's target is unidentified and phenotype screening is needed. Second, it's used for target-based screening, in which researchers attempt to alter the action of a known protein of interest by activating or inhibiting it. HTS robotics can speed up the process of drug discovery through automated screening. Similarly, mass spectrometry (MS) is a technique that uses observations of interactions between small-molecule proteins to detect the smaller molecules more thoroughly. MS and fragment-based drug discovery (FBDD) work as a powerful tool in discovering drugs at initial points.

Along with MS, many other techniques for FBDD are surface plasmon resonance (SPR), X-ray crystallography, nuclear magnetic resonance (NMR), and isothermal titration calorimetry (ITC). Through the FBDD technique, tiny molecules (fragments), about half in size than the size of standard drugs, are recognized and then spread or joined together to produce drug leads. Magnetic resonance mass spectrometry (MRMS) was earlier known as Fourier transform mass spectrometry (FTMS). This technology's fragmented screening capabilities are improved many folds (Sally-Ann Poulsen, Professor of Chemical Biology, Griffith Institute for Drug Discovery (GRIDD), Griffith University, 2019).

1.7 Heading Toward Precision Medicines (PM) Through Innovation and New Technologies

The frequent and everyday use of companion diagnostics (CDX) and biomarkers (BMs) has the capability of shifting from empirical medicine to precision medicine (PM) (Steensberg and Simons 2015; Seyhan and Carini 2014). Precision medicine is designed according to the individual patient's genetics or biochemistry, which relies on measurements of particular, objectively quantifiable biomarkers in patient samples to match treatments. Biomarkers may be predictive, prognostic, or both for each specific disease. In precision medicines, treatment is provided only to patients who have compatible chemistry for that particular drug, thus avoiding the stress in noncompatible patients taking non-required treatments and getting any potential toxic side effects. It thus also helps in saving high costs related to such treatments. The efficacy of precision medicines is proved by their ability to treat the diseases such as cancer and autoimmune conditions, which remained unresponsive to traditional therapies. Novartis developed a tyrosine kinase inhibitor, also called Gleevec (imatinib). It is an excellent example of the success of precision medicine. Patients who were suffering from chronic myeloid leukemia were treated with Gleevec as a first-line treatment. Their survival rate was enhanced by 83.3% as they lived for 10 more years as compared to the 43–65% survival rate with earlier treatments.

Similarly, Herceptin (trastuzumab) has dramatically boosted the survival rates of patients suffering from early and metastatic HER2-positive breast cancer. With chemotherapy, the survival rate used to be 75.2%, which increased to 84% after the use of Herceptin (Roy et al. 2006; Kantarjian et al. 2012; Hochhaus et al. 2017; Medicine 2019). To develop precision medicines, it is imperative to combine the clinical indices with molecular profiling so that diagnostic, prognostic, and therapeutic strategies could be established. Therefore, it is crucial to interpret the data accurately for best use of PM ecosystem as it integrates "omics" and clinical data to find the best course of action to be taken for each specific patient group (Seyhan and Carini 2019) (Fig. 1.3).

1.8 Summary and Conclusion

Presently the multidisciplinary research teams are working on a plethora of many different new emerging and advanced technologies for the discovery of modern drugs. It is essential to use advanced technologies such as experimental and computational approaches in integration with the drug discovery programs as it puts high synergistic effects on the selection and optimization of bioactive compounds. The technologies such as VS, HTS, SBDD, LBDD, QSAR, and so on have their mutual goals and are thus complementary. Therefore, the integration of both empirical and silico efforts is feasible at many different lead optimization levels and new chemical entity (NCE) discovery. The biomedical community is now applying artificial intelligence (AI) and machine learning algorithms to manage and study huge available data. The development and application of wearable medical devices (e.g., wearable watches), mobile health applications, and clinical outcome data contributed to the accumulation of substantial data through various molecular profiling (genetic, genomic, proteomic, epigenomic, and others) efforts of patient samples. The technological advancement generates new research opportunities in predictive diagnostics, virtual diagnosis, precision medicine, patient monitoring, and drug discovery and delivery for targeted therapies. Along with the scientific communities, the academics, industry researchers, and regulatory agencies are filled with enthusiasm with these new advancements, and physicians are being provided with novel tools. Thus, the application of new medical practices can be enhanced by the use of these highly advanced new age technologies, such as BMs, omics data, artificial intelligence, and deep machine learning.

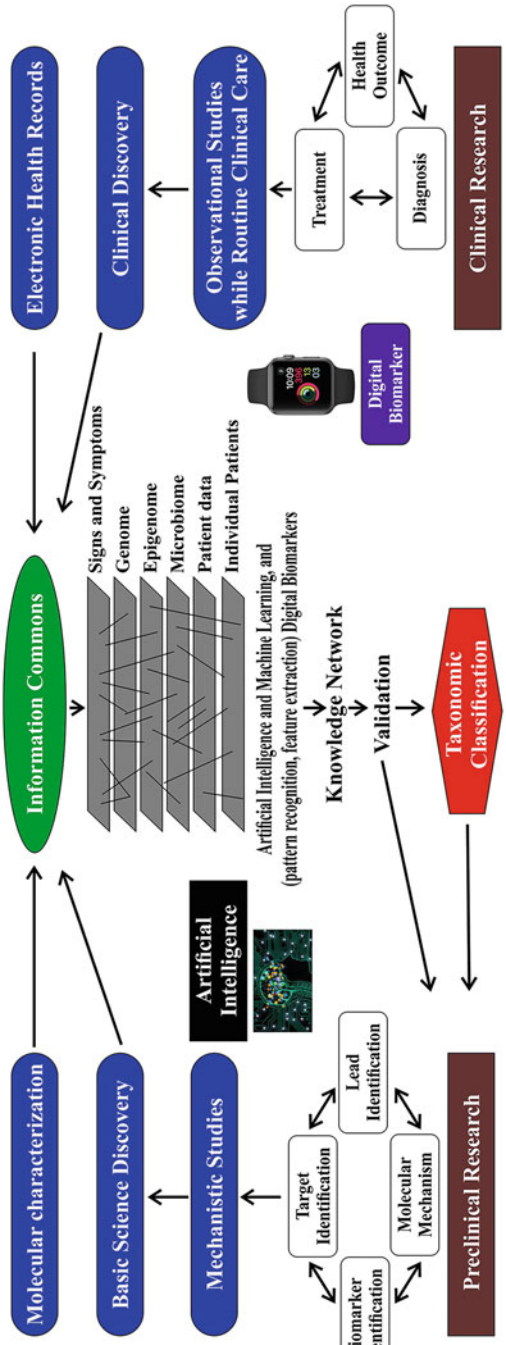

Fig. 1.3 The way comprehensive biomedical knowledge network supports a new taxonomy of disease

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Polypharmacology: New Paradigms in Drug Development

2

Tammanna R. Sahrawat and R. C. Sobti

Abstract

Polypharmacology is the term coined for the interaction of drug molecules with multiple targets and is responsible for the side effects and toxicities of drugs. The advent of various in silico computational approaches would help to identify multi-target activates and anticipate potential selectivity issues early in the iterative drug design process. This would not only prevent costly failures but also offer the possibility of identification of novel applications of existing drugs through drug repurposing. These in silico approaches can be classified broadly into ligand- and structure-based approaches, data mining and network systems biology. This review intents to summarize the various computational techniques that have been developed to study polypharmacology in the drug development process. Polypharmacology has emerged as a new paradigm in drug discovery as it promises to provide novel avenues to rationally design the next generation of more effective but less toxic therapeutic agents.

Keywords

Polypharmacology · Off-targets · Multi-target · In silico · Drug discovery

T. R. Sahrawat (✉) · R. C. Sobti
Centre for Bioinformatics and Structural Biology and Department of Biotechnology, Panjab University Chandigarh, Chandigarh, India
e-mail: tammanna@pu.ac.in

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2.1 Introduction

In the past decades, the predominant paradigm in drug discovery was the "one drug, one target", in which highly selective drugs were designed for individual targets. This methodology was extremely successful for diseases with well-defined mechanisms, aetiology and pathophysiology (Hopkins and Groom 2002; Zambrowicz and Sands 2003). But a major pitfall of such a reductionist approach of drug design was that one drug acts on a single receptor, "is blind" to other processes which are inevitably connected in view of the hierarchical nature of biological systems (Maggiora 2011). The mechanisms of some diseases such as cancer are much more complicated as they stem from multiple genetic alterations, and therefore, addressing a single target is usually insufficient to cure or contain such diseases. Treatment of cancer with as single kinase inhibitor has been shown to be insufficient in case of cancers of lung, breast, colorectal, pancreatic and prostate (Yang et al. 2017). Therefore, the development of drugs that targets multiple proteins or pathways holds more promise in the treatment of multi-target complex diseases.

The approval rate of new drugs has been decreasing in the recent times, and some marketed drugs had to be withdrawn due to their unexpected side effects (Connolly et al. 1997; Rothman et al. 2000). An interaction with unintended targets is one of the main reasons behind drug side effects and toxicities. For instance, terfenadine, an H1 receptor antagonist, launched in 1982, was later withdrawn from the market as it caused a life-threatening ventricular tachyarrhythmia, torsades de pointes, due to its interaction with human ERG causing its blockage (Du-Cuny et al. 2011). Therefore, identification of possible off-targets of drugs during early stages of drug discovery may go a long way to prevent costly failures, since drug discovery is a complex, time-consuming and expensive process.

Numerous drugs are known for their multi-targeting activities. An illustrative example is aspirin that has been clinically used as an analgesic or antipyretic which has been found to acts as an anti-inflammatory medication to treat rheumatoid arthritis, pericarditis and Kawasaki diseases. Additionally, it has been also used in the prevention of transient ischemic attacks, strokes, heart attacks, pregnancy loss and even cancer (Reddy and Zhang 2013). In recent years, there is shift of drug design paradigm towards polypharmacology, which is the ability of small molecules to interact with multiple proteins. It is of much interest, as it has implications in therapeutic efficacy, anticipating adverse reactions of drugs and to discover the unknown off-targets for the existing drugs (also known as drug repurposing) (Connolly et al. 1997; Reddy and Zhang 2013; Sahrawat and Chawla 2016). For instance, the blockbuster drug sildenafil (Viagra), a phosphodiesterase (PDE) inhibitor, was initially developed for hypertension and ischemic heart disease. During phase I clinical trials, its side effect of inducing penile erections was reported, and after phase II clinical trial failure, sildenafil was repurposed for the treatment of erectile dysfunction (DeBusk et al. 2004) and received FDA approval in 1998.

The concept of polypharmacology has been receiving unprecedented attention in recent years. Using the keyword "polypharmacology" in a Google Scholar search generated 8570 hits in the first week of April 2019 as compared to 3840 results as of

March 16, 2016, reported by Tan et al. Polypharmacology approach of drug design involves systematic integration of the data derived from different disciplines, such as computational modelling, synthetic chemistry, in vitro/in vivo pharmacological testing and clinical studies (Yamanishi et al. 2008; Dar et al. 2012) and is encouraging the shift to experimental and computational multi-target approaches (Hopkins 2007).

2.2 Polypharmacology Studies Using In Silico Approaches

In recent times, a number of computational approaches, such as bioinformatics, ligand- and structure-based methods, ligand binding site similarity comparison, network systems biology and data-mining-based methods, have been applied to the study of polypharmacology (Tan et al. 2016). This review aims to summarize some of the recently developed computational tools, databases and web servers that are being used to study polypharmacology to identify possible off-targets of drugs and for repurposing of known drugs.

2.2.1 Ligand-Based Methods

The basic principle of ligand-based target identification methods is that similar receptors bind similar ligands. Over the past decade, there has been a rapid growth in biological databases and biology-related web resources that makes huge amount of chemogenomics data freely available to the research community. Databases such as ChemBank and Chemical Entities of Biological Interest (ChEBI) contain information of biologically important small molecules; UniProtKb and Protein Data Bank (PDB) contain protein information, whereas protein-ligand interactions are present in BindingDB, Therapeutic Target Database (TTD) and ChEMBL. These databases contain an enormous amount of complex data matrices, which cannot be analysed using traditional computational tools for studying target-ligand interactions. Therefore, to handle the "big data" problem, ligand-based target fishing approaches are used that are based upon machine learning models or similarity-based screening. In the former approach, compounds are classified on the basis of activity prediction using Binary kernel discrimination, naive Bayesian classifier, artificial neural networks and support vector machine (SVM) (Lavecchia 2015), and a training data set with known characteristics (active or inactive) is essential. In similarity-based target fishing, the protein targets for screening are initially determined followed by identification of ligands to represent those targets and finally the similarity method for comparing ligands is selected. The ligand-based approaches have advantages such as not being dependent upon the availability of 3D structure information of the target and faster descriptor calculations. Their disadvantages include false-positive results due to high similarity of inactive and active compounds, or no hits may be obtained in the absence of ligand-target interaction information in the databases.

2.2.2 Similarity Search Based on 2D and 3D Descriptors

In a 2D representation, the molecule is represented as a graph, without spatial coordinates of the atoms. The atoms are represented as nodes and the bonds as edges. A number of approaches such as SPiDER (Reker et al. 2014), self-organizing maps (SOM) and similarity ensemble approach (SEA) have been developed for in silico identification of ligand-target interactions. SEA is used to identify molecular targets based on set similarities of their respective ligands (Keiser et al. 2007), and using this approach, Lounkine et al. had predicted the activity of 656 marketed drugs on 73 unintended side-effect targets, and nearly 50% of these predictions were later experimentally confirmed (Lounkine et al. 2012).

A major determinant of biological activity are the 3D characteristics of a molecule, as drug pairs that share high 3D similarity but low 2D similarity (i.e. a novel scaffold) were found to exhibit pharmacologically relevant differences in terms of specific protein target modulation (Yera et al. 2011). In chemogenomics research for 3D similarity searching, most commonly used measures are shape- or pharmacophore-based similarity (Willett 2009; MacCuish and MacCuish 2014). Rapid overlay of chemical structures (ROCS) developed by OpenEye Scientific Software is a commonly used shape-based platform that has been used for drug repurposing studies (Méndez-Lucio et al. 2014), in reprofiling existing FDA-approved drugs (Vasudevan et al. 2012) and to identify off-targets for several drugs (Abdul Hameed et al. 2012). Recently, there has been a surge in the development of computational tools for 3D similarity search, which include Gaussian ensemble screening (GES), computational polypharmacology fingerprint (CPF) and feature point pharmacophores (FEPOPS) (Jenkins et al. 2004; Pérez-Nueno et al. 2012, 2014).

2.2.3 Structure-Based Methods

These methods predict the binding of a ligand to the target whose 3D structure has been obtained experimentally by X-ray crystallography or NMR. In their absence, homology-based models may be used, but due to their low reliability, the off-target predictions are less accurate. Using the 3D atomic coordinates of the target, molecular docking predicts binding orientation and binding affinity of molecules.

2.2.4 Inverse Docking

The technique of inverse/reverse docking, i.e., docking ligands against a variety of targets is being used for target prediction, and subsequently, the ligands are scored according to their binding affinity scores with the targets (Rognan 2010; Koutsoukas et al. 2011). Tools such as idTarget, INVDOCK, TarFisDock and DRAR-CPI have been designed for inverse docking to predict the targets and/or side effects of various ligands (Chen and Ung 2001; Li et al. 2006; Luo et al. 2011;Wang et al. 2012).

INVDOCK performs docking of low-weight ligands into cavities of each target using a computer-automated search for potential protein and nucleic acid targets. It ranks the ligands based on molecular mechanic interaction energy and competitive binding analysis (Chen and Zhi 2001). TarFisDock is another inverse docking tool (Li et al. 2006) and ranks the ligand-protein interaction in terms of binding energy (Shoichet and Kuntz 1993). idTarget optimizes search space by dividing the potential target into small boxes based on the size of the ligand followed by identification of binding sites using an optimized MEDock algorithm (Wang et al. 2012). Conventional docking programs such as AutoDock Vina and Glide software (McMartin and Bohacek 1997; Morris et al. 1998) have also been adapted to incorporate the feature of reverse docking (Rognan 2010).

2.2.5 Multi-Target Drug Design (MTDD)

This approach is promising for neurological disorders and cancers that are complex multifactorial diseases. Better therapeutic efficacy and safety is known to be achieved by designing individual new chemical entities that can simultaneously target different points of a given pathogenic cascade. MTDs have been shown to have a higher synergistic effect as compared to a combination of drugs (Bottegoni et al. 2012). They are developed using either of the two available strategies: a fragment-based approach, involving the combination of pharmacophores from selective, single-target ligands, and a single, multitasking computational model, involving screening of compound collections to identify compounds with a suitable combination of activities by simultaneous application of multiple computational models. A hybrid molecule with a dual mode of action that has been designed is donecopride, which is a novel drug candidate for Alzheimer's disease that has been shown to exhibit dual binding site inhibitory effects (Lecoutey et al. 2014).

2.2.6 Multi-Target Virtual Ligand Screening (VLS)

Rational drug design project to identify multi-target hits can begin following the identification and validation of a suitable combination of targets. High-throughput screening (HTS) can be successfully used to identify initial hits but is time-consuming and expensive for even one target and much more cumbersome when multiple targets are to be considered simultaneously. Therefore, as an efficient and faster alternative to HTS, virtual ligand screening (VLS) is being used for processing large libraries of compounds (Abagyan and Totrov 2001). In VLS, every molecule in the library is tested against an ideal model of activity, and they are ranked by assigning each compound a predicted activity score. Only the top-ranking fractions are analysed using further testing (Jenwitheesuk et al. 2008). VLS applied to multi-targets thereby helps to identify hybrid molecules that can simultaneously bind to the selected targets. Wei and co-workers successfully identified novel anti-inflammatory candidates displaying activity against phospholipase A2 (PLA2) and human

leukotriene A4 hydrolase (LTAH4-h), by designing a common pharmacophore having the combined relevant features from both targets followed by structure-based VLS (Wei et al. 2008).

2.3 Data Mining to Identify Novel Targets from "Big Data": A Network Systems Biology Approach

The explosion in the amount of biological data being generated and freely available to the research community has shifted focus of attention to the development of new techniques for data mining. Data mining involves retrieval, extraction and filtering of valuable data from the "big data" available online, and in polypharmacology, target identification was the first application of data. Ozgur et al. used support vector machine (SVM) methods to construct a gene-disease interaction network and were able to successfully confirm high association between the predicted candidate genes and prostate cancer (Özgür et al. 2008). Similarly, other researchers have used data- and structure-based data mining approaches to predict novel cancer targets and also identify potential targets for cancer imaging and therapy (Pospisil et al. 2006, 2007).

Data mining has also been used to identify unknown relationships between genes and disease using systems biology approaches to analyse polypharmacology. Cheng and colleagues developed a web server, PolySearch, to provide related genes, proteins, metabolites and drugs based on a given disease, or vice versa (Cheng et al. 2008). Other data mining tools such as GeneWays that focuses on Alzheimer's disease and GenCLip are also based upon gene interactions present in molecular networks (Krauthammer et al. 2004; Huang et al. 2008; Wang et al. 2014). Polypharma, a novel database, has 953 ligands complexed with more than two structures of distinct protein families in the RCSB Protein Data Bank (PDB). It has provided some interesting insights into ligand-target interactions, such as multi-target ligands are slightly more hydrophobic and tend to have lower molecular weights (<200 Da) than single-target ligands (Reddy and Zhang 2013; Reddy et al. 2014).

2.4 Drug Repurposing

A direct application of polypharmacology is drug repurposing/repositioning, i.e. identifying a new clinical use for an existing approved drug (Ashburn and Thor 2004; Aubé 2012). A closely related concept is drug rescue, as for the case of sildenafil (Viagra) (DeBusk et al. 2004). In many instances, drug repurposing has occurred by serendipity (Paolini et al. 2006), but now concerted efforts are being made to conduct drug repurposing systematically by envisioning three general strategies, namely, chemical, biological and data mining (Boran and Iyengar 2010). Drug repurposing is primarily a retrospective approach, which offers manifold benefits to the pharmaceutical industry, such as lower drug development costs and reduced time for approval, as shelved drugs can be quickly marketed for new

indications (DeBusk et al. 2004). It is advantageous to the patients as medications for diseases, which were earlier not treatable, can be easily accessed, as knowledge about possible side effects, pharmacokinetics and interactions with other drugs already exists in various online resources. Further, rare diseases wherein no drugs have been developed or discovered can also be targeted with known drugs. To this effect, the US Food and Drug Administration (FDA) launched a database of approved drugs, which are promising to be repositioned to orphan/rare diseases (Schenone et al. 2013). Also, a "high-throughput" in vivo pharmacology platform theraTRACE1 has been developed for drug repurposing (Boran and Iyengar 2010). Repurposing of chemicals and natural products that differ from drugs, such as herbal remedies or compounds used in traditional Chinese medicine (TCM), has led to the advent of TCM database (Baron 2012). Therefore, the availability of a large number of online resources is opening up newer avenues to search for targets of active components using computational approaches.

2.5 Concluding Remarks

Polypharmacology aims to identify all the possible targets of a given compound. However, it is highly impractical to experimentally test the binding affinities between each drug-target pair for all the possible compounds, genes and proteins. Therefore, drug target prediction using computational technologies plays a significant role to sift through the big data by development of accurate, fast and robust algorithms. These in silico tools based upon integration of knowledge and technologies from varied disciplines, such as cheminformatics, network-systems biology and data mining, have been successfully used to predict possible off-target of drugs that account for their reported side effects and can be used for drug repurposing and design of combination therapies. The fact that several drugs exert their effect through the interaction with multiple targets is shifting the drug discovery paradigm from the one target-one drug model to a multiple-target approach. This has also been necessitated by the multi-faceted nature of various complex diseases, such as neurodegenerative disorders and cancer. In spite of tremendous challenges that lie ahead, in the years to come polypharmacology would have a major role in transforming next-generation drug discovery and development.

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Drug Repurposing in Biomedical Research: Benefits and Challenges

3

Aashish Sharma and Jagdeep Kaur

Abstract

Drug repurposing is establishment of new medical uses for already known drugs. This is the new trends/options to combat the difficult diseases with already existing drugs as the development of new drugs is lengthy and cost-effective process. The unexpected success paved way to support repurposing of existing experimental, approved, discontinued, or shelved drugs for several diseases. This article provides a brief synopsis about repurposing and particularly evokes its most recent scientific basis used to render it more efficient.

Keywords

Drug repurposing · Itraconazole · Digoxin · Nitroxoline · RNA viruses · DNA viruses

3.1 Introduction

The repositioning of an active pharmaceutical ingredient that is already available in the market for a new indication is referred to as drug repurposing. Albeit a number of drawbacks and challenges associated with this strategy, it still possesses many advantages, which include overcoming the abrasion currently associated with the field of drug discovery (Pushpakom et al., 2018). Several repositioned drugs, including some very old drugs, have been used throughout the course of medicine historically. This repositioning of drugs was purely through serendipity in those times. In the present scenario, novel methods based on data mining have been

A. Sharma · J. Kaur (✉)
Department of Biotechnology, Panjab University, Chandigarh, India
e-mail: jagsekhon@yahoo.com

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developed for identification of new candidates for drug repurposing (Mercorelli et al., 2016, 2018). The success of drug repositioning in providing benefits in certain diseases brought the global attention on the potential off-target effects of some of the drugs (Yan et al., 2014; Yeo et al., 2018). In view of the fact that these existing drugs have been used previously in humans, their dose regimen with favorable pharmacokinetics and pharmacodynamics properties, including any side effects, is already in public domain, making these old drugs useful in new drug discovery.

3.2 Benefits Associated with Drug Repositioning

Drug repositioning has various advantages that are interrelated in nature. The simplification associated with the regulatory procedures involved in introduction of a previously approved drug is the most essential part of it. As the data on the safety and toxicity of the drug is available, this makes the initial development phase of drug repositioning considerably faster (Nosengo, 2016) and therefore cheaper with increase in chances of it being introduced on the market. Although the level of safety required for a particular drug is heavily dependent upon its indication, hence, the acceptance of the adverse effects of a drug when repurposed for a less severe disease than its original indication will be proportionately less acceptable (Cavalla, 2017).

3.3 Challenges Associated with Drug Repositioning

The major challenge faced by the companies working on drug repositioning is the relatively weak intellectual property protection on these products, which can lead to reduction in return on their investment, further discouraging these companies from developing these drugs (Rastegar-Mojarad et al., 2015; Talevi and Bellera, 2020). The drug that is being repositioned had already been patented as a new chemical entity, making the task of protecting it even more difficult involving a new application patent based on a new formulation process. The scope of application patents is narrower in comparison to a new chemical entity in terms of coverage of their therapeutic uses. These patents are also tough to defend from a legal perspective as it can be challenged that the new indication proposed for the drug was predictable from the already available data in the scientific literature. However, there are certain advantages for the companies working on repositioning of drugs for the treatment of orphan diseases (defined in Europe as those with prevalence no higher than 5 in 10,000), such as reduction in fees and guaranteed market exclusivity for a period of time (Xu and Coté, 2011).

3.4 Drug Repositioning Intended for Anticancer Applications

3.4.1 Itraconazole

Itraconazole was developed in 1980s as a triazole antifungal drug and was effective against a variety of systemic fungal infections like other members of the azole family of antifungal drugs. The well-established mechanism of antifungal activity of itraconazole involves the inhibition of cytochrome P450-dependent lanosterol 14-α-demethylation (14DM) in pathway associated with ergosterol biosynthesis in fungi (Vanden Bossche et al., 1988). Antiangiogenic property of itraconazole leads to its usage either alone or in combination with other anticancer drugs in various preclinical models, including medulloblastoma, non-small cell lung cancer (NSCLC), and basal cell carcinoma (Kim et al., 2010; Aftab et al., 2011; Tsubamoto et al., 2017).

3.4.2 Digoxin

Digoxin, a cardiac glycoside isolated from foxglove, has been historically used for treating heart failure and arrhythmia (Hollman, 1996). Digoxin is a potent inhibitor of Na+/K+-ATPase pump in cell membrane (Rossi et al., 1982). The regulation of sodium ion gradient across the cell membrane by Na+/K+-ATPase leads to intracellular Ca^{2+} ion efflux. The increase in intracellular Ca^{2+} concentration in myocardiocytes and pacemaker cells, resulting in lengthening of the cardiac action potential, is associated with the inhibition of Na+/K+-ATPase by digoxin (Belardinelli et al., 1979). It was later demonstrated that this cardiac glycoside triggered immunogenic demise of the cancer cells (Kepp et al., 2012).

3.4.3 Nitroxoline

Nitroxoline, an antibiotic which has been widely used almost throughout the world since 1960s, is specifically used in the treatment of urinary tract infections (UTI) due to its unique pharmacokinetic property. Oral administration of nitroxoline leads to rapid absorption into the plasma followed by excretion via urine (Mrhar et al., 1979). The long retention time of nitroxoline in urine makes it an ideal candidate for UTI treatment. The mechanism of action of nitroxoline works on its ability to chelate divalent metal ions, such as Mg^{2+} and Mn^{2+}, resulting in its possible antibacterial activity (Pelletier et al., 1995). Recently, the anticancer activity of this antibiotic has been demonstrated in prostate cancer (Chang et al., 2015).

3.5 Drug Repurposing for RNA Virus Infections

3.5.1 ZIKV and Other Flaviviruses

ZIKV is an arbovirus that leads to a large outbreak in Latin America recently. Usually, ZIKV causes a self-limiting disease associated with neurological disorders (such as Guillain–Barré syndrome and others). However, in the case of pregnancy, severe congenital defects, including microcephaly and ophthalmological alterations, have been observed in newborns. The capability of ZIKV to spread from human to human through vertical (transplacental) and sexual transmission makes it a possible candidate of global concern for pandemic (Wikan and Smith, 2016).

3.5.2 Ebola Virus

Since the discovery of EBOV in the late 1970s, several outbreaks have been attributed to it. The most alarming of these outbreaks was in 2014–2016 based on its size and spread, causing an international health emergency while being accidentally imported to nonendemic geographical areas, such as Europe and the USA. The lethal disease caused by this virus is characterized by acute hemorrhagic fever and has fatality rate of 90%. The manipulation of EBOV for developing antiviral drugs requires high level of biocontainment (BSL-4) that hampers the process of vaccine development. The last outbreak of this deadly virus leads to several drug repurposing (DR) studies (Sweiti et al., 2017; Bixler et al., 2017). A DR-based approach was made in clinical trials amid the last outbreak of EBOV by evaluating several drugs in infected patients for testing their ability in protecting for the lethal EBOV. These included the viral RNA polymerase inhibitors—favipiravir (approved in Japan for treating influenza A virus) (Sissoko et al., 2016; Liu et al., 2017), GS-5734 (an adenosine analog actively targeting highly pathogenic coronaviruses) (Warren et al., 2016; Sheahan et al., 2017), and amodiaquine (an antimalarial drug widely used in Africa).

3.5.3 Coronaviruses

Coronaviruses (CoVs) are RNA viruses responsible for respiratory, gastrointestinal, and neurological diseases in animals, including zoonotic infections in humans. The potential of these viruses of cross-species transmission is well established in domesticated animals, which further act as intermediate hosts for infection in humans. Severe acute respiratory syndrome CoV (SARS-CoV), a highly pathogenic CoV, emerged in China in 2002–2003 and was responsible for causing a pandemic with over 8098 infected people with mortality rate of 10%. Trezza et al. used a robust in silico drug repurposing strategy to identify spike protein–ACE2 interaction inhibitors and identified simeprevir and lumacaftor that demonstrated high binding affinity to the receptor-binding domain of the spike protein and prevented ACE2

interaction (Trezza et al., 2020). Recently, studies have demonstrated that the repositioning of chloroquine and hydroxychloroquine for treatment of COVID-19 has proved efficacious (Khuroo, 2020; Oscanoa et al., 2020).

3.5.4 Influenza Virus

Influenza virus belonging to the family *Orthomyxoviridae* is a pathogen of global public health concern as it causes seasonal, pandemic, and zoonotic influenza disease outbreaks. The absence of innate immunity against the pandemic and zoonotic influenza strains makes them a concern, owing to their high potential for transmission among human population. The antiviral treatment for influenza infections includes some options as opposed to other RNA viruses referred previously. It involves usage of adamantanes and neuraminidase inhibitors; however, the rapid emergence of drug-resistant viral strains pose a serious challenge to this approach (Loregian et al., 2014). DR-based approach leads to identify some previously approved drugs demonstrating anti-influenza properties, such as BAY 81-8781 (approved as intravenous aspirin), and demonstrating antiviral activity via blockage of NF-kB pathway activation (Droebner et al., 2017), such as dapivirine (a non-nucleoside inhibitor of HIV-1 retrotranscriptase (Hu et al., 2017), naproxen (which targets influenza nucleoprotein (Lejal et al., 2013)), and the antibiotic clarithromycin. Clarithromycin and naproxen in combination with oseltamivir have been evaluated in phase 2b/3 clinical trial and have shown efficacy in the treatment of severe influenza (Hung et al., 2017).

3.6 Drug Repurposing for DNA Virus Infections

Novel antiviral strategies involving DR have been directed against DNA viruses responsible for latent, lifelong infections that can lead to high morbidity and also be life-threatening for at-risk populations. Human cytomegalovirus (HCMV), a β-herpes virus, causes persistent infection, and in immunosuppressive individuals it can also reactivate (Mercorelli et al., 2016). Several approved or investigational drugs have been identified using DR-based approach for the treatment of HCMV infections that work on different anti-HCMV mechanisms (Gardner et al., 2015; Mercorelli et al., 2016), such as the statins (Ponroy et al., 2015), cardiac glycosides (Kapoor et al., 2012), the antiparasitic drugs emetine and nitazoxanide (Mercorelli et al., 2016; Mukhopadhyay et al., 2016), kinase inhibitors (Arend et al., 2017), and the antihypertensive drug manidipine (Mercorelli et al., 2018). Identification of inhibitors for hepatitis B virus (HBV) using DR campaign identified calcium-channel blockers, antifungal terbinafine, and dopamine receptor antagonist as promising candidates as inhibitors of HBV RNA transcription and DNA synthesis (van de Klundert et al., 2016). Virus-targeting drugs such as HIV integrase inhibitors, demonstrating broad-spectrum activity against various herpesviruses by causing the inhibition of the viral terminase (Nadal et al., 2010; Yan et al., 2014), and

the HIV protease inhibitors lopinavir/ritonavir, which were clinically evaluated for potential treatment of human papilloma virus (HPV)-related preinvasive cervical malignancies (Hampson et al., 2016), also showed potential for repurposing.

3.7 Potential Drugs for Repurposing Against Infectious Agents

3.7.1 Anticancer Drugs Repurposed Against Bacteria and Fungi

The drugs used against cancer have also been demonstrated to exhibit antibacterial activity, particularly against gram-positive pathogens (Soo et al., 2016). Floxuridine and streptozotocin, FDA-approved drugs used in colorectal cancer treatment and pancreatic islet cell cancer, have exhibited activity against *S. aureus* by causing the inhibition of SaeRS two-component system (TCS) (Yeo et al., 2018). Besides causing significant changes in the transcription of *S. aureus* genes, these drugs also inhibited the transcription of other virulence regulatory systems of *S. aureus* (Yeo et al., 2018). Clofazimine (CZM), a drug used for the treatment of leprosy, was repurposed for use against MDR-TB.

3.7.2 Immunomodulatory Drugs Repurposed Against Bacteria and Fungi

Several anti-inflammatory and immunomodulatory drugs have also demonstrated comparatively higher antibacterial activity against gram-positive as compared to gram-negative bacteria and fungi. Celecoxib is a nonsteroidal anti-inflammatory drug (NSAID) showing antibacterial activity by causing the inhibition of DNA and RNA replication, protein synthesis, and cell wall formation while simultaneously reducing the levels of IL-6, TNF-α, IL-1β, and MCP-1 (monocyte chemoattractant protein-1) against various pathogens, including *S. aureus, Bacillus anthracis*, *B. subtilis*, and *M. smegmatis*. With the exception of linezolid, celecoxib has demonstrated synergistic effects with several topical and systemic antimicrobials used against *S. aureus* (Thangamani et al., 2015). Also, antidiabetic drug metformin (MET) was reported to inhibit the intracellular growth of mycobacteria (Singhal et al., 2014).

3.8 Concluding Remarks

The repositioning of drugs for a therapeutic indication besides the one originally associated with their marketing is an upward trend these days. The foremost objective of drug repurposing is to fight against the attrition and high costs that have a dramatic effect on the number of new drugs that are entering the pharmaceutical market, although this approach must be an add-on rather than being an alternative to the quest for the search of novel drugs.

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Computational Methods for Drug Repurposing

4

Sailu Yellaboina and Seyed E. Hasnain

Abstract

Drug repurposing also called drug repositioning or therapeutic switching refers to finding new uses to existing drugs. This method offers an economically efficient pipeline to identify new indications for existing drugs. The candidates for repurposing are essentially marketed drugs or drugs that have been discontinued in clinical trials for reasons other than safety concerns. To date, repurposed drugs have been the consequence of serendipitous observations. However, recent advances in sequencing and high-throughput technologies lead to the generation of enormous amounts of genomic data such as transcriptomics, proteomics and genetic variation leading to paradigm shift in the drug discovery process. Currently, there are a large number of candidate disease genes identified through genome-wide association studies (GWAS) and other approaches. Also, there is a growing amount of data on FDA-approved drugs to treat the disease and several other drugs which are not toxic to humans but failed to treat the diseases. In silico approaches for analyzing and integrating large-scale genomic datasets have been incorporated in the drug repurposing methodologies. Here, we focus on describing existing genomic datasets and computational methods for drug repurposing.

S. Yellaboina (✉)
Department of Biochemistry, All India Institute of Medical Sciences Bibinagar, Hyderabad, Telangana, India
e-mail: bio.sailu@gmail.com

S. E. Hasnain
National Science Chair, Indian Institute of Technology Delhi, Hauz Khas, New Delhi, India

Sharda University, Greater Noida, Uttar Pradesh, India
e-mail: seyedhasnain@gmail.com

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Keywords

Drug repurposing · Therapeutic switching · Serendipitous observations · Genomic datasets · GWAS

4.1 Historical

The sildenafil, the active ingredient in Viagra, was originally developed by Pfizer for the treatment of hypertension and angina pectoris (chest pain due to heart disease). The drug was meant to dilate the heart's blood vessels by blocking an enzyme called phosphodiesterase type 5 inhibitor (PDE-5). The discovery that sildenafil could lead to a penile erection was germinated during clinical trials for treating hypertension, when the nurses saw men with embarrassment lying on their abdomen to hide their penile erections (Krishnappa et al. 2019). It appeared that the blood vessel's dilation was not in the heart but rather in corpora cavernosa by reducing cyclic guanosine monophosphate (cGMP) degradation and thus increases arterial blood flow into penile sinusoids for erection (Boolell et al. 1996a). Subsequent systematic clinical studies on men, with erectile dysfunction without an established organic cause, showed sildenafil to enhance the erectile response to visual sexual stimulation, thus highlighting the important role of the drug in human penile erection (Boolell et al. 1996b; Goldstein et al. 1998).

4.2 Introduction

Drug repurposing involves the investigation of marketed drugs or drugs that have been discontinued in clinical trials for reasons other than toxicity concerns for new therapeutic purposes. In comparison to drug repurposing methods, the traditional drug discovery is laborious, time consuming, expensive, and with a low success rate (Fig. 4.1). The striking benefit of drug-repurposing method over traditional drug discovery is that, for an existing drug, not only preclinical information but also various clinical profiles such as therapeutic index, pharmacokinetic (PK), pharmacodynamic (PD), and toxicity (TD50) are already available; as a result, it reduces the risk of failure at the terminal stage of drug development. Therefore, the drug compound can rapidly enter terminal stage clinical trials, which involves testing of the efficacy to treat the new disease. Due to the rapid growth of computational methods, computing infrastructure, and the explosive large-scale growth of genomic data such as protein-protein interactions, gene expression, and disease gene association data, the cost of drug repurposing is dramatically decreasing. Here, we focus on recent progress in the area of various genomic datasets (Table 4.1) which can be exploited for developing new computational methods and identifying repurposed drugs.

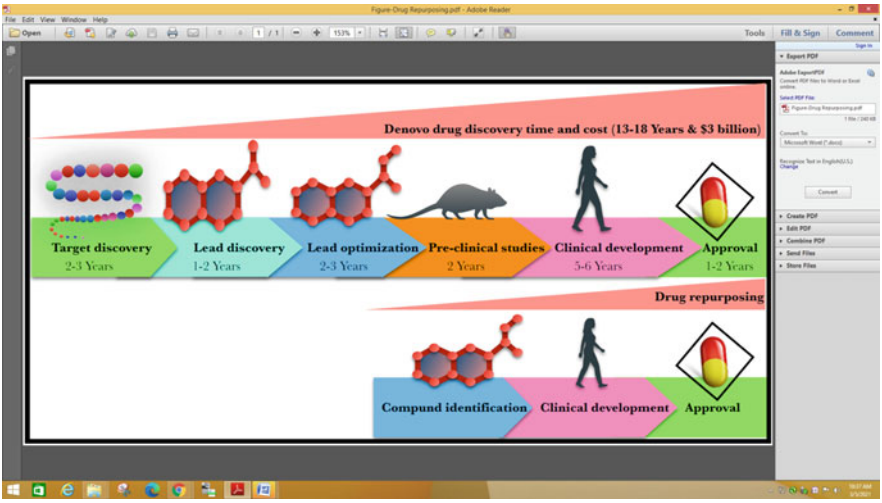

Fig. 4.1 Various stages of drug discovery and the time: De novo drug development strategies generally include five stages: target and lead discovery, lead optimization, and preclinical studies (Phase I studies) which involves toxicity and safety studies, clinical research for efficacy (Phase II and III studies), and FDA review. However, there are only three steps in drug repositioning: compound identification and acquisition, clinical development (clinical research for efficacy), and FDA review. Therefore, drug repurposing begins with target discovery for an existing drug, directly followed by clinical trials on humans, while animal and Phase 1 clinical studies were not conducted as results for these studies are already available for an existing drug. Therefore, de novo drug discovery takes 13–18 years with an approximate cost of $3 billion whereas drug repurposing dramatically reduces the time and cost

4.2.1 Protein-Protein Interactions

The recent advances in high-throughput proteomic and mass-spectrophotometry techniques have led to the rapid generation of large-scale protein-protein interactions. Biological General Repository for Interaction Datasets (BIOGRID) is an interaction repository data compiled from literature (http://www.thebiogrid.org). The database contains protein-protein interactions, protein complexes, genetic interactions, and post-translational modifications from major model organisms. The database IntAct provides a list of curated or user-submitted protein-protein interactions and protein complexes along with analysis tools (Kerrien et al. 2012). The Human Protein Reference Database provides a list of literature curated human protein-protein interactions and post-translational modifications (Mishra et al. 2006). Another database called molecular interactions (MINT) focuses on experimentally verified protein-protein interactions mined from the scientific literature by expert curators (Chatr-Aryamontri et al. 2007). The International Molecular Exchange (IMEx) consortium is an international collaboration between major publicly available protein-protein interaction data providers to share the data and make a

Table 4.1 List of proteomic and genomic datasets useful for drug repurposing

Pathway databases		
BioGRID	Breitkreutz et al. (2007)	http://www.thebiogrid.org
IntAct	Kerrien et al. (2012)	https://www.ebi.ac.uk/intact/
HPRD	Mishra et al. (2006)	http://www.hprd.org/
MINT	Chatr-Aryamontri et al. (2007)	https://mint.bio.uniroma2.it/
iMEX	Orchard et al. (2012)	https://www.imexconsortium.org/
Protein-protein interactions		
KEGG	Kanehisa and Goto (2000)	https://www.genome.jp/kegg/
Reactome	Croft et al. (2014)	https://reactome.org/
BioCyc	Karp et al. (2005)	https://biocyc.org/
MSigDB	Liberzon et al. (2011)	https://www.gsea-msigdb.org/gsea/msigdb/
Drug-target interactions		
DrugBank	Wishart et al. (2018)	https://www.drugbank.ca/
BindingDB	Liu et al. (2007)	https://www.bindingdb.org/
ChEMBL	Bento et al. (2014)	https://www.ebi.ac.uk/chembl/
DGIdb	Griffith et al. (2013)	http://www.dgidb.org/
STITCH	Kuhn et al. (2007)	http://stitch.embl.de/
PharmGKB	Hewett et al. (2002)	https://www.pharmgkb.org/
TTD	Chen et al. (2002)	http://bidd.nus.edu.sg/group/cjttd/
Drug-induced gene expression databases		
CMap	Lamb et al. (2006)	https://clue.io/cmap
LINCS	Keenan et al. (2018)	http://www.lincsproject.org/
Disease-gene interactions		
HPO	Köhler et al. (2017)	https://hpo.jax.org/app/
OMIM	Sherry et al. (2001)	https://omim.org/
PheGenI	Ramos et al. (2014)	https://www.ncbi.nlm.nih.gov/gap/phegeni
GTEx	Lonsdale et al. (2013)	https://www.gtexportal.org/home/

non-redundant dataset of protein interactions available in a single resource (http://www.imexconsortium.org/).

The consortium has developed common curation rules, and a central registry is used to manage the selection of articles to enter into the database (Orchard et al. 2012). In addition to the aforementioned experimental datasets, there were several computationally predicted protein-protein interactions in human, mouse, and other species (Yellaboina et al. 2008; Szklarczyk et al. 2015).

4.2.2 Pathway Databases

There are several different resources of human pathway databases slightly different from each other in content. The pathway database Kyoto Encyclopedia of Genes and Genomes (KEGG) consists of integrated genomic, chemical, and system level functional information. In particular, individual genes from completely sequenced

genomes such as human and mouse are linked to higher-level functions of the cell and the individual organisms (Kanehisa and Goto 2000). It contains a collection of manually drawn pathway maps representing knowledge on the biochemical pathways, pathways of genetic information processing, pathways of environmental information processing, organismal systems, human genetic disorders, and drug development. The pathway database, BioCyc, consists of predicted pathway information from model organisms and provides various tools for pathway analysis (Karp et al. 2005). Reactome contains a manually curated open-source data of human pathways, reactions, bioinformatics analysis, and visualization tools (Croft et al. 2014). The Molecular Signatures Database (MSigDB) contains gene-sets belonging to particular pathways, chromosomal locations, protein complexes, and transcription factor binding (Liberzon et al. 2011).

4.2.3 Drug-Target Interactions

The database DrugBank is a unique genomics and cheminformatics resource that integrates drug and nutraceuticals data with all-known drug target information. The recent version of DrugBank (version 5.1.6) contains information on 13,579 drugs that consists of 2635 approved small molecule drugs, 131 nutraceuticals, and over 6375 drugs in discovery phase (Wishart et al. 2018). The database BindingDB is a publicly available repository of experimentally measured binding affinities related to interactions between small, drug-like molecules and drug-targets. BindingDB contains 1,881,721 binding data, for 7548 protein targets and 833,792 small molecules (Liu et al. 2007). The Drug-Gene Interaction database (DGIdb) mines existing data from 30 disparate sources that provides information about how mutated genes could be targeted therapeutically or prioritized for drug development. The database also provides a web interface for searching a set of genes against a collection of drug-gene interactions and potential druggable genes (Griffith et al. 2013). The Pharmacogenomics Knowledge Base (PharmGKB) consists of the database on the effect of various genetic variations on drug response (Hewett et al. 2002). The database ChEMBL is a repository of bioactive drug-like small molecules, two-dimensional structures, computed physicochemical properties, and bio-activities such as binding constants, pharmacology, and ADMET information (Bento et al. 2014). Therapeutic Target Database (TTD) is a repository that provides information about the known and predicted protein and nucleic acid targets, drugs directed at each of the targets, and pathway information on the targets. In addition, the database also consists of links to other relevant databases containing information about target function, ligand binding properties, enzyme nomenclature and drug structure, therapeutic class, clinical development status, target sequence, and 3D structure (Chen et al. 2002). Another database, search tool for interactions of chemicals (STITCH), integrates information about crystal structures, binding experiments, drug-target relationships, and interactions from metabolic pathways (Kuhn et al. 2007). STITCH also contains the network of chemical relations depending on chemical similarity and information for over 70,000 different

chemicals, including 2200 drugs, and connects them to genes and their interactions from the STRING database.

4.2.4 Genetic Variation and Disease-Gene Interactions

OMIM is the primary repository of curated information on human genetic disease/disorder phenotypes along with associated gene mutations. The database provides interactive access to the knowledge repository, including genomic coordinate searches of the gene map, views of genetic heterogeneity (multiple genes associated with single diseases), and pleiotropy (Amberger et al. 2019). The database ORPHANET contains inventory, classification, and encyclopedia of rare diseases (disease that affects less than 200,000 individuals) and their associated genes and orphan drugs (Weinreich et al. 2008). The database also offers services to the needs of patients and their families, health professionals, and researchers. Database of Single Nucleotide Polymorphism (dbSNP) is a free publicly available resource (Smigielski et al. 2000) for genetic variation within and across different species (https://www.ncbi.nlm.nih.gov/snp/). Even though the database contains the majority of the variants as SNPs only, it also contains a range of other molecular variations: (1) short InDels (insertions and deletions), (2) microsatellite markers or short tandem repeats, (3).multi-nucleotide polymorphisms, (4) heterozygous sequences, and (5) named variants. The database also contains genomic and RefSeq mapping information for both common variations and clinical mutations (Sherry et al. 2001).

The Human Phenotype Ontology (HPO) provides a systematic vocabulary of phenotypic abnormalities encountered in human diseases. Each term in the Human Phenotype Ontology describes a phenotypic abnormality and the Human Phenotype Ontology identifiers are cross-linked to SNOMED-CT, Orphanet, DECIPHER, and OMIM identifiers (Köhler et al. 2017). The Phenotype-Genotype Integrator (PheGenI) database combines NHGRI genome-wide association study (GWAS) catalog data with several other databases housed NCBI including gene, database of Genotypes and Phenotypes (dbGaP), Online Mendelian Inheritance in man (OMIM), dbSNP, and expression quantitative trait loci (eQTL). The database enables deeper examination of SNPs associated with a variety of traits, facilitating the identification of the relationships between genetic variations and various diseases of the human (Ramos et al. 2014). The Genotype-Tissue Expression (GTEx) database is a public resource that consists of tissue-specific gene expression and regulation. It includes whole genome sequencing, whole exome sequencing, and RNA-seq data from different non-diseased tissue sites across nearly 1000 individuals. It provides access to data including gene expression, quantitative trait loci, and images of histology (Lonsdale et al. 2013).

4.2.5 Drug Induced Gene Expression Signatures

High-throughput screening of drugs has been greatly enhanced by the development of computational methods and various genomic resources such as connectivity map (CMap) (Lamb et al. 2006) and the Library of Integrated Network-Based Cellular Signatures (LINCS) (Keenan et al. 2018). CMap and LINCS are large-scale gene expression databases based on drug perturbation of many cultivated cell lines. Both datasets serve as reference datasets for drug perturbation profiles of thousands of chemical compounds. These large scale data resources provide an important platform to characterize signatures of gene expression changes induced by drugs and small molecules. Such signatures of drug perturbation have been used to identify the interactions, similarities, or dissimilarities among drugs, diseases, genes, and pathways.

4.3 Major Techniques of Drug Repurposing

Advanced high-throughput technologies in proteomics, gene expression, sequencing, and genome-wide association studies have been generating large amounts of data on protein-protein interactions, gene expression, and disease gene interactions. In addition to docking-based methods (Kumar et al. 2019; Hasnain et al., US 2020/0188477 A1, 2020), there were several genomics and cheminformatics-based computational methods developed for drug repurposing by exploiting aforementioned datasets. Here, we discuss some of the computational methods.

4.3.1 Connectivity Map

The connectivity map (CMap) and Library of Integrated Network-Based Cellular Signatures (LINCS) are comprehensive, large-scale drug perturbation databases containing transcriptomic profiles of dozens of cultivated cell lines treated with thousands of bioactive chemical compounds serving as reference databases for drug-induced gene depression signatures (Lamb et al. 2006; Subramanian et al. 2017). The resource can be used to find connections among small molecules sharing a common mechanism of action, diseases, and physiological processes. Particularly, the reference data resource can be used in drug discovery to find out the small molecules which could possibly suppress or reverse the disease-induced gene expression signature based on anticorrelation between small molecule-induced gene expression and disease-induced gene expression signature of interest. Several groups have used the cMap drug discovery feature to identify the potential candidate drugs for various diseases such as cancer and Crohn's disease to name a few (Cheng et al. 2014; Dudley et al. 2011; Kwon et al. 2020). The gene expression was obtained from Gene Expression Omnibus (GEO). The disease gene expression signatures were identified by differential expression analysis of genes between the disease affected (Crohn's disease and ulcerative colitis) and healthy control samples. The

disease gene expression signatures were compared with drug-induced gene expression profiles obtained from the connectivity map (Lamb et al. 2006) to derive a therapeutic score. Drugs with significant negative scores have gene expression patterns that are anti-correlated with disease-specific gene expression patterns and therefore represent putative novel therapeutic indications (Dudley et al. 2011).

4.3.2 Side Effect Similarity

Drug side effects are the result of complex phenotypes that arise due to a number of molecular interactions including the interaction with the primary target or off-targets (Campillos et al. 2008). Although off-target interactions of the existing drugs are generally undesired and harmful, they can occasionally be useful and can lead to development of new therapeutic options for drugs (e.g., sildenafil). The drugs lacking chemical similarity can cause similar side effects due to their common off-targets implying a direct correlation between off-target binding and side-effect similarity (Fliri et al. 2005). Thus, additional targets for FDA-approved drugs, often implicated in entirely different therapeutic options and disease processes, can be proposed. A method was developed to identify molecular activities of drugs that are completely based on side effects but not implicit by their chemical similarity or the sequence solely of their known protein targets (Campillos et al. 2008). The method was able to identify alternative targets for many FDA-approved drugs, often implicated in different therapeutic classes. The authors have used the relations between side effect terms using Unified Medical Language System (UMLS) ontology (Lindberg et al. 1993) to capture similarities between drugs. Finally, chemical similarity is combined with side effect similarity to provide a final score for assigning a probability to any pair of drugs to share a target.

4.3.3 Network-Based Approach

In this method, a comprehensive human protein-protein interactome was built from fifteen commonly used resources with evidence from multiple types of experiments (Cheng et al. 2018). Further, genes belonging to different types of cardiovascular disease types were identified by Medical Subject Headings and Unified Medical Language System vocabularies (Lindberg et al. 1993). For each cardiovascular event, disease-related genes from eight frequently used databases were collected. In addition, drug-target interactions on FDA-approved drugs from six frequently used databases were assembled, and the interactions were weighted using reported binding affinity data between drug and protein: inhibition constant, dissociation constant, median effective concentration, or median inhibitory concentration. Drug-target interactions were acquired from the DrugBank database (Wishart et al. 2018), the Therapeutic Target Database (Chen et al. 2002), and the PharmGKB databases (Hewett et al. 2002). The bioactivity data of drug-target pairs were collected from ChEMBL (Bento et al. 2014), BindingDB (Liu et al. 2007), and

IUPHAR/BPS Guide to Pharmacology (Pawson et al. 2014). Finally, the protein-protein interaction data, disease gene data, and drug target were combined to calculate the network distance between all the drugs and a given disease (Cheng et al. 2018).

4.3.4 Chemical Similarity

The chemical similarity ensemble approach (Keiser et al. 2007) compares target proteins by using the chemical similarity of the ligands that bind to them, represented as e-values, adapting the basic local alignment and search tool algorithms (Altschul et al. 1990; Hert et al. 2008). The structural similarity between each drug and each target's ligand set was quantified as an e-value using the similarity ensemble approach (Keiser et al. 2007). It can be used to quickly search large ligand databases and to identify similarity maps among target proteins in large scale. The method is different from traditional bioinformatics methods for identifying similarity between proteins that uses the sequence of amino acids or three-dimensional structural similarity among target proteins. A total of ~3600 drugs were compared against ~65,000 ligands organized into 246 targets from the MDL Drug Data Report database (Schuffenhauer et al. 2002), generating 0.9 million drug-target comparisons. Most of the drugs had no significant expectation values to most of the ligand sets. Along all possible pairs of drugs and ligand sets, ~6900 pairs of drugs and ligand sets were similar, with significant e-values. Predicted off-target proteins with strong similarity ensemble expectation values are evaluated for novelty using the literature.

4.4 Summary

Thanks to the emerging innovations in technologies, the low-cost sequencing and high-throughput technologies are resulting in the generation of a massive number of genomic datasets in biology and medicine. Currently, there are a large number of candidate disease genes identified through GWAS and other approaches. Massive data on single cell transcriptomics is enabling us to precisely identify the cell types and associated gene expression signatures involved in different diseases. There is a growing amount of data on FDA-approved drugs to treat the disease and several other drugs which are not toxic to humans but failed to treat the diseases. Integrating the current datasets on single cell transcriptomic, genotype-phenotype, pharmacogenomic, protein-protein interactions and pathways could ultimately result in identifying drug action mechanisms, disease mechanisms, and new uses of existing drugs. However, the current methods to deal with the massive amount of high-dimensional genomic (big data) data are very limited. There is a need to develop new statistical and computational methods to deal with rapidly growing, high-dimensional, and heterogeneous genomic datasets and use these methods for drug repurposing.

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Genomic Approaches for Drug Repositioning

5

Isra Ahmad Farouk, Zheng Yao Low, Ashwini Puniyamurti, Nur Zawanah Zabidi, Mohammad Khusni Ahmat Amin, and Sunil Kumar Lal

Abstract

Drug treatments of certain diseases that are either rare, complex, or novel may not always be available due to high cost of drug development and research. Drug repositioning (DR) is an alternative approach to usurp already available drugs or drug candidates with FDA approval, which have been initially developed for specific diseases and re-established their use for other diseases. Modern genomic methods for drug repositioning involve the usage of computational programs and online tools to analyse and ultimately deduce targets with high specificity to be considered as candidates for repositioning. Gene, protein, disease, and drug databases are built from high-throughput experimental, in vitro, in vivo, and clinical data, thus providing a reliable basis for drug target acquisition purposes. Key experimental and in silico approaches for modern drug repositioning, namely, signature matching, molecular docking, genome-wide association studies, and network-based approaches aided by artificial intelligence will be described in this chapter along with research examples that have used these methods. Drug repositioning for certain diseases, such as Alzheimer's disease, cystic fibrosis, and SARS-CoV-2 disease, will be discussed in this chapter. Lastly, we will discuss the challenges faced and the future perspectives of DR. Genomic computational approaches for drug repositioning presents much potential in identifying drug targets more efficiently and effectively, which

I. A. Farouk · Z. Y. Low · A. Puniyamurti · N. Z. Zabidi · M. K. A. Amin
School of Science, Monash University, Selangor, Malaysia

S. K. Lal (✉)
School of Science, Monash University, Selangor, Malaysia

Tropical Medicine and Biology Platform, Monash University, Selangor, Malaysia
e-mail: sunil.lal@monash.edu

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provides the opportunity to fulfil the gap for treatment of diseases with little or no cure.

Keywords

Drug repositioning · FDA approved · Disease · Genomic approaches · Drug target · In silico approaches · Computational · Artificial intelligence

5.1 Introduction

Drug development is a process wherein a new drug aimed to alleviate or treat disease symptoms is introduced into the pharmaceutical market upon identification of a lead compound via drug discovery. Discovery of lead compounds holding therapeutic activity is traditionally found through classical and reverse pharmacological approaches. The former involves the screening of natural products or chemical libraries of synthetic small molecules in vitro or in vivo, whereas the latter relies on high-throughput screening of large compound libraries against a cellular pathway of interest. Across the recent years, drug discovery has shown a staggering decrease in productivity because of its lengthy, risky, tedious, and financially straining process. Statistics show that a conventional route from drug discovery to drug delivery may cost a span of approximately 10–15 years with an average expense of US$12 billion (Xue et al. 2018). Most drug leads fail to enter clinical trials due to the lack of safety and efficacy. Some may go as far as phase II and III clinical trials only for it to be retracted because of insufficient validation or suboptimal setup of clinical trials (Everett 2015).

The United Nations General Assembly has recently designed a blueprint enlisting the Sustainable Development Goals (SDGs) to be achieved by 2030. SDG 3 calls for an improvement in health services to promote healthy lives and well-being amongst citizens of all ages (The 17 Goals 2021). In alignment with rapid technology advancements moving towards a digital era, we foresee a revolution in digital healthcare. Consequently, there is a dire need to understand in depth how we may establish a novel framework in drug development for long-term sustainability. An increasingly popular trend currently is drug repositioning (DR). Evidently, a query of publications associated with the term “drug repositioning” on PubMed began in 2006 with only 1 publication, followed by an exponential growth with up to more than 1000 publications in 2021.

DR, synonymously known as drug repurposing, drug recycling, drug redirecting, drug re-tasking, or drug reprofiling, is defined as measures looking into approved or investigational drugs for new indications of other diseases aside from that originally intended (Fig. 5.1). Instead of solely relying on top pharmaceutical industries, more extensive collaboration and networking through the partnership of these industries, biotechnology companies, and academia will encourage a wider exchange of information while garnering financial support. Considering that these drugs have been de-risked, the investment and cost in time may be reduced while reaping the benefits

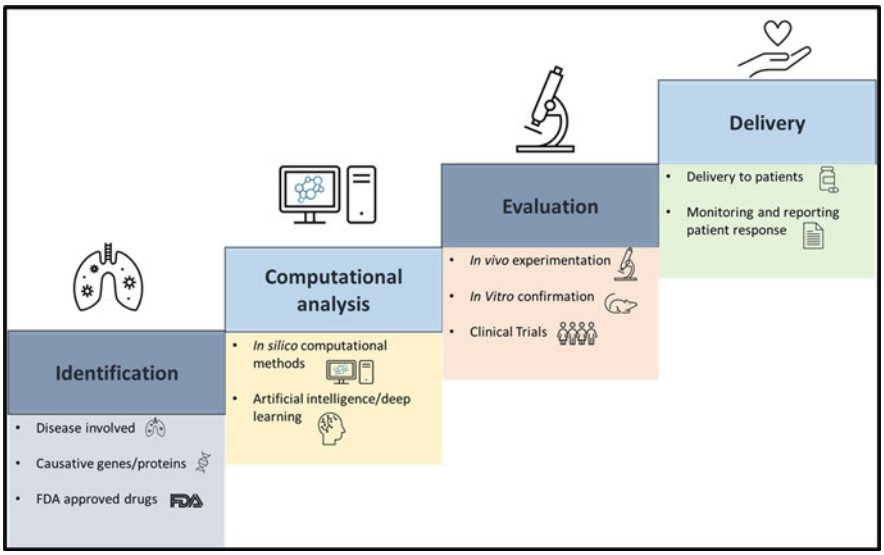

Fig. 5.1 Schematic diagram representing the pipeline of DR, beginning with the identification of a drug candidate through a disease- or drug-based approach, followed by its analysis and evaluation and, finally, delivery to the patient

of success to finding more cures. Old drugs that were previously withdrawn for safety reasons may also be re-evaluated as potential candidates for the treatments of other diseases (Everett 2015). Another great advantage is the innovation of drugs for orphan diseases—rare diseases that affect only a minute fraction of the population, therefore not yielding a large enough marketing avenue and, unfortunately, not gaining the deserving medical resources (Govindaraj et al. 2018). Furthermore, quicker solutions may be found in response to sudden outbreaks, such as that observed in the recent COVID-19 global pandemic, to reduce morbidity and mortality (Serafin et al. 2020). Based on the genetic make-up, patients and diseases can be stratified into molecular subtypes for better tailoring of personalized medicine with more specific drugs carrying the least risk (Li and Jones 2012). From today's standpoint, we will describe the experimental, in silico, and modern genomic approaches of DR, as well as its challenges and prospects.

5.2 Experimental and In Silico Approaches to DR

The benefits to DR, such as reduced risk, time, and cost, makes it a widely favourable approach. For example, in the recent SARS-CoV-2 outbreak, drugs that were investigated against severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) in the past were being examined against COVID-19. Drugs that are currently in clinical trials to be repurposed are remdesivir, danoprevir/ritonavir, interferon β-1b, nitazoxanide, and others (Parvathaneni and

Gupta 2020). There are usually two strategies involved in DR—on-target and off-target. The pharmacological mechanism is known in an on-target profile, whereby the same target is used for a different disease. In contrast, the target is completely new in an off-target profile. Table 5.1 describes FDA-approved drugs that have been successfully repositioned classified according to these two strategies. There are many approaches taken towards DR, especially with the boom of technology in the past decade, with the two majors being experiment-based and in silico approach (Rudrapal et al. 2020).

5.2.1 Experimental Approaches

DR through an experiment-based approach is dependent on experimental assays. Experimental methods used to identify target interactions are binding assays (affinity chromatography and mass spectrometry) and phenotypic screenings (high-throughput screening using in vitro or in vivo models) (Pushpakom et al. 2018). A group of investigators used an in vitro screening method with US Food and Drug Administration (FDA)-approved drugs and discovered that ceftriaxone and harmine can upregulate transported GLT-1, a previously unknown effect (Rothstein et al. 2005). This implication is significant to the development of amyotrophic lateral sclerosis (ALS). Although the clinical trials of ceftriaxone were eventually halted in 2012, this method was shown to rapidly advance a drug into a clinical testing phase. In vivo models such as zebrafish models are also used for DR studies. In a study by Cousin et al., a larval zebrafish model was used to screen 39 FDA-approved drugs for tobacco dependence and found that 8 drugs from 5 different classes can modify nicotine behavior (Cousin et al. 2014).

A key player in phenotypic screening is a platform known as theraTRACE® developed by Melior Discovery that utilizes 40 mouse disease models in the areas of inflammation, metabolic disease, immunology, allergic reaction, regenerative, psychotherapeutic, neurology, neurodegenerative disease, pain, gastrointestinal, cardiovascular, and urogenital (Cavalla 2013). From this platform, a compound called MLR-1023, also previously known as tolmidone, discovered by Pfizer was identified as a candidate for type 2 diabetes through insulin sensitization (Saporito et al. 2012). This drug was initially developed for gastric ulcer but was discontinued as it was not efficacious. Another drug, gefitinib, which is an epidermal growth factor receptor (EGFR) inhibitor, commonly prescribed to lung and breast cancer patients was studied using affinity chromatography and mass spectrometry. Through this study, more than 20 different protein kinases and other cellular proteins that are unrelated to EGFR inhibition were identified (Godl et al. 2005). High-throughput screening using micropillar arrays (BIMA) to screen for compounds that promote remyelination discovered that the drug clemastine, widely used for its antihistamine properties, was a likely candidate for human remyelination trials (Mei et al. 2014). Additionally, the drug did undergo clinical trial (NCT02040298) as a treatment for patients with multiple sclerosis (MS), and findings suggested that the drug was safe and myelin repair could be achieved (ClinicalTrials.gov 2021; Green et al. 2017).

Table 5.1 List of FDA-approved repurposed drugs classified according to on- and off-target approach indicating their cellular targets and original and repositioned indications

Strategy	Drug	Cellular target	Original indication	Repositioned indication	Reference
On-target	Sildenafil	• PDE5	Hypertension, angina	Erectile dysfunction	Ghofrani et al. (2006), Unegbu et al. (2017), Sildenafil (2021)
	Thalidomide	• TNF-α	Erythema nodosum laprosum (ENL)	HIV/AIDS	Kim and Scialli (2011), Thalidomide (2021)
	Duloxetine	• Serotonin, noradrenaline	Depression	Stress urinary incontinence (SUI)	Ashburn and Thor (2004), Gupta et al. (2007), Duloxetine (2021)
	Finasteride	• Type II 5-α-reductase	Benign prostatic hyperplasia	Male pattern hair loss	McClellan and Markham (1999), Henriksen et al. (2011), Finasteride (2021)
	Trastuzumab	• Receptor tyrosine-protein kinase erbB-2	HER-2-positive breast cancer	HER-2-positive metastatic gastric cancer	Boekhout et al. (2011), Rogers et al. (2014), Trastuzumab (2021)
Off-target	Furosemide	• Carbonic anhydrase 2 • G protein-coupled receptor 35 • Solute carrier family 12 member 1	Oedema associated with congestive heart failure	Moderate to severe hypertension	Divorty et al. (2018), Furosemide (2021)
	Mifepristone	• Progesterone receptor • Glucocorticoid receptor	Pregnancy termination	Hyperglycaemia secondary to hypercortisolism in Cushing's syndrome	Jang and Benet (1998), Fjerstad et al. (2009), Mifepristone (2021)

(continued)

Table 5.1 (continued)

Strategy	Drug	Cellular target	Original indication	Repositioned indication	Reference
	Topiramate	• Gamma-aminobutyric acid receptor subunit α-1 • Voltage-gated sodium channel α-subunit • Carbonic anhydrase	Partial-onset seizures, primary generalized tonic-clonic seizure epilepsy	Mood disorders, migraine, weight management	Arnone (2005), Wenzel et al. (2006), Topiramate (2021)
	Mirtazapine	• 5-Hydroxytryptamine receptor 2A • 5HT3 serotonin receptor • α-2A adrenergic receptor • Histamine H1 receptor	Major depressive disorder	Reverse weight loss, improve sleep	San and Arranz (2006), Maletic et al. (2017), Mirtazapine (2021)

5.2.2 In Silico Approaches

The in silico approach has garnered more attention in the recent decade. This approach requires a computer and available databases with drug, disease, or pathway information. This approach comprises many different types of methods, such as network analysis, data mining, ligand/structure based, and molecular docking (March-Vila et al. 2017). Network analysis allows for the modelling of functional similarities between drugs, proteins, genes, and other biological systems (Tuerkova and Zdrazil 2020). Networks can be classified into two categories—homogenous and heterogenous. A homogenous network is defined as protein-protein interaction networks that can be used to identify drug targets involved in multiple pathways, whereas a heterogeneous network incorporates different information, such as genomics, proteomics, and metabolic pathways to create a multilayer relationship model (Xue et al. 2018).

Data mining allows the generation of novel hypotheses through a method known as the "ABC model" discovered by Swanson (Weeber et al. 2005). In this model, it states that if A and B are related and B and C are related, then it can be hypothesized that A and C are indirectly related. This model is said to be a pioneer in literature-based discovery (LBD) (Kim et al. 2016). One example of DR through LBD is pirlindole (BVA-201), where a new indication to treat MS was found when it was initially used as a chronic treatment of depression and anxiety disorders (Lekka et al. 2011). There has been an increase in text mining tools with the development of natural language processing (NLP) techniques. A summary of the tools and respective descriptions can be found in this review by Xue et al. (2018). Using the network analysis approach, human immunodeficiency virus (HIV) protease inhibitors were observed to inhibit the phosphoinositide 3-kinase (PI3K)/Akt pathway, a pathway that is activated in many types of cancer. As a result, nelfinavir, a HIV protease inhibitor, is undergoing clinical trials to be repositioned as an anticancer agent inhibiting Akt (Gills et al. 2007).

Molecular docking is a method that visualizes the binding of a drug inside a three-dimensional target structure. In 2001, another method known as "inverse docking" was proposed to investigate one drug against multiple protein binding sites (Li and Jones 2012). In a study by Kumar et al., molecular docking was used to screen a library of available antipsychotic drugs and found that benperidol interacted with different target proteins involved in Alzheimer's disease (AD), showing its potential as a possible candidate for treating the disease (Kumar and Kumar 2019).

The computational approach is still growing, and more methods that are considered "newer" and more advanced are being utilized. The sections below will dive deeper into the more current computational approaches focusing on the genomic and network approaches. Fig. 5.2 below also provides a brief summary of various computational methods and their strategies.

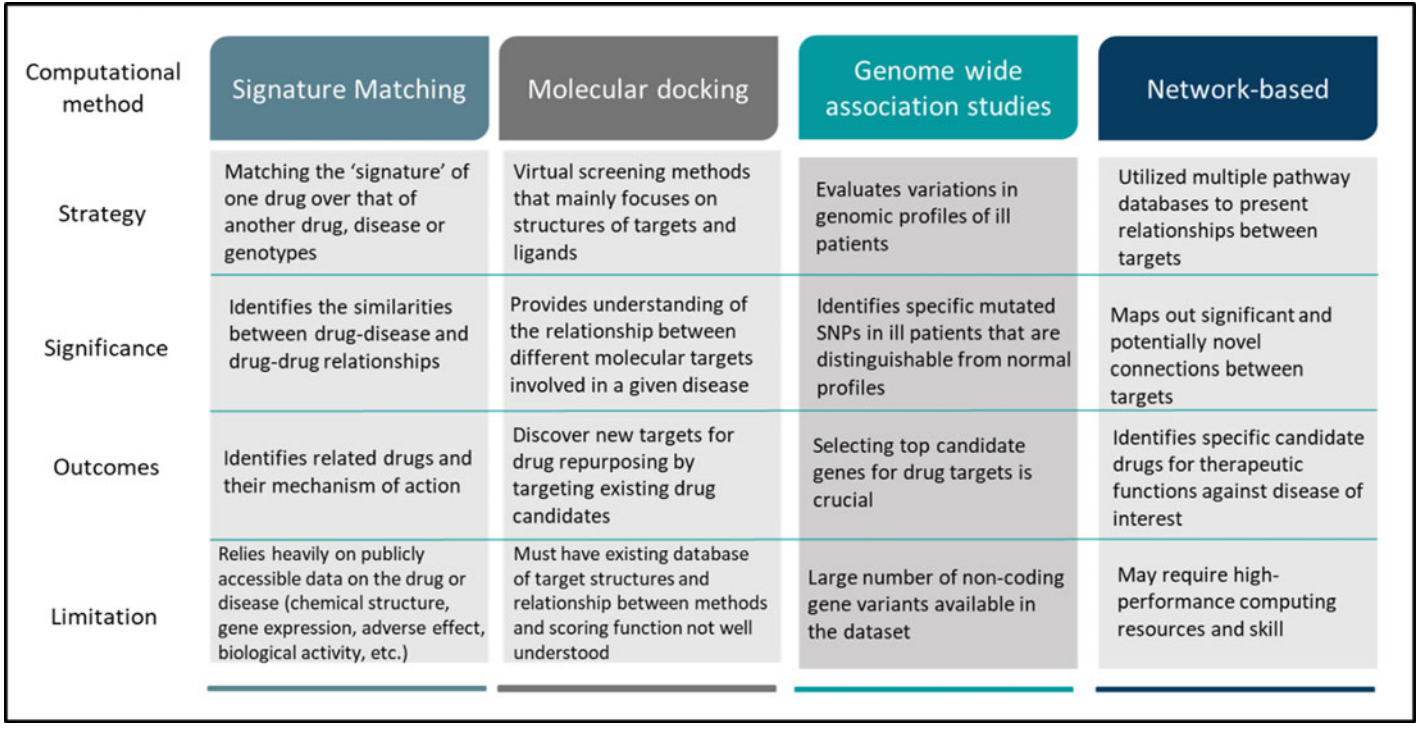

Fig. 5.2 A summary of the strategy, significance, outcomes, and limitations of various computational approaches used for drug repositioning

5.3 Genomic Approaches to DR

Human genetics is a broad field of study that, in part, identifies genetic risk factors that are common amongst complex, rare, or common diseases (Bush and Moore 2012). Disease being a no stranger term denotes harmful deviations from the normal state of structure or function of an organism. This entails the state of complete physical, mental, and social well-being. There are generally four main types of disease: genetic, infectious, deficiency, and physiological diseases. Genetic disease, in particular, has both hereditary and non-hereditary scenarios. Genetic disease or more commonly known as genetic disorder is a health condition where there are abnormalities in the genome of an individual. A genetic disease may manifest in various forms ranging from a single gene mutation or chromosomal mutation, which entails multiple genetic mutations, thus affecting many body systems and causing great damage. Medicines are usually designed to treat genetic diseases via two main approaches—restore normal levels of genes with a loss-of-function mutation or inhibit excessive gene expression in those that have a gain-of-function mutation (Sun et al. 2014).

Apart from the difficulty in accurate diagnosis, a genetic disease often has no effective treatment or no treatment at all. This is often caused by changes in genes which are very complex, life-debilitating, and sometimes rarely occurring; albeit, the latter is not an absolute (FAQs About Rare Diseases 2021). For genetic conditions, most treatment and management strategies are only in place for alleviating the symptoms. For example, a bone marrow transplant for sickle cell disease limits the intake of certain substances that are potentially toxic for individuals with a metabolic disorder, which otherwise will be normally broken down by digestive enzymes in healthy individuals.

Although it is not always the case, a genetic disorder can sometimes be described as "rare" or as an "orphan disease". It often affects minute portions of individuals instead of the mass public like the current COVID-19 pandemic. The biggest hurdle to tackle genetic diseases lies within the complexity and rare occurrence, in which no effective treatment is available (Dunoyer 2011; Muthyala 2021; Sardana et al. 2011). To complicate the situation, some genetic changes may increase the risk of health problems, such as breast cancer BRCA1 and BRCA2 gene mutations. Additionally, the low availability of clinical trial subjects is a limiting factor for drug development, drawing interest away from researchers and pharmaceutical industries as the investment of time and money may not be profitable (Wästfelt et al. 2006). To date, there are more than 7000 genetically associated orphan diseases, and still, the number is continuously rising (Xu and Coté 2011).

In response to this perturbing challenge, DR might be the solution to curb life-threatening and debilitating genetic diseases. With DR, the ever-concerning cost and time issue for a low demand drug, especially for complex genetically associated diseases, can be resolved by exploring new avenues from existing or abandoned drug therapies. To date, DR plays an important role in bridging the gap closer over access to medicine by broadening the availability of drug treatment for various diseases within a population. Indeed, there have been a few notable successes for rare/orphan

diseases attained by numerous computational approaches and drugs with previous functional indication.

5.3.1 Linking Target Genes to Clinically Approved Drugs

With the rapid rise of technology, there have been significant upgrades in the study of human genetics and usage of the output data. Many different programs now exist to capture genetic information and analytical tools to identify key genetic risk factors (Bush and Moore 2012). Forming a link between disease traits such as disease-causing genes and clinically approved drugs provides a greater chance of DR success as compared to a proposed repositioning with no link to a genetic target (Nelson et al. 2015). Transcriptional data reflect gene regulation profiles of human cells in responses to disease, toxins, drugs, and more. Such data provides closer insight to specific isolated situations, thus enhancing the understanding of relationships between the host, drug, and disease (Iorio et al. 2013). The regulation of specific genes or disease markers may serve as a basis for successful DR. Inhibitors, stimulators, or other types of approved drugs may be used to specifically counter the disease-causing effects by moderating those responses.

5.3.2 Usage of Computational Methods

Modern in silico research for DR employs a combination of databases, software, and analysis tools to elucidate specific and theoretically functional annotations to serve as targets or potential therapies (Talevi 2018). With the large amount of information being gathered from genetic studies, it is important to highlight that no particular stringency of computational workflow suits all. Rather, fluidity in research methodology is dependent on factors such as the analysis required to determine disease-causing gene variants known as causal genes, data availability of known pathways related to the gene/disease, and the choice of databases and software. Selecting the most suitable workflow for computational methods can narrow down the search time and provide more reliable drug candidates for other diseases.

5.3.2.1 Genome-Wide Association Studies (GWAS)

As the role of genetics in medicine has become more evident in the past years, DR against specific targets using computational approaches have also grown in popularity. A genome-wide association study (GWAS) is a research approach involving the evaluation and scanning of genomic profiles of different individuals to identify genetic markers or specific genetic variations that may be associated with a particular disease (Genome 2021). Gene variation in GWAS is identified from single-nucleotide polymorphisms (SNPs) of ill individuals. In the past, the clinical application of GWAS was criticized as being limited due to the large number of non-coding gene variants available in the data set. As an implication, when considered for certain treatments, they may have reduced potential as efficient targets (Pritchard et al.

2017). Nevertheless, the large data available from GWAS is indeed valuable and contains great potential for future therapy. The gap between GWAS and DR may be bridged closer using additional methods to identify the most relevant genes and deduce drug candidates using drug-drug, drug-disease, and biological pathway mapping and analysis (Lau and So 2020).

Most current studies use GWAS-based prioritization methods to create lists of top potential causal genes for a disease of interest. A recent study (Zheng et al. 2020) used a series of computational methods to identify plausible candidate genes for coronary artery disease (CAD). These methods included computational programs, such as Sherlock, NetWAS, SMR, GWAB, TWAS, Prix fixe, DAPPLE, and DEPICT. GWAS summary data that has been narrowed down using variables, such as co-functionality, gene-functionality, and tissue-specificity factors, is subsequently fed into all or some of the eight computational programs. The resulting hits are then further analysed for biological functions using GO/KEGG enrichment analysis, tissue-specific gene expression, and interactions with other cellular pathways and crosstalk analysis (Zheng et al. 2020). The significance of such an analysis ultimately provides mechanistic insights into the disease pathogenesis and narrows down the pool of drug targets that can be repositioned for the studied disease.

The general flow of GWAS begins with identifying the basic unit of genetic variation, SNPs, as markers of a particular genomic region of interest. Disease indication SNPs are distinguishable from common SNPs in the human genome, which makes a strong basis for study. Linkage analysis is a technique which aids in the identification of gene mutations such as the cystic fibrosis transmembrane conductance regulator (CFTR) gene known to cause CF. This technique, however, does not work well for diseases where the genetic mechanisms are influenced by external factors such as heart disease (Bush and Moore 2012). GWAS obtains genomic data from diverse diseases according to the type of disease through customizing certain parameters, tools, and analysis criteria (Bush and Moore 2012). The array of GWAS data contains a large potential pool of novel targets that can be used for DR (Pritchard et al. 2017), albeit GWAS alone is not the final answer. Deeper analysis of GWAS is required to identify key target genes for DR, including functional genomic techniques, identification of existing drugs, and preclinical validation of drug targets.

5.3.2.2 Network-Based Approaches and the Support of Artificial Intelligence (AI)

Network-based models of DR extract information from diverse databases and provide results of key connections between the information. In the form of coded nodes and edges, the data output is commonly seen in the form of a network of connections. The nodes commonly represent either a drug, disease, or gene, while the edges represent the interactions between them (Yella et al. 2018). These approaches use GWAS data, cellular pathway mapping, and drug database information to output networks by either entirely knowledge-based inputs (information from databases) or computationally inferred information from existing inputs (aided by

artificial intelligence (AI)). These representations include interactions presented between genes, proteins, targets, drugs, and diseases in various combinations (Yella et al. 2018). Novel information can arise from such analysis as formerly unstudied, yet potentially accurate interactions may result, hence increasing the opportunity for drug candidates that were previously not considered.

There are many network-based methods that have been developed for pathway analysis (Nguyen et al. 2018). These are commonly found to be topology-based commercial tools such as MetaCore and iPathwayGuide. Individual databases that can be used to perform in silico analysis for DR include disease-based databases such as the Cancer Genome Atlas (TCGA) and the Cancer Cell Line Encyclopedia (CCLE) (Kwon et al. 2019). These data sets compile and hold the gene expression profiles and additional research information obtained from in vitro, in vivo, and clinical samples. Some of the drug-based databases include CMap, LINCS, and CTRP, which contain much information of drug features and efficiencies (Kwon et al. 2019). Knowledge-based databases include the Gene Ontology, KEGG, and MSigDB, which display all studied mechanisms or pathways available in literature (Kwon et al. 2019). To bring these data sets closer to DR, wet-lab-based DR tools are available, which allow the exploration of the aforementioned data sets altogether. Despite this, such tools often require proficient computer skills and high-performance computing resources (Kwon et al. 2019). Examples of such tools include the CLUE, L1000CDS^2, and DeSigN. Another online tool of such nature is Gene2Drug, which uses pathway annotations from multiple sources (CMap, GO BP, GO MF, CP, KEGG, Biocarta, Reactome, CGP, TFT, CORUM) to identify one or more candidate drugs that are able to modulate a therapeutic target for the disease of interest (Napolitano et al. 2018).

DR using in silico methods are now improved with the aid of AI. The machine learning algorithms that have been incorporated into some online tools have allowed for enhanced analysis of large omics data sets (Koromina et al. 2019). Some well-known tools and online platforms that are being used for DR include Biovista (Biovista 2021) and drug repurposing (Koromina et al. 2019; Drug Repurposing Online 2021). These AI-supported tools provide assistance in pinpointing non-obvious correlations between targets of interest, thus predicting high potential candidates for DR.

A recent study (Zeng et al. 2021) developed their own network-based deep-learning approach named deepDR for the purpose of in silico DR. deepDR integrates 10 networks of drugs, diseases, and side effects to predict specific associations and highly specific drugs. Several predictions using deepDR have already been validated by the ClinicalTrials.gov database for some diseases such as Parkinson's and AD (Zeng et al. 2021). Other studies have integrated the deepDR as a basis to model their DR usage. The deep2CoV framework model was developed to effectively search for potential drugs for COVID-19 to reduce redundancies in clinical trials (Liu et al. 2020). This heterogeneous network was designed to predict candidate drugs using drug-drug, drug-disease, and drug-target data sets. Using deep2CoV, the following drugs were predicted as candidates for COVID-19: ceftriaxone, ciprofloxacin,

piperacillin, amphotericin B, and doxycycline (Liu et al. 2020). Every drug candidate listed was supported with scientific literature for reference.

Tools and platforms enhanced with the aid of network-based approaches and AI allow researchers to come up with candidate drugs more efficiently while scanning through massive data sets simultaneously. In addition, the outcomes of these analyses and methods are considered to have higher potential. This is due to the integration of more stringent variables within each search and wider data coverage. Connections are also more reliable as data sets mostly include validated experimental findings. Figure 5.3 provides some of the popular databases, tools, and platforms that can be used for DR.

5.3.3 Examples of DR for Genetic Diseases

5.3.3.1 Alzheimer's Disease

AD is an age-related neurodegenerative disease (Alzheimer disease 2021). The development of this disease is irreversible and progressive, causing slow disruption to the thought process, memory, and motor performance, typically after age 65 (Alzheimer disease 2021). About 75% of AD cases are believed to be sporadic, with no history of the disorder in their family and 25% are from familial inheritance (Alzheimer disease 2021). The progression of AD is complex and not fully understood. From what it is known, the disease manifests by an accumulation of abnormal amounts of amyloid proteins and tau proteins in the brain, affecting neuronal function, therefore resulting in a progressive loss of brain function (Tackenberg et al. 2020).

In the context of DR, numerous literature and computational approaches are preferred. A study conducted by Zhang et al. has reported 244 genetic variations, 14 epigenetic modifications, 98 proteins, and 86 metabolites associated with AD by analysing "omics" data comprising genomics, epigenomics, proteomics, and metabolomics data from the GWAS Catalogue, PubMed, and HMDB databases (Zhang et al. 2016). Subsequently, DrugBank and Therapeutic Target Database (TTD) were used for drug-target data extraction. With an in-house developed anti-AD ranking algorithm, two best candidates for drug target (i.e., CD33 and migration inhibitory factor (MIF)/CD74 receptors) and seven potential existing drug repurposing candidates were found (Zhang et al. 2016). CD33 leads to the impairment of microglia-mediated clearance of Aβ, resulting in an accumulation of amyloid plaques in the brain (Jiang et al. 2014). Hence, an anti-CD33 inhibitor like gemtuzumab ozogamicin, which was first approved in 2000 for CD33-positive acute myeloid leukaemia, now holds a significant therapeutic potential for AD (Zhang et al. 2016; Jiang et al. 2014).

5.3.3.2 Cystic Fibrosis

CF is an autosomal recessive and hereditary disease that affects the lungs and digestive system (Delavan et al. 2018; De Boeck et al. 2017). It is life-threatening and affects more than 70,000 individuals worldwide, primarily Caucasians (About

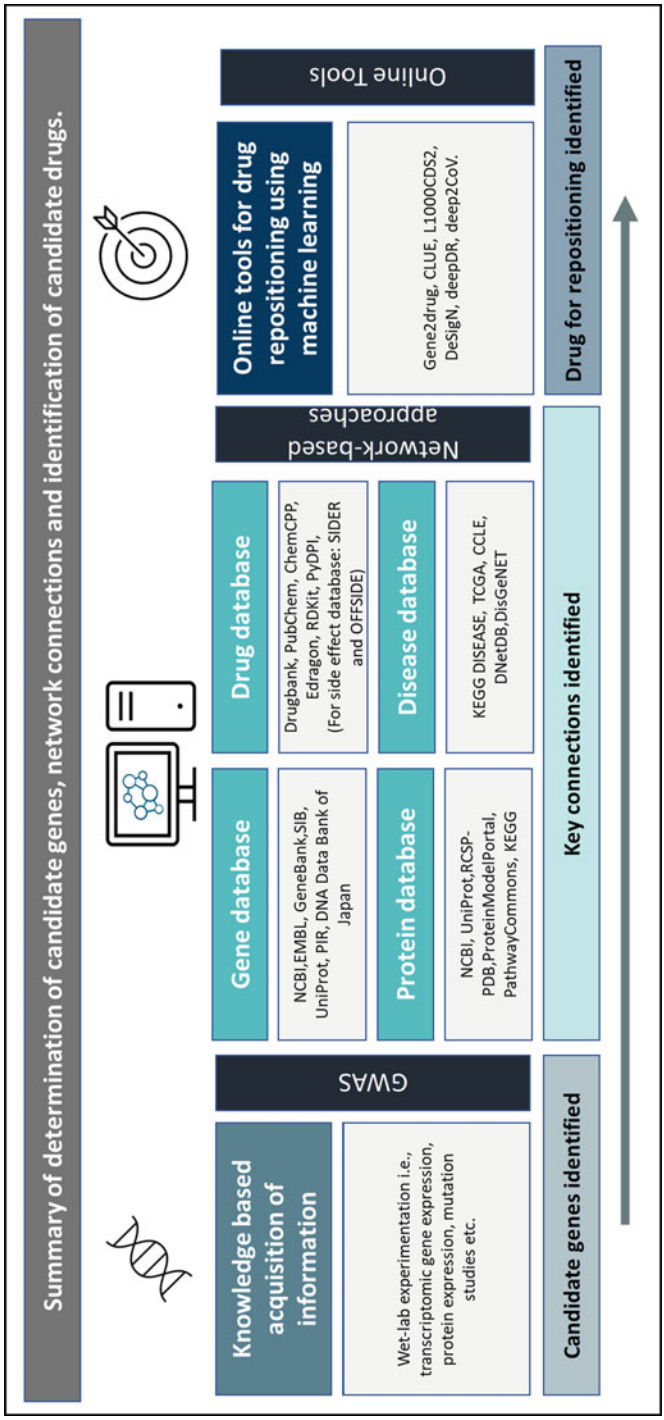

Fig. 5.3 Summary of determination of candidate genes, network connections, and identification of candidate drugs

Cystic Fibrosis 2021). CF is attributed by the mutation in the CFTR protein, a cAMP-regulated chloride channel, primarily expressed at the secretory epithelia in the airways, intestine, and other tissues (Rowe and Verkman 2013). These mutations lead to thick mucus production in lung airways, blocking the air passage, and, consequently, increased infection risk and repeated inflammation. There are more than 2000 identified genetic mutations of the CFTR gene in CF patients (Delavan et al. 2018; Rowe and Verkman 2013). Amongst the identified CFTR mutations, F508del and G551D mutations are the major mutations found in more than 90% of CF patients (Delavan et al. 2018). F508del is associated with the impairment of CFTR folding, compromising the stability at the endoplasmic reticulum, plasma membrane, and chloride channel gating (Delavan et al. 2018). On the other hand, the G551D mutation is related to the alteration of channel gating (Rowe and Verkman 2013).

To date, the FDA-approved drug, ivacaftor, targets only 6% of CF patients with G551D mutation (Delavan et al. 2018). To ease CF condition, it mainly uses nebulized inhaled therapies. This includes hyperosmolar inhaled therapy such as hypertonic saline, mucolytic inhaled therapy such as rhDNase, and inhaled antibiotic therapies such as colistin, tobramycin, and aztreonam if infection or inflammation is present (Hurt and Bilton 2014). Therefore, the need for a more effective DR approach is very crucial. Recently, a drug named Bronchitol or more commonly known by its active ingredient, mannitol, was successfully repositioned for the treatment of CF (De Boeck et al. 2017). Mannitol was firstly approved in 1964 by the FDA for multiple reasons, such as the management of cerebral oedema, increased intracranial pressure, and removing excess water and toxins in kidney failure patients (De Boeck et al. 2017). Being a diuretic drug, its capability of drawing water molecules through epithelial aquaporins confers benefits. In the ventilation airways of CF patients, the drug reduces the thick and sticky mucus, easing its clearance. Also, Bronchitol powder can be administered using an inhaler, making the drug administration much easier, tentatively bringing down the treatment cost significantly (Hurt and Bilton 2014). In 2020, Bronchitol gained approval by the FDA and is expected to be available in March 2021 (Chiesi USA, Inc 2020).

5.3.3.3 SARS-CoV-2 Disease

Amidst the rapid progression of SARS-CoV-2 disease (COVID-19), a new yet effective therapeutic approach is crucial. The time-consuming, laborious, and costly methods via conventional drug discovery do not cut it. Therefore, a significant consideration for DR is indispensable. To no surprise, DR involves numerous computational methods, in which will be discussed in the following context.

For instance, five proteins of SARS-CoV-2 were selected and generated for DR target via SWISS-MODEL workspace, namely, the 3-chymotrypsin-like protease (3CLpro), papain-like protease (PLpro), cleavage site, heptad repeat (HR) 1, and receptor binding domain (RBD) in the S protein (Mahdian et al. 2020). Subsequently, the Protein Data Bank (PDB) was used to coordinate with human angiotensin-converting enzyme (ACE)-2. Following that, 2471 compounds obtained from the DrugBank database were screened against the cleavage site and RBD in S

protein via AutoDock Vina. From the 2471 drugs, 128 FDA-approved drugs were found to be suitable, with 18 drugs reported with antiviral effects. Virtual screening was performed on the 18 drugs with ACE-2, 3CLpro, PLpro, HR1, and TMPRSS2 via PyRx 0.8 software. Of the 18 drugs, 7 drugs were most favourable and deemed as promising DR candidates, namely, glecaprevir, simeprevir, ledipasvir, paritaprevir, glycyrrhizic acid, Hesperidin, and TMC-310911.

Using the LigPlot program, glycyrrhizic acid and hesperidin showed the highest number of H-bond interaction with ACE2 and RBD amongst all candidates. Glycyrrhizic acid, an active ingredient obtained from the roots of the licorice plant, has been proven to show positive effects in numerous viral diseases, such as herpes simplex type 1 (HSV-1), varicella zoster virus (VZV), HIV, SARS, and Epstein-Barr virus (EBV). On the other hand, hesperidin is a flavonoid extracted from citrus fruit, which has been demonstrated to inhibit replication in influenza A virus (IAV). Apart from that, hesperidin exhibits potent 3CLpro inhibition, an effective target for SARS-CoV-2. Conclusively, the importance in computational approach for DR is once again affirmed, especially in an urgent pandemic such as the current ongoing COVID-19.

5.4 Challenges of DR

5.4.1 Optimization of Repositioned Drugs, Intellectual Property, and Compound Availability

Although DR offers great benefits and opportunities over the de novo drug discovery, there are also some drawbacks, gaps, and challenges that come along the way. According to Pritchard et al. (2017), if the repositioned drug is intended for a new population, or if the dosage and delivery method of the drug needs to be optimized, clinical trials including animal models, followed by a clinical program, are still required to test its safety. Normally, phase III of a clinical trial is a very lengthy process, presenting itself as one of the obstacles in the development of repositioned drugs. In unprecedented cases, the duration may be reduced when the number of incidences is relatively low during the AD recruitment process of a clinical trial. For example, the clinical trial conducted for COVID-19 in China using the repositioned drug, remdesivir, was halted due to an inadequate number of infected patients to achieve the requirement of a trial recruitment target (Wang and Xu 2020).

Concerns regarding intellectual property (IP) also arise as repositioned drugs need to endure a strenuous process to be patented, because it has been publicly unveiled. However, the eligibility for obtaining IP protection varies between jurisdictions across the globe (Lexology 2021). Consequently, it may discourage pharmaceutical companies from the opportunity to generate more revenue and retrieve as much profitable development costs of the repositioned drug to resemble that of a de novo drug discovery (Rastegar-Mojarad et al. 2015; Nosengo 2016). Companies may experience financial distress, eventually resulting in interference of the drug development process. Therefore, it is crucial to seek clarity on up-to-date

patent policies and offer more incentives from investors to companies focusing on DR. Additionally, it is very common that safety information of developing drugs, including repositioned drugs, is lacking at the time of authorization. This is due to the nature of clinical development, where emphasis is placed on drug efficacy at the early stages. A more robust risk evaluation plan is highly recommended to address any safety uncertainties during the post-authorization and post-marketing phase (Sultana et al. 2020).

In certain cases, some pharmaceutical companies or drug inventors seem inferior or hesitate to release their compounds/chemical libraries outcomes (e.g. shelved drugs) to be further explored by other drug developers over its possible applications or novel indications. This situation stands as one of the barriers to the progress of DR prospects, especially when the potentially repurposed drug targets diseases that are beyond the organization's expertise area. It is also highlighted by Talevi and Bellera (2020) that regardless of collaborative works between large and smaller firms in the DR market, it is crucial to establish proper administrative procedures (e.g. agreement on chemicals/compounds distribution and its subject matter) to ensure benefits to both parties.

5.4.2 Data Availability and Computational Tools

With the development of high-throughput technology, an enormous amount of biomedical data has been generated and uploaded on online databases such as drug-related databases (e.g. DrugBank, PubChem, CTD, and SIDER), disease-related databases (e.g. Disease Ontology (DO), MalaCards, Online Mendelian Inheritance in Man (OMIM), and DisGeNET), and protein/gene-related databases (e.g. UniProtKB, BioGrid, HPRD, and PDB). However, public access to certain types of valuable and essential information is still very limited. Some of the data may be missing or insufficient to be processed by modern or classical approaches. The integration of appropriate databases using computational approaches will enhance the quality of the analysis and ease the course of identifying new indications for existing drugs (Talevi and Bellera 2020).

Despite there being various types of computational (based on different algorithms) and experimental methods, each technique has its own applicability, drawbacks, and limitations. Thus, none of these methods alone will be able to decode the complex interaction between drugs, targets, and diseases. Generally, the most common techniques in computational methods are network- and machine learning-based for DR (Le and Nguyen-Ngoc 2018; Wang et al. 2020). Nevertheless, these techniques only employ a single measurement to analyse the information similarity of the drugs and disease association in predicting new indications of existing drugs. In fact, resemblances between the drug-disease interaction are very multifaceted and must be evaluated from different angles to produce a more precise quantification of drug-drug and disease-disease relationships. Furthermore, there is still patchy information about the drug-disease relation in a form of an adjacency

matrix or a bipartite network for computational approaches, which can affect the prediction performance (Le and Nguyen-Ngoc 2018).

Integration of one or more repositioning approaches is required to meticulously manoeuvre the heterogeneous amount of available data into an incorporated workflow. More importantly, the new integration methods must be able to extend its domain of applicability. Indeed, it is generally recognized that computational techniques offer a more systematic repositioned plan and indubitably, a cost-effective means to discover new interactions between drugs and diseases compared to conventional approaches like in-lab experimental techniques. Nonetheless, in scientific research, the outcomes from computational approaches will subsequently always entail proper validation using experimental work to strengthen the findings.

5.5 New Horizons of DR

5.5.1 Neglected Conditions and Orphan Diseases

Indeed, DR has brought into spotlight the rare pathophysiological conditions/diseases (e.g. orphan disease), which are often poorly characterized and receive less attention from pharmaceutical companies to develop drug treatments. This is because only a small grant or budget is allocated in mainly developing countries to create new or repositioned drugs to combat the disease. According to the World Health Organization (WHO), an orphan disease is defined as the prevalence of an illness being less than 6.5–10 in 10,000 people (Aronson 2006). These types of disorders have recently gained more attention and interest, since there are now several orphan drugs available in the market such as haem arginate (for porphyria—acute intermittent, variegate, and hereditary), ibuprofen (for patent ductus arteriosus in neonates), and N-acetylcysteine (for paracetamol poisoning) (Hift and Meissner 2005).

Although there are approximately 8000 existing orphan diseases (Statista 2021), increasing numbers of pharmaceutical industries are taking the initiative to formulate repositioned drugs as compared to a decade ago, where there was only around 5% of participation (Sardana et al. 2011). This is because of the latest development of technologies (e.g. computational approaches) and the offered incentives and assistance provided under the Orphan Drug Act (ODA) 1983. To encourage and ease the burden of pharmaceutical and research organizations, the ODA 1983 has outlined some benefits, including the following: (1) tax credits, (2) financial aids for research, (3) fastening the marketing authorization process, and (4) marketing exclusivity (Lavandeira 2002).

5.5.2 Personalized Medicine

Personalized medicine aims to improve the treatment course of some disorders by utilizing genomic findings to integrate biomedical research and clinical medicine.

The wide expansion of biological data from the Human Genome Project and the introduction of next-generation sequencing has permitted the customization of healthcare and incorporation of electronic medical information to design the appropriate treatment for each patient based on their intrinsic biological profile (Chen et al. 2015). Both personalized medicine and DR offer to improvise the productivity of drug treatment for some pathological conditions, which consume a lengthy time and an enormous cost until the discovery of a new indication. This approach is also particularly relevant to study rare diseases or disease subtypes for patients who are resistant to certain therapies and have still not found any cure (Li and Jones 2012).

Previous studies have unveiled the essential involvement of DR linked to the strategy of personalized medicine to find tailored therapies for individual patients. For example, crizotinib was initially used for anaplastic large-cell lymphoma disorder and was further diagnostically tested as the repositioned drug subset for non-small-cell lung cancer (NSCLC) patients (Shaw et al. 2011). Another study reported a metastatic colorectal cancer patient who resisted chemotherapy and radiation undergoing whole-genome sequencing. Two proto-oncogenes, namely, FOS and JUN, were differentially expressed and resulted in the repurposing of the antihypertensive angiotensin II receptor antagonist, irbesartan, as an anticancer therapy to inhibit the renin-angiotensin system (Jones et al. 2016).

5.5.3 System Medicine and Combination of Repositioned Drugs

System medicine, also known as network pharmacology, is a healthcare approach closely related to personalized and stratified medicine. It is based on computational models to further understand disease mechanisms and design multitarget therapeutics against a particular condition. A combination of synergistic drugs using approved drugs from DR may also expand the spectrum of its usage and effectiveness. For example, nifurtimox was initially developed for cancer treatment, whereas eflornithine for American trypanosomiasis in the late 1970s. Later, it was found that a dual combination of these drugs showed a new indication in managing advanced stages of sleeping sickness (Alirol et al. 2013). This has not only allowed easier administration but also a reduction of treatment duration as compared to using eflornithine alone. Active compounds in a single-drug therapy may potentially display weak activities or low potency, limiting their immediate action to combat certain pathological conditions. The synergistic effects of a multidrug therapy will thus enable compensation in areas where a drug has weaker activity, thus enhancing its therapeutic effects (Talevi and Bellera 2020; Zheng et al. 2018).

5.6 Conclusion

DR prioritizes establishing treatment for diseases that urgently require them. Novel drug development often focuses on high-priority diseases or diseases that affect a large sum of individuals, whereas DR provides an opportunity to establish

treatments for diseases that are rare, complex, or novel. In the past, DR was considered tedious, as target identification required manual selection through literature searches. Modern approaches have advanced to incorporating knowledge-based and machine learning information into databases that can provide valuable output of target molecules and networks and ultimately lead to the identification of a functional FDA-approved drug target to reposition for the disease of interest. Some challenges of DR include concerns regarding intellectual property and the integration of the multitudinal approaches for DR, in particular, to incorporate vast amounts of data across various computational tools and databases with advanced expertise. DR is being recognized to provide hopeful outcomes for neglected and orphan diseases as well as significant potential for the future of personalized medicine. Overall, genomic approaches confer a more efficient and effective establishment of drug targets for DR.

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Organ-on-a-Chip: Novel In Vitro Model for Drug Discovery

6

Geeta Aggarwal, Gaurav Kaithwas, Manjari Singh, and Ramesh K. Goyal

Abstract

In conventional drug discovery process, the attrition in clinical development as a consequence of late clinical trial failure has been very high. One of the main reasons for high attrition rate during clinical trials is the limitation of animal models used for preclinical testing of drugs, which are not able to clearly predict drug response in patients. In spite of advancements in use of preclinical and in vitro models, viz., cell cultures, computational models, animals, and humanized animals during drug discovery process, there is a need to develop a human-specific model to bridge this gap between animal-based models and human clinical trials.

In vitro disease models can provide an excellent alternative to the animal models as they minimize the use of animals and understand the cellular and molecular aspects of various diseases. One of the important techniques for development of three-dimensional (3D) in vitro model is 3D bioprinting, which makes realistic in vitro disease models and mimics the actual cellular arrangement of any human tissue or organ. Organ-on-a-chip as 3D in vitro model has shown its potential to understand the disease mechanism along with evaluation of new therapeutic compounds. Further, multiple organs on a chip are utilized to understand drug–drug interactions and pharmacokinetic profile of new drugs and thus show potential to predict safety and efficacy of drug in patients in a more realistic

G. Aggarwal · R. K. Goyal (✉)
Delhi Pharmaceutical Sciences and Research University, New Delhi, India
e-mail: goyalrk@gmail.com

G. Kaithwas
Babasahem Bhimrao Ambedkar University, Lucknow, Uttar Pradesh, India

M. Singh
Department of Pharmaceutical Sciences, Assam University, Silchar, Assam, India

R. C. Sobti, N. S. Dhalla (eds.), *Biomedical Translational Research*,
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way. The organ-on-a-chip as in vitro model will prove to be a potential platform in the development of personalized medicines in future.

Keywords

In vitro model · Liver model · Kidney model · Lung model · Bioprinting · Human-on-a-chip

6.1 Introduction

The pharmaceutical's industry is the most demanding, and the most challenging aspect is drug discovery that involves development, identification, characterization, and optimization of new drug chemical entity (Hughes et al. 2011; Harper and Topol 2012). Several problems continually faced by drug developers related to adverse effects of novel lead molecules and then to carry new drug candidate/entity on to the market in a systematic and progressive manner (Bhusnure et al. 2017). In vitro conventional cell models are generally used to examine and observe a variety of signal molecules, viz., enzymes, receptors, and ligands, which are associated with biological and physiological processes (Booth and Kim 2012). Nevertheless, there are several drawbacks of conventional in vitro models like they do not mimic the complexity of interactions of cells within the body. They do not detect the time-changing signalling molecules (mechanical or chemical) due to its static conditions and require excessive quantity of nutrients, which are vital for the normal cellular functions (Frohlich et al. 2013; Griep et al. 2013; Polini et al. 2019). Further, these models do not imitate the extracellular mechanical environment (Bhusnure et al. 2017).

Due to the poor predictive power of existing animal models used in preclinical research, several proposed drug molecules fail in clinical studies during the new drug research and discovery process. This problem arises due to the use of conventional animal testing models that only detect the investigational drugs in animals and in human cells. In addition, another complexity comes from the genetic variability among patients that can change the pharmacokinetic and pharmacodynamic behaviour to a drug.

Thus, there is always an urgent demand by the pharmaceutical companies in the development of novel testing approaches, to produce the authentic and reliable information of drug candidate *w.r.t.* its safety and toxicity profile clinically in human beings. To overcome these challenges, microfabrication and microfluidics based on micro-engineered cell culture models are used nowadays. Development of advanced cell culture models opens a new era in the drug discovery, i.e. organ-on-a-chip, which is a more reliable and precise approach in the human biological processes and can transfigure the outlook of drug development process and methods (Esch et al. 2015). This advanced technique when compared to conventional models assist for the selection of right drug molecule and its concentrations in a time efficient manner (Booth and Kim 2012). Moreover, they can generate different

mechanical/chemical stimuli and its concentration gradients of all signalling molecules that can be applied electronically in a time-controlled manner. Nowadays, pharmaceutical industry focussing on the latest advancement in the development of the newest microdevices and bio-microelectromechanical systems (BioMEMS), collectively known as 'organ-on-chips'. The 'organ-on-chip' has shown its potential to bridge the difference between preclinical to clinical studies. It can enumerate the complexity between cell-to-cell interactions, providing cellular micro-environment in a time-controlled manner, and signals are transmitted into the cellular constructs with high precision (Frohlich et al. 2013; Griep et al. 2013; Jang and Suh 2010; Polini et al. 2014; Chen et al. 2012).

On the other hand, 2D/conventional cell culture simulations do not provide information about the structural complexity within and outside the cells in a consistent and realistic manner. Organ-on-chips make the best selection possible by utilizing 3D cell culture versions due to their superior capability to imitate tissue design, structure, and function (Chen et al. 2012). Gap junctions, which are required for cell-to-cell information exchange, tissue integrity, and architecture, are also more prevalent in 3D. Further, 3D cell culture is fully grown and binds compactly to cells, preventing or decreasing drug diffusion and permeability, which is not possible in 2D cell culture models. Thus, 3D cell culture models such as organ-on-a-chip are more capable in microfluidics than 2D cell models when it comes to the discovery of new drug molecules and their associated studies (Guido et al. 2011; Arrowsmith and Miller 2013).

This chapter looks at how rapid advances in '3D bioprinting' of tissues and organs, particularly 'organ-on-a-chip' in vitro technologies, have opened new possibilities for improving human condition modelling. It discusses that organ-on-chip-based 3D models can be a possible replacement for animal modelling and might be helpful in the transition of conventional preclinical techniques and models into novel research and brings a new platform in the modern drug research for developing in a cost- and time-effective manner. In the chapter, development and applications of various organ-on-chips are discussed along with challenges in the use of these novel in vitro models.

6.2 Potential of In Vitro Biological Models in Drug Discovery

Drug discovery process is a time-consuming and costly process. Several drugs fail during clinical trials (phase 2 and phase 3) due to its low pharmacological efficacy profile and its safety concern, i.e. inadequate therapeutic index (Langhans 2018). Currently used preclinical models in drug discovery do not provide better precision data, and hence, attrition rate is high during development of new lead molecules. There is always a need to consider new technologies/testing models with enhanced precision in the process of drug development. In vitro models are found to be crucial in drug research because they provide insight into the behaviour of cells and microorganisms. Conventional in vitro models may not be able to forecast the impact

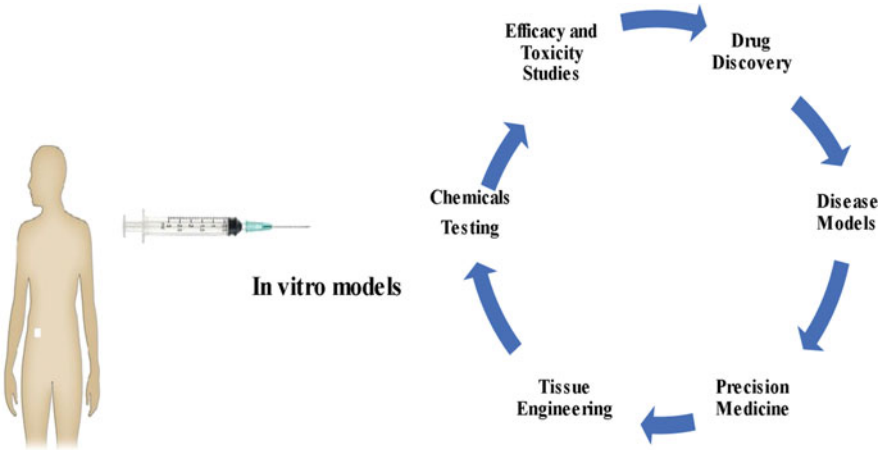

Fig. 6.1 Illustration of application of in vitro models for different phases of drug research

on the whole organism, since these cells are separated from their biological environment.

In vitro models including organ-on-a-chip architecture ensure progress in this field. Cells cultured in microfluidic chip are assumed in the 'organ-on-a-chip'. The chip generates artificial organs by simulating bioactivities, dynamics, and physiological behaviour of organs or organ systems. So far, multiple cell lines have been used for each artificial organ (Nelson and Bissell 2006). The most used cell lines for the lungs are 16HBE, Calu-3, A549, and NHBE, while the most used cell lines for the liver are Hep 3B, HepG2, and TPH1 (Nikolic et al. 2018; Langhans 2018). They demonstrated significance of extracellular matrix in cell performance and are used widely in culturing cells in 3D systems (Nikolic et al. 2018). Choosing the right in vitro biological model in the different phases of drug discovery (Fig. 6.1) establishes a solid foundation for the entire research and development process, and combining insights from advanced in vitro and in silico methods early in drug development will increase clinical success rates. Rather than using the traditional 'bench to bedside' method, researchers should begin at the bedside, where patient characteristics, tissue type, and physiological goals are well defined. As a result, researchers will be able to reverse engineer the drug production pipeline and make more informed decisions about which biological models to employ. Using an integrated approach to build in vitro biological model systems, researchers can obtain more accurate results while saving time and money.

A study conducted by Pan et al. reported results of preclinical studies for 155 drugs that were progressed to clinical studies on humans as part of the regulatory approval steps. Out of 155, 27 drugs were accepted as monotherapy for treatment of lung cancer, but 128 drugs declined at some point during clinical trial (Pan et al. 2020). Despite their limited predictability, animal models are still the favoured approach in drug safety studies. Numerous drugs previously found ineffective in

animal models have shown promise in vitro or in human cell models. It indicated that in vitro models can be used in conjunction with more traditional in vivo toxicological and pharmacokinetic evaluations throughout the drug development process. These validated in vitro models can be classified as in vitro screens. The utility of these screens for drug research process is evaluated in terms of their standardization, validation, human tissue uses, iteration level with in vivo studies, regulatory status, and cost-effectiveness (Kačarević et al. 2018).

6.3 Organ-on-a-Chip as Novel In Vitro Disease Model

Different organs have been built using on-a-chip technology in recent years. One of the most recent advancements in in vitro disease models is 'organ-on-a-chip technology', which uses microfabrication techniques (Derakhshanfar et al. 2018; Zhang et al. 2019). This technique is becoming more popular as a tool for studying drug metabolism (Mengus et al. 2017). Due to this 'human-on-a-chip' technology, researchers will gain a better understanding of drug metabolism, pharmacokinetics, and toxicity in humans. Additionally, researchers have been able to detect metabolic changes associated with the disease in humans. Organ-on-chip systems require only a small amount of patient tissue to replicate the organ, implying that the development of personalized medicine may be possible (Kimura et al. 2018).

In several of these models, living cells resulting from tumours and tissues are refined in conditions designed to mimic disease states and processes. However, since every structure is only as powerful as the cells it is made with, choosing the right cells to form the model's base is a major challenge. Increasingly, human primary cells are being used to build physiologically relevant in vitro cell model systems. Primary cells carefully extracted from human blood and tissue closely resemble the functions and processes of the tissues from which they were derived.

On the other hand, efficiently isolating and purifying primary cells is a challenging task that necessitates a thorough understanding of cell and tissue biology. Given the complexities, many laboratories would profit from working with vendors to acquire primary cells that have already been characterized, enabling them to construct the right model more confidently for their needs. After being isolated, cells must be checked for viability and functionality, as well as for the presence of common laboratory pathogens. Tissues must also be collected in an ethical manner from donors who have filled out the required paperwork and given their permission (Cooley et al. 2002; Rodriguez-Garcia et al. 2020).

To characterise the safety of lead drug compounds by predicting how the drug will interact with an organ in vivo, 'organ-on-chip' microfluidic procedures are increasingly being used. While assay robustness and model sophistication appear to be impeding progress towards wider acceptance, these sophisticated models have the potential to increase predictivity, allowing for more confident drug candidate selection.

6.4 Advantages of Using Organs-on-a-Chip as In Vitro Model

Organs responsible for respiratory system, central nervous system, hepatic system, and cardiovascular system, as well as lymph junction and mammary gland, have all been created using 'organ-on-a-chip' technology. Several researchers are trying to construct a 2D, 2D+, or 3D architectural design with oxygenation to better simulate the in vivo environment. They may, however, have some major advantages over conventional safety, efficacy, and toxicity testing methods:

1. Biology in 3D is superior to 2D biology.
2. Ideal for use as a barrier.
3. Replicates hormonal flows.
4. Extracellular matrix can be thick for drug/factor binding.
5. Support the relationships between organs.
6. Sufficient tissue for tens to thousands of variable multi-omics studies.
7. Minimal media volumes are needed.
8. It is possible that drug prices will drop in the future due to less failures in clinical trials.
9. It is possible that 1 day a separate-patient miniature will be designed.
10. It is possible that animals-on-chips would be possible in the future.

6.5 Role of 3D Bioprinting in Developing Organ-on-a-Chip and Its Uses

A significant advancement in a key engineering, manufacturing, education, as well as design and health sectors has come from the widespread use of 3D (or 'additive') printing. Biocompatible components and biointeractive cell parts can now be 3D printed to create living tissues. To meet the demand for transplantable tissues and organs, 3D bioprinting is used in tissue regeneration engineering (Chen and Liu 2016). 3D bioprinting is more complicated than non-biological printing due to substrate choice, types of cells, proliferation and differentiation variables, and technical difficulties associated with the susceptibilities of living cells or tissues construction. To address these issues, engineering, biomaterials research, cell biology, physics, and medicine must all be combined (Doke and Dhawale 2015). Several tissues, including multilayered skin, bone, vascular grafts, tracheal splints, cardiac tissue, and cartilaginous structures, have already been created and transplanted using 3D bioprinting. Other applications include the creation of elevated 3D-bioprinted in vitro studies for experimentation, drug development, and toxicology (Yan et al. 2018). Figure 6.2 depicts the process of 3D bioprinting.

Charles Hull, an American engineer, invented the first 3D printer in the early 1980s, which used computer-aided way to design solid objects (CAD). By depositing various combinations of an acrylic-based photopolymer and crosslinking them concurrently with UV light, the printer created a solid 3D object. A fundamental technique, stereolithography (SLA), has revolutionized the additive manufacturing

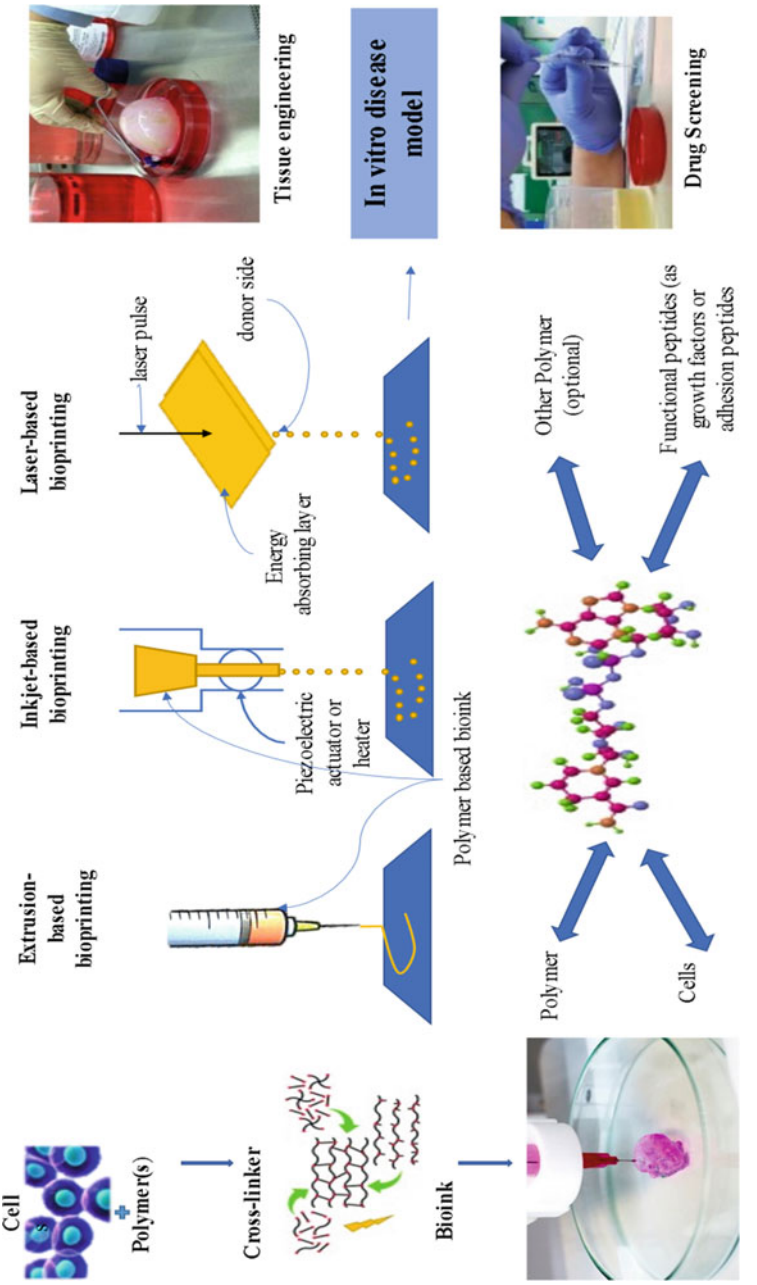

Fig. 6.2 Process of 3D bioprinting and its applications

industry. 3D printing had entered the medical field by the late 1990s, with surgeons printing dental work, custom prostheses, and renal sacs. As a result, the term '3D bioprinting' was coined to describe the 3D printing of living organisms, materials science, or active molecules using a material known as 'bioink'. As with additive manufacturing, '3D bioprinting' utilizes layer-by-layer deposition of bioink to create 3D structures such as tissues and organs (Gu et al. 2020).

There are three types of 3D bioprinting: 'extrusion droplet bioprinting', 'inkjet-based bioprinting', and 'laser-based bioprinting'. Extrusion-based biomimetic deposits 'bioinks' in filaments via pneumatic, hydraulic, or solenoid sprayer systems, whereas inkjet-based bioprinting generates 'bioink' droplets via heat energy, microvalve, or electrodes. Structures are 3D printed using a photopolymerization hypothesis in laser-based bioprinting methods like stereolithography (SLA). In laser direct-write and laser-induced forward energy transfer, it can also be used to precisely position cells (LIFT). Each of these bioprinting techniques necessitates a different set of 'bioinks' with different viscosity, flowability, crosslinking chemical properties, and bioactivity. Shear-thin bioinks are required for extrusion-based bioprinting, whereas low viscosity funds are needed for inkjet bioprinting. To meet the growing demand for new 'bio-printable' materials, 'bioink' layout and synthesizing have advanced significantly in recent years. Making 3D structures with low viscosity 'bioinks', for example, has always been tricky. These 'bioinks' can now be compacted into a granular structural with strain rate hydrogels. These hydrogels harden around the extruded structure, trying to prevent it from collapsing and thus fixing the issue. Bioprinting is used to create in vitro tissue designs for drug test, clinical diagnostics, and a variety of other in vitro applications, in relation to printing organs (Ashammakhi et al. 2019).

In case of use of 3D bio-printed organs in regenerative medicine and tissue engineering, Kim et al. described a tubular tracheal graft made of two layers of polycaprolactone that was 3D printed. This tracheal graft seeded with induced pluripotent stem cell (iPSC)-derived mesenchymal (MSCs) and chondrocyte stem cells assisted the regeneration of tracheal mucosa and cartilage in a rabbit model of a segmental tracheal defect (Kim et al. 2020). Galarraga et al. used a norbornene-modified hyaluronic acid (NorHA) macromer as a symbolic bioink for cartilage tissue engineering. Printed structures containing MSCs increased compressive moduli and expressed biochemical content like native cartilage tissue after long-term culture (Galarraga et al. 2019). Vidal and his team used 3D printed adjustable calcium phosphate scaffolds with and without a vascular pedicle to treat large bone defects in sheep. To model the microenvironment of the oesophagus (Vidal et al. 2020). Nam et al. used a bioink made of decellularized matrix from the mucosal and muscular layers of native oesophageal tissues. Using gelatin-based bioinks. Leucht et al. investigated vasculogenesis in a bone-like microenvironment (Nam et al. 2020; Leucht et al. 2020). To resemble the different layers of osteochondral tissue, Kilian in his lab used a calcium phosphate cement (CPC) and an alginate-methylcellulose-based bioink containing primary chondrocytes. In case of drug testing and drug discovery, 'liver-on-a-chip' was used successfully to assess the drug-drug interaction and cytotoxic analysis of acetaminophen (Kilian et al. 2020). The results

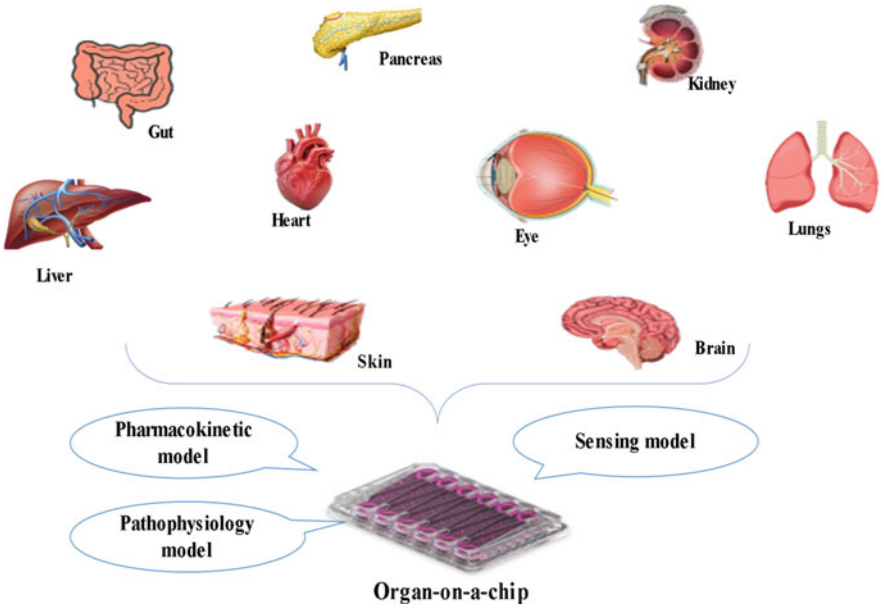

Fig. 6.3 Types of organ-on-a-chip

revealed that due to drug-drug interaction, hepatotoxicity was 14.88% for combination of acetaminophen-omeprazole, 17.15% for combination of acetaminophen-rifampicin, and 19.74% for acetaminophen when taken with ciprofloxacin. It showed that this 3D printed liver model can be used to analyse hepatotoxicity of newly discovered drug and drug-drug interactions (Deng et al. 2019). In another study, the renal proximal tubule was bio-printed to analyse the nephrotoxicity of cyclosporine A in place of preclinical studies. This bio-printed proximal tubule was proved to be useful for testing of newly discovered drugs and in vitro disease modelling (Homan et al. 2016).

Biglari et al. during a study developed a skin 'wound-on-chip' model to analyse the anti-inflammatory efficacy of dexamethasone and macrophages during wound healing. The researchers were able to predict the mechanism of wound healing process of macrophages and dexamethasone. This 3D 'bio-printed model' can provide insight into the mode of action of a newly discovered drug/new drug substance for tissue regeneration and can improve preclinical models, according to the scientists (Biglari et al. 2018).

The studies in literature reveal that the 3D printed 'organ-on-a-chip' is a micro-scale device that mimics the human body's environment. One of the main goals of organ-on-a-chip is to develop human tissue models for disease modelling and drug research. Microfluidics and cells are used to create physiological and mechanical conditions like those found in the human body. Various types of 'organs-on-a-chip' are depicted in Fig. 6.3, and their development procedures and uses are discussed in greater detail further down.

6.6 Development and Usage of Various Types of Organ-on-Chip Technologies in Drug Discovery

6.6.1 Lung-on-a-Chip

In humans, lungs are the main part of lower respiratory system. The composition of lungs includes airways, branched blood vessels, conducting zone (air entry zone), and respiratory zone (zone for exchange of oxygen and carbon dioxide). The respiratory parts include bronchus, bronchiole, and alveoli. The smallest unit of lung is the alveolus, which is functional and provides a sufficient surface area for gas exchange. The creation of tissue of lungs is challenging but can help to understand the effect of new drugs, toxins, and pathogens in airways and mechanism of infectious diseases affecting the respiratory function (Benam et al. 2016; Zepp and Morrisey 2019).

In this area of research, the microfluidic lung-on-a-chip device was developed by Harvard scientists at the 'Wyss Institute for biologically inspired engineering'. It incorporated the flow of lung fluid and breathing patterns like those of a human-on-a-microchip. The membrane was permeable between two distinct layers of lung cells, with separate upper and lower canals between them. For cyclic mechanical breathing, the chambers were evacuated to get a greater range of motion and stretch. It was demonstrated that the 'alveolus-on-a-chip' or 'lung-on-a-chip' can be used to conduct experiments on natural breathing, and it can be used to understand the safety and efficacy of new dosage forms like nanoparticles. It is also possible to do experiments on new and novel types of drugs and lung cancers. Asthmatic and non-asthmatic bronchial epitheliums were grown on the air/non-asthmatic cells, respectively, to create 3D models of asthmatic and non-asthmatic tissue (Huh et al. 2012).

The Wyss Institute worked on several microchips to inspect the capillary-alveolar membrane system of the human lung (Huh et al. 2010). It was experimented with a variety of configurations, one of which used a living alveolar-capillary unit. Mimicking the human's lung's functional alveolar unit, the biomimetic microsystem was developed to assess the effectiveness of drugs (Jain et al. 2018).

A thin alveolar septum, built on a bioengineered 'lung-on-a-chip', was freshly found to be capable of accurately reproducing lung dynamic respiratory complexity. As one can see, the main goal of lung tissue regeneration is to help to grow epithelial and endothelial cells in an environment that simulates human respiration. The researchers showed that involuntary stress affects the epithelial wall porousness using a bronchial epithelial cell line. Furthermore, the cell culture outperformed a static model, it can mimic the lungs, and it helps in explaining how the lungs work and can be used to simulate pulmonary disease. Results revealed that toxicity testing and a new drug development are also possible using this micro-device (Stucki et al. 2015).

A drug development study made use of the model, resulting in finding out the effect of interleukin during pulmonary oedema in patients. Research has shown that intercellular junctions are opened synergistically with the endothelial-lung junctions

in the alveoli during respiratory movement. In fact, the mechanical forces increased tissue permeability three times over that time in combination with IL-2. Additional research revealed that IL-2 successfully inhibited vascular leakage completely when given with angiotensin-1 (Xu et al. 2013).

The 'lung-on-a-chip' model for asthma and chronic obstructive pulmonary disease (COPD) was used to substantiate claims that tofacitinib, a medication employed to treat rheumatoid arthritis, could suppress lung inflammation. Additionally, it was discovered that inhibiting neutrophil adhesion with a bromodomain-containing protein 4 (BRD4) inhibitor could reduce lung infection by nearly 75% under complex flow conditions (Benam et al. 2016).

6.6.2 Liver-on-a-Chip

The liver has a multitude of roles, such as protein synthesis, hormonal balance, glycogen storage, and detoxification. It is an important toxicity target in human drug interactions as well as a player in the drug induction. The liver is extremely active in metabolism and thus essential to life. Metabolic activity in the liver is very high, and without it, we would not have a metabolism. Besides its amazing regenerative capabilities, chronic diseases and viral infections cause significant damage to tissue, as well. Hepatocytes are tied together by blood vessels and Kupffer cells into the hepatic lobule, which performs all the major functions of the liver. Hepatocytes are difficult to keep alive in the lab. Hepatic cell microsphere system was specifically created to study hepatic interactions in 3D cultures. Other experimentation that may be conducted includes but is not limited to drug testing, pathogenesis, human physiology, and toxicity, and screening with a liver-on-chip (Dixon et al. 2018).

The liver is used in human and animal models to study the metabolic rate and toxicity of chemicals and medications, as well as to assess the efficacy of treatments. Primary cells are obtained from patients or cell banks, such as the 'American Tissue Culture Collection', and subjected to a battery of tests to determine whether they exhibit the properties of human tissue when cultured. They may use cell cultures to better understand how drugs move through the body. The ability to discover new drugs is harmed when normal human liver cell activities such as transport and drug breakdown are lost in cell culture. Liver-on-chip systems have been proposed as a new generation of in vitro drug screening models. Some liver-on-chip systems include biophysically preprefigured or 3D bio-printed matrices to enable 3D organ building (Bhatia and Ingber 2014; Miranda et al. 2021).

On the one hand, hepatology research and drug discovery depend on in vitro models of the liver. An essential part of these models is the cell source. Human, animal-derived, and hep-derived stem cells are the three major cell types employed to generate real liver tissue in the laboratory. Researchers currently utilise a variety of human hepatic cell outlines like hepatocytes, sinusoidal endothelial (Hep2), and hepatic stem cells for toxicological assessments (HepaRG). Hepatocytes are the liver's parenchymal cells that help maintain liver functions, making them the most active cells in the lobules. Also known as the reticuloendothelial, the system, the

reticuloendothelial cell is involved in both homeostasis and immunity regulation of the liver. Instead of primary cells, the immortalized liver cell lines HepG2 and HepG2B can be used for drug screening and toxicological tests. p53 and Nrf2 proteins, which are nuclear transcription factors required for drug metabolism, are found in hepatocellular carcinoma cells, which are frequently employed to measure drug toxicity (Deng et al. 2019; Rodriguez-Garcia et al. 2020).

The HepaRG is the best cell line for studying a slowly excreted able drug and uncovering xenobiotic exposure mechanisms. Acute liver injury, which results in the withdrawal of approved drugs from the market, is another toxicity concept that should be included in the drug assessment. Promising advancements in molecular pharmacology and toxicology have created more relevant cell interactions within their own environment, which allow for a more enabling long-term culture of livers and better re-creation of cellular responses to toxicants (Maschmeyer et al. 2015; Gori et al. 2016).

In research conducted by Bavli et al., the mitochondria and the sensor helped the liver respond to impairment. To monitor the levels of glucose and lactate over time, the sensor was designed. The world's first micro-liver-on-a-chip was also created to determine the effects of opioids on the liver. The objective of the study was to try to reconstruct the 3D cellular arrangement of the hepatic sinus. The hepatocyte culture was conducted for an additional 4 weeks and permitted the assessment of new drugs for potential toxicity (Bavli et al. 2016).

6.6.3 Kidney-on-a-Chip

Filtration and preservation of essential combinations, as well as blood pressure, can all be used to regulate kidney function. Additionally, the kidneys function as a waste disposal system, metabolizing drugs and excess materials. Due to these functions, drug testing is typically conducted in the kidneys. As kidney function declines in the elderly, glomerulonephritis, pyelonephrosis, and high cholesterol crystal disease can occur. Most people suffer from problems with their ability to excrete (Paoli and Samitier 2016). The primary goal of kidney chip studies is to identify nephrotoxicity, which occurs when various treatments/drug products are administered.

The kidney integrates several different tissues with the proper environmental conditions, making it difficult to model. Interstitial, glomerulotubular, proximal, ascending, distal tubule, and renal endothelial cells make up the kidney. Using four different co-cultures instead of a single kidney model allows both morphology and biochemical conditions to be utilized as well as the proximal, distal, and distal tubules. This is a simplified procedure to study drug toxicity, which mimics the renal nephrons on the chip. This model aids in drug filtration and understanding of the rate of molecules' reabsorption. The chip serves as the basis for the convoluted proximal tubule, the 'nephron's glomerulus', and the 'loop of Henle'. A single silicon chip was rumoured to contain a three-component artificial nephron function. Numerous factors preclude the use of [kidney] pods in the fabrication of kidney-on-chips, including the absence of human podocytes, the primary element of the glomerulus.

This problem has recently been dealt with by reprogramming pluripotent stem cells from human sources and conjugating them with primary cells from rat kidneys. This type of platform is ideal for preclinical drug research as it mimics the human and appears to reproduce human functions (Lee et al. 2018).

The work of several research teams has been successfully concluded on 3D cell culture has become a reality. Jang et al. developed a 'kidney-on-a-chip' through microfluidic device. Lumenal interstitial cells were placed in separate compartments, each separated by membrane flow. However, compared to nonfluid, transwell, dual chamber (membrane-separated), cells show increased primary pleomorphism, NaK-ATPase expression, and glucose uptake, albeit less than in vivo (Jang et al. 2013). Creative explanation: As an alternative technique, Jansen et al. established bioengineered tubules composed of hPTECs grown on polyether's hollow membranes with continuous perfusion and the basolateral cells were additionally fixed to the outer surface of the hollow fibres to provide perfusion-enabled basolateral delivery as an alternative technique. The organic anion transporter 1 protein was increased sixfold when seeded in Transwell compared to freshly isolated cells (Jansen et al. 2016).

A recently discovered kidney on a microchip has been developed, based on human-induced pluripotent stem (hiPS) cells in podocytes. Anhydrous basement membrane (AMBA) was used to create a 3D glomerular-endolite matrix associated with human endothelial cells. Through their experiments, they were able to produce glomerular filtration wall physiology in the lab as well as they were being able to replicate the patient's findings with pharmacological podocyte injury and albuminuria. The short part of the proximal tube was utilized to make a probe for drug and nephrotoxicity (Wilmer et al. 2016).

6.6.4 Gut-on-a-Chip

Natural models are often unreliable for studying the human gastrointestinal tract due to a severe lack of microenvironmental conditions. Microfluidic flexible channels were constructed from Caco-2 intestinal cells, which incorporated parastatistical movement (Xiang et al. 2020). They were able to create rippling epithelium columns with polarized 'Caco-2' cells and multiple distinguishable intestinal types of cells using the conditions described. Enteroendocrine tissues, Paneth cell types, and distinct goblet cells, all of which secrete a substantial portion of saliva in the living intestinal tract, were all replicated in this study. The same community then concentrated on the inflammation caused by intestinal bacterial overgrowth. They were able to study pathophysiology for several weeks as part of the project, which is an excellent model for a variety of medical applications (Jing et al. 2020; Costa and Ahluwalia 2019).

To test the medication's ability to damage human cells, the medications are irradiated from mice and then expose' to specific levels of ultraviolet (UV) light. The researchers' test system modelling of radiation injury in the gut is viable since it maintains consistency and results in comparable outcomes (Tang et al. 2020).

In the replication of *Coxsackie virus* infection, another use for the 'gut-on-a-chip' is being studied. This virus also affects the pancreas and the liver in newborns. Replicating the microenvironment allows for successful human experiments on polarized systems, which creates a pressing challenge in this research field. The research by Sosa-Hernández et al. highlights the application of this model to other enterovirus studies, as well as the benefits of running dynamic systems in the form of culturing cells on demand (Sosa-Hernández et al. 2018).

To test for movement along the villi-crypt axis, researchers created a 3D porous substrate, and the cell migration was easy to observe by incorporating lactic acid (polylactic acid) to help the co-culturing of different cell lines (Wang et al. 2017a, b; Costello et al. 2014).

6.6.5 Skin-on-a-Chip

The greatest part of the human body is the skin, which keeps the internal organs on or after heat and cold, among other things. Under a wide range of stressors, this cell reacts with a wide range of responses. While trying out experiments to see how different conditions affect things in vitro and in people, the outcomes are variable. Thus, skin-on-a-chip becomes the key to study the effect of topical/transdermal formulations, and it can reduce the animal usage in drug testing during product development and discovery process.

Reverse ageing skin is becoming a more widely accepted in vitro model. One was conceived using epidermic, cutaneous, and endothelial layers to mimic the infection and oedema associated with eczema. The skin is more complicated than a simple distinction between the epidermis and dermis. Wrinkles are a natural occurrence that occur because of use and external pressure. Other factors may contribute to wrinkles, and it has been demonstrated that using a magnetic field, skin-on-a-silicon-microchip can be stretched out. This experiment may prove useful for testing cosmetics and pharmaceutical products (Wufuer et al. 2016; Zhang et al. 2018).

In scientific paper by Sriram and his team, it was discussed that creating a full-depth fibrin-matrix skin chip for 3D scaling, as well as epithelial collapse and dermal matrix, is useful in testing of new drugs (Sriram et al. 2018). Human intestinal micro-like biofilms were used to examine intestinal radiation damage in a microfluidic gut chip device. During radiation treatments, the continuity of the junctions is compromised, the epithelial function is diminished, and mucus production is inhibited (Jalili-Firoozinezhad et al. 2019).

6.6.6 Brain-on-a-Chip

The human brain and animal brains do not have the same functioning and genetics. Since the disorders exhibited by animal brains cannot be shown to correspond to those of humans, animal models are of not much use for research on brain lesions. Numerous 'brain-on-a-chip' systems have been established with the goal of

simulating fundamental connections and validating applications. One of the researches was focussed on the principles governing the development of highly complex brains, as well as their interactions with other body parts. Combining fundamental pharmacological approaches to degenerative diseases with an understanding of the brain's structure and drug interactions can result in a pharmacological approach to degenerative disorders. It is being developed by mimicking the central nervous system with pluripotent human cells that maintain an intact blood-brain barrier. A portion of the procedure was focussed on the interaction of human foetal stem cells with an adult mouse model. Then, in neuro-oncology studies, a chemotactic gradient was used.

A proof of concept in Alzheimer's disease has been done on a chip neurospheroids, in a flow-controlled environment. The results were recorded, both before and after blood flow. The research studies suggested that dynamic conditions encouraged the growth of neurons. There was another group who had been successful in constructing a brain-on-a-chip, mimicking the characteristics of the brain. They used both the electrodes that measure changes in a specific brain region as well as all that region's network activity to make a representation of disease in the research of new molecule (Bang et al. 2019).

6.6.7 Heart-on-a-Chip

Cardiovascular in vitro models typically establish a monolayer soft tissue within a complex geometry under static conditions. The tissue forms a flat layer with random cell alignment, no movement, and a physiological state that is not present in vivo. At the start of 'heart-on-chip' studies, similar situations were applied, but the physiology gradually progressed. A system that uses 'micro-engineered cardiac tissues' (ECTs) to sustain a 3D beating development that helps up of human cardiomyocytes is an innovation. There was a high degree of coupling in both the mechanical and electrical responses. The platform stimulated the cells mechanically during culture, promoting maturation and increasing mechanical and electrical pairing. The device was also used to measure the amounts of various isoprenalines. Cell proliferation with high alignment and morphology was aided by the interaction of oxygenation conditions and exosystemic geometry.

Engineered nanomaterials were used to create a 3D chip influenced by mussels to assess cardiac contractility. The extracellular matrix was made from gelatin, titanium oxide, and silver nanoparticles. In vitro, the method allowed investigators to measure the contractile effects of nanoparticle cardiotoxicity on sarcomere calcium signalling. A 48-h test has been used as anti-pharmacological quality control assay. Preclinical trials were also used to compare the efficacy and safety of the drug in this case. An 'Integrated Heart/Cancer on a Chip' (iHCC) was created using human cells. To simulate heart dynamics and evaluate the anticancer drug doxorubicin, the microfluidic sensor was outfitted with a micropump and hydraulic valves (Kamei et al. 2017).

6.7 Human-on-Chip-Related Microfluidic Chips

'Human-on-chip' measurements take place in the microfluidic setting. The use of 'multi-organ chips' and 'human-on-chip' models will be useful for drug development in the biopharmaceutical and pharmaceutical industry. According to prior evidence, animal-based and multi-organ research paradigms provide human-centred and single-organ perspectives. The high-level and low-throughput screenings are extremely valuable in this kind of study. Additionally, microfluidics has become more precise because of modern techniques, such as gel electrophoresis, homogeneity regulation, chemical gradients, and time-dependent media effects. 'Human-on-a-chip' technology enables researchers to examine physiological, chemical, molecular, and functional factors in a simulated environment. It contributes to a better understanding of compounds' pharmacodynamics and pharmacokinetics (Syama and Mohanan 2021).

6.8 Challenges of Organs-on-a-Chip

Numerous scientists are advancing our understanding to the next level of complexity in relation to in vivo tissues/organs. Different organs were successfully tested and worked on, yielding favourable results in literature. 'Organ- or tissue-on-chip' systems have a variety of applications in biomedical innovations, but it is important to note that they are still in their infancy and need to be refined before they are widely used. It is important to associate these instruments with medications that have undergone extensive ADME testing and to validate their relevance to clinical efficacy and toxicity. In addition, the upkeep of these procedures necessitated specialized preparation. Selection, fabrication, and maintenance of these products are also necessary skills. In addition to concerns about fabrication and cell maintenance, researchers are sceptical that any in vitro cell culture would ever completely embody the complexity of whole animal systems. The endocrine, skeletal, and nervous systems' adaptive immune responses, as well as complex system level behaviours, have not been studied. There is also the problem of toxicity, which is complicated by the fact that in vitro studies only use a few cell or tissue types, but toxicity can occur in areas of the body where the drug is not targeted.

Companies like 'TissUse GmbH', 'Emulate, Inc.', 'MIMETAS Inc.', 'Nortis, Inc.', 'AlveoliX AG', and 'Hesperos, Inc.' have emerged in the last 5 years, demonstrating the critical nature of 'organ-on-a-chip' technology in research. The US Food and Drug Administration (FDA) announced in April 2017 that it had entered into a multi-year collaboration agreement with 'Emulate Inc.' (a spin-off of Harvard University's Wyss Institute for Biologically Inspired Engineering) to conduct a series of trials utilizing 'organ-on-a-chip' technology to develop a toxicological safety assessment testing system (Isoherranen et al. 2019). These findings show that 'organ-on-chip' systems can be used to evaluate human health. 'Organ-on-a-chip' system will eventually be able to incorporate stem cell modern technologies, microenvironment, and personalization parameters (such as respiratory rate, cardiac

output, and drug abuse), allowing for the development of models of various gender groups, territories, age groups, and infections down to minute biological differences, advancing the advancement of precision health (Van Den Berg et al. 2019).

The resolution of this question is yet to be established: despite advancements in organ-on-chip models, whether organ workable reproduction is limited by cell source. Researchers described the substantial difficulties in culturing human primary alveolar and epithelial type II cells (Shiraishi et al. 2019; Weiner et al. 2019). Because of the limited number of primary cells and need of expanded supply, the organ-on-chip method increases cost and makes the technology more difficult to spread to the general population. Many bio-on-chip devices are made with polydimethylsiloxane (PDMS). PDMS chips can be carefully mounted on a conventional incubator and optical microscopes for use with cell culture. It was argued that that PDMS blocks the effect of the supplement, while enhancing the involvement of the protein, and has many limitations in its use (Wang et al. 2017a, b).

6.9 Future Scope

'Lab-on-a-chip' and 'multi-organs-on-a-chip' are two recent developments in microfluidic-based computer manufacturing. Several of those microfluidic-based programs have been designed in recent years with the goal of improving in vitro and in vivo prototype predictability. Furthermore, when compared to traditional cell culture methods like flask culture, dish culture, and well plate culture, the microfluidics-based cultured cell study claims a thorough insight into the interaction between cell culture variables and microenvironmental aspects that traditional cell culture methods lack. Microfluidics' flexible multifunctional characteristics, such as accurate positioning over microenvironmental components, provide new avenues for next-generation drug development.

The development of a 'human-on-a-chip' is critical because animal models in investigation and the healthcare industry will eventually be replaced. The 'human-on-a-chip' technology allows for expansion without the need for external assistance by adding more fully functional tissues. A truly autonomous approach, according to this proposal, requires that each tissue be capable of performing its physiological function adequately. To begin, extended cell viability must be maintained. In vivo studies of the interaction between tissues and microfluidic channels are required.

The electrochemical biosensors are being integrated into human-on-chip environments, which is a rising practise in the design world of electronic components. Physiological and physical implementations must be considered when designing the system, including suitable flow parameters, and biochemical equations are necessary. As with all experiments, these in vitro models should be understood within the context of their limitations.

Advanced and state-of-the-art research has made us closer to human-on-a-chip technology, as well as sensors for detecting various drugs and hormones. Thus, it was possible to demonstrate that the biophysical environment plays a critical role in assisting sperm in reaching the egg through the construction of a woman's

reproductive tract. Cancer metastasis, tumour metastasis, and physiology are additional important topics being studied with human organs on a chip. Clinically relevant in vitro parameters and effects must be correlated with chip measurements to maximize the utility of 'human-on-a-chip' testing. During drug development, this will also create a link between conventional cultured cells and non-animal clinical testing techniques.

6.10 Organ-on-a-Chip for Personalized Medicine

The scarcity of adequately predictive preclinical models of human origin is one factor contributing to the pharmaceutical industry's high attrition rate. The invention of human (induced) pluripotent stem cells (hPSCs) and their ability to distinguish into a range of cell types have sparked interest in developing more stable in vitro models, as well as for further investigation of their potential in personalized medicine. Extensive testing has revealed great promise for these models. For example, hepatocytes derived from hPSCs exhibited phospholipidosis and steatosis after 14 days of exposure to hepatotoxic compounds, both of which are indicative of chronic long-term toxicity. Another example is the production of functional neuronal cells from induced pluripotent stem cells and their application in Alzheimer's and epilepsy research. Investigators have also been able to investigate new therapeutic options for autosomal dominant-negative diseases, which is a big step forward. This was demonstrated elegantly by using RNA interference (RNAi) to rescue the diseased phenotype of hPSC-derived cardiomyocytes carrying a long QT syndrome mutation or a phospholamban mutation causing either dilated or arrhythmogenic cardiomyopathy.

The goal of personalized medicine is to determine the optimal medication and dosage for each patient. Because traditional methods are inefficient or time-consuming, they are unsuitable for this task. However, using patient samples, the physical-biological features of each patient's disease can be reproduced in an 'organ-on-a-chip'. The cells can be extracted directly from the patient's biological fluids and cultured on the microfluidic device before being exposed to various drug dosages. To reveal the cell types to the drugs in a manner consistent with their natural microenvironment, the medication should be mixed with blood. Other critical tissue-related variables, such as oxygenation, motion, and flow behaviour, can be altered in response to health data to design the physiological, structural, and chemical micro-environment around the cells. The 'organ-on-a-chip' technology, as an in vitro model, connects translational and reverse translational research for the advancement of personalized medicine (Fig. 6.4).

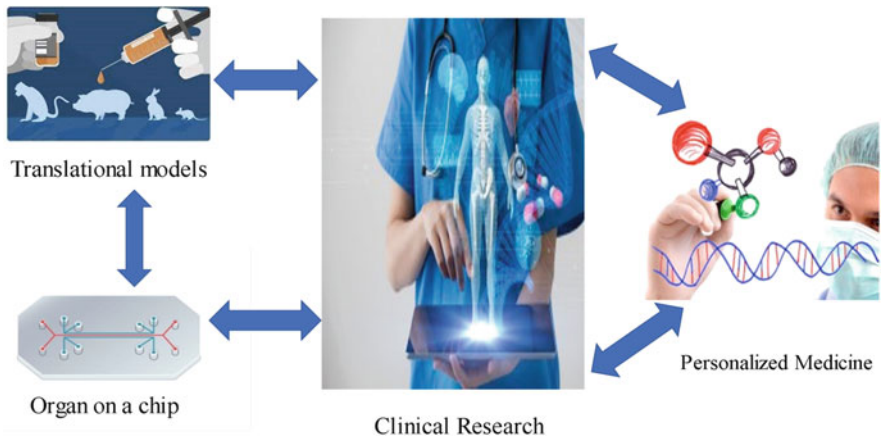

Fig. 6.4 Organ-on-a-chip as in vitro model for development of personalized medicine

6.11 Market Potential of Organ-on-a-Chip Technique

Companies that sell organs-on-chips for drug production, toxicity testing, and individualized medicine research make up the 'organ-on-a-chip' industry. 'Organs-on-chips' are a viable substitute to animal models, because they mimic the cellular physiology of human organs. It is expected to grow at a CAGR of 63.2% over the next 5 years, from 2016 to $5 million by 2023.

Growth during the historical period was fuelled by the increased demand for personalized medicine, a growing desire to minimise financial losses associated with late-stage drug failure, and robust public and private funding for organ-on-chip start-ups and research organizations. Previously, production was slowed by the high cost of this technology and the limitations of PDMS, the most used substrate material for organ-on-a-chip fabrication. The worldwide organ-on-a-chip industry is poised to increase at a 39.9% compound annual growth rate between 2020 and 2026, from USD 41 million in 2020 to USD 303.6 million in 2026, according to a report published by Global Opportunity Analysis and Industry Forecast, 2021–2026 (Bajaj 2017).

Regaining dominance in the industry has been helped by major players such as Emulate and others like Organovo. As well as expanding their market shares, the report highlights the fierce competition in the industry. Some of the main participants in the market are 'AXO Technologies', 'CN Bio Innovations', 'Hurel Corporation', 'InSphero AG', 'MIMETAS B.V.', 'Nortis, Inc.', 'Tara Biosystems', and 'Ascendance Biotechnology, Inc'.

6.12 Conclusion

By combining pathology and tissue engineering expertise, organ-on-a-chip-based in vitro models enable scientists to investigate disease under controlled conditions and to mimic a variety of human pathological conditions. These in vitro models have been found to be helpful in bridging the gap between preclinical and clinical trials during the drug research phase and lowering attrition rates during the drug development process. Numerous chip-based in vitro models have been developed previously for nearly every organ of the body. The application of 3D bioprinting technology for creation of complex 3D tissue analogues has resulted in new insights into the microenvironmental role in treatment and cause of disease. This 3D printing technology, when combined with tissue engineering, enables the formation of effective models of different tissue/organ dysfunction that can mimic some of the defining features of human disease. It is also useful for the future improvement of patient-specific biomarker models for any specific illness, as well as customized drug testing on these models during drug discovery.

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Precision Radiomolecular Oncology: Challenging the Classical Statistical Evidence-Based Medicine

7

Baljinder Singh, Harneet Kaur, Ashwin Singh Parihar, Ankit Watts, and Vikas Prasad

Abstract

Evolution and survival instinct have been the principal forces behind the existence of any biological system in nature. *Homo sapiens* are no exception to it. Cancer cells, evolving from the "normal cells" of *Homo sapiens* are thus governed by the same rules. This is one of the reasons why, despite the advances in modern oncology, cure is not achievable in several cancers. Driven by the challenges posed by cancerous tissue, several new multipronged strategies have been envisaged. At the center of the new strategical development in oncology is the understanding that we need to outsmart cancer cells by evolving much powerful diagnostic tools and highly specific targeted therapy. Nuclear medicine, at the forefront of precision theranostics, has realized the challenges and offered appropriate and well-timed diagnostic and therapeutic options to improve and optimize patient management. This chapter will highlight the pitfalls of classical evidence-based medicine and show the way forward to more individualized/personalized approach, i.e., by amalgamating radiomolecular oncology with specific molecular profiling and gene panels, texture analyses, and better understanding of the cancer phenome.

Keywords

Evolution and survival instinct · Biological system · Modern oncology · Therapeutic options · Personalized approach · Radiomolecular oncology · Cancer phenome

B. Singh (✉) · H. Kaur · A. S. Parihar · A. Watts
Department of Nuclear Medicine, Postgraduate Institute of Medical Education and Research, Chandigarh, India
e-mail: drbsingh5144@yahoo.com

V. Prasad
Department of Nuclear Medicine, University Hospital Ulm, Ulm, Germany

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7.1 Introduction

The prediction of tumor behavior and the process of neoplastic transformation require a careful understanding of tumor biology, cellular metabolism, cell–cell and cell–matrix interactions, and the interpretation of genotype–phenotype alterations in the mutated cells (Vander Heiden and DeBerardinis 2017). Neoplastic transformation leads to several changes in the cellular metabolism, with the more common ones being an increased metabolic and nutrient demand, high turnover (especially with aggressive tumors), and loss of regulatory signals restricting cell growth and multiplication. Several of these metabolic changes can be used to image these tumors, e.g., the use of ^{18}F-FDG to target cells with high utilization of glucose, and several agents targeting the differential receptor expression over neoplastic cells (^{68}Ga-PSMA, ^{68}Ga-DOTATATE, 68Ga-DOTA-Exendin) (Parihar et al. 2018j, k). Targeting these processes not only aids tumor detection but also helps in prognostication and prediction of response to specific treatments in a wide variety of malignancies.

Currently, we live in a dichotomized world, one where the developed nations face cancer and non-communicable diseases such as cardiovascular ailments as the primary foe whereas the under-developed countries still face the highest morbidity and mortality from infectious diseases. Indeed, as the countries' transition from a primarily infectious diseases afflicted population to one that is challenged by the non-communicable diseases, access to affordable, accessible, and quality healthcare becomes vital (Are et al. 2013).

The current challenges in oncologic practice can be broadly divided into three planes—screening, diagnosis, and treatment. The diseases must be identified preferably in the pre-cancerous stage (dysplasia) or at least in early stages of non-metastatic disease. This would necessitate development of suitable screening techniques, which are already in place for several malignancies, such as colonoscopy for colorectal cancers and breast self-exam and mammography for breast cancers. Next comes the importance of diagnostic tests that can yield an accurate and sufficiently detailed diagnosis as non-invasively as possible. Majority of the nuclear medicine diagnostic armamentarium would fall into this category. Finally, the need for precise, personalized, and evidence-based medicine that is affordable and has the most favorable efficacy and safety profile cannot be overemphasized.

Nuclear medicine can address several of these challenges. Radiopharmaceuticals offer the advantage of specific targeting of various bodily functions in a relatively noninvasive way that often detects changes earlier than conventional anatomic imaging. Further, the radiolabels can be altered to use the same targeting agents to deliver a therapeutic dose of radiation, i.e., *theranostics*.

Going forwards, we discuss the avenues for diagnostic and therapeutic applications of these radiopharmaceuticals in oncology.

7.2 Conventional Treatment Regimes

Cytotoxic systemic chemotherapy and external beam radiation therapy have long been at the forefront of treating cancers. As an example, the majority of the approved therapies in metastatic castration-resistant prostate cancer that have significant survival benefits are next-generation anti-hormonal therapies such as abiraterone and enzalutamide, chemotherapeutic agents such as docetaxel and cabazitaxel, and radiation therapy in the form of external beam or brachytherapies (Nuhn et al. 2019). The introduction of ^{223}Ra which conferred not only palliation of metastatic osseous pain, but associated survival benefits as well, led the way for molecular-targeted endoradiotherapies.

The studies evaluating prostate-specific membrane antigen (PSMA) as a theranostic target in prostate cancer brought about a revolution in both disease detection and therapy. Starting with monoclonal antibodies, and later with small and mostly urea-based PSMA inhibitors, the targeting of PSMA has expanded significantly (Vahidfar et al. 2019; Deb et al. 1996). The data on efficacy and safety of PSMA-based radioligand therapies continues to provide solid evidence for their introduction in the management algorithm of metastatic prostate cancer (Ahmadzadehfar et al. 2015, 2020; Heck et al. 2019; Kratochwil et al. 2015; Satapathy et al. 2021; Santoni et al. 2014). A multi-center analysis of chemotherapy-naïve mCRPC patients undergoing ^{177}Lu-PSMA-based radioligand therapy showed a significantly better overall survival in comparison to those that had undergone prior chemotherapies (Ahmadzadehfar et al. 2020).

7.3 Targeted Therapy

^{18}F-FDG, the ubiquitous molecule for PET imaging, targets glucose metabolism and is used for various oncologic and non-oncologic applications (Parihar et al. 2018a, b, c, d, e; Jain et al. 2018; Moadel et al. 2003); however, its non-specificity for tumor cells and the lack of a theranostic pair prompted the development of new ligands with greater tumor specificity and availability of suitable radionuclide theranostic pairs.

Targeted theranostics, when coupled with suitable indications and adequate preselection of patients among other parameters, have proven to be safe and effective in majority of cases (Ahmadzadehfar 2016; Baum and Kulkarni 2012; Kwekkeboom et al. 2008; Strosberg et al. 2017). The safety and efficacy of peptide receptor radionuclide therapy (PRRT) in neuroendocrine tumors and PSMA-based radioligand therapy (RLT) have been prospectively confirmed in multiple large-scale studies, even with multiple therapy cycles (Yordanova et al. 2017a, b, c; Ahmadzadehfar et al. 2016).

Nuclear medicine-led theranostics have proven valuable in several malignancies. Neuroendocrine neoplasms—NENs (e.g., pheochromocytoma, paragangliomas, neuroblastoma, gastro-entero-pancreatic NENs, pulmonary NENs, etc.)—have been at the center of fruitful research for a long time (French et al. 2013; Matthay et al. 2007; Yanik et al. 2015; Yordanova et al. 2017a, c; Baum et al. 2008;

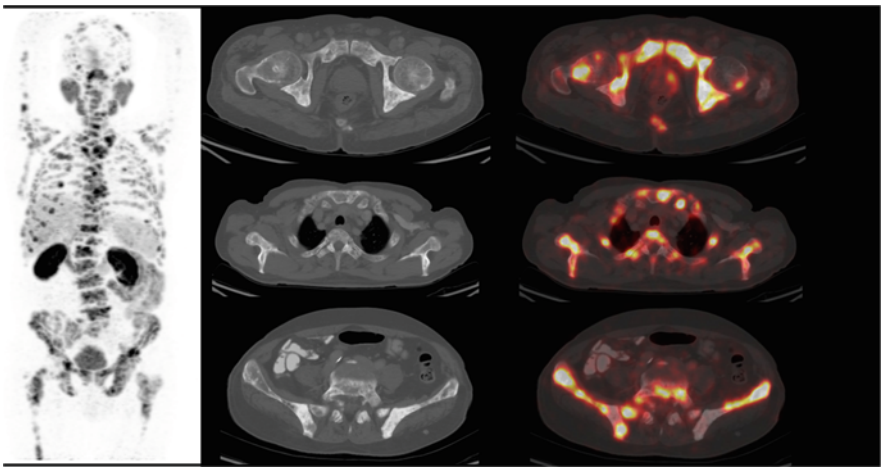

Fig. 7.1 A known case of castration-resistant metastatic prostate carcinoma with ^{68}Ga-PSMA-11 PET/CT showing diffuse disseminated skeletal metastases involving the multiple bones of the axial and proximal appendicular skeleton

Ashwathanarayana et al. 2017). A bicentric study by Prasad et al. (2010) indicated the superiority of ^{68}Ga-DOTANOC PET/CT in the detection and management of NENs with unknown primary sites (Prasad and Baum 2010).

Metaiodobenzylguanidine (mIBG) coupled with the theranostic pair of ^{123}I/^{131}I has been of immense utility in the detection and treatment of NENs, especially neuroblastomas and pheochromocytoma-paragangliomas (Bombardieri et al. 2010; Decarolis et al. 2013; Schmidt et al. 2008). Treatment of advanced neuroblastomas with ^{131}I-mIBG has shown response rates of 20–40%, with an acceptable toxicity profile with/without chemotherapy followed by stem-cell rescue (Zhou et al. 2015).

As previously discussed, the development of PSMA-based ligands for diagnosis and therapy has been a game changer in prostate cancer. The primary PSMA-based ligands in current use include PSMA-11 (^{68}Ga-based), PSMA-I&T (imaging and therapy), PSMA-1007 and DCFPyL (^{18}F-based), ^{99m}Tc-PSMA, and PSMA-617 (therapy with ^{177}Lu), among others (Prasad et al. 2016a; Singh et al. 2021). A biodistribution study in prostate cancer using ^{68}Ga-PSMA-11 is shown (Fig. 7.1). A randomized controlled trial of mCRPC patients treated with either 177Lu-PSMA-617 radioligand therapy (RLT) or cabazitaxel showed more frequent PSA-based responses in the RLT arm (66% vs 37%) with lesser incidence of Grade 3 or 4 adverse events (33% vs 53%) (Hofman et al. 2021).

Angiogenesis which is an essential step in tumor growth and metastasis is a useful pathway for molecular targeting. $\alpha_v\beta_3$ integrin targeting using RGD peptides (arginine-glycine-aspartate), labelled with ^{68}Ga, is used for the detection of neo-angiogenesis (Fig. 7.2) and can be more accurate in detection of malignancies such as breast and thyroid carcinoma in comparison to ^{18}F-FDG (Parihar et al. 2018f,

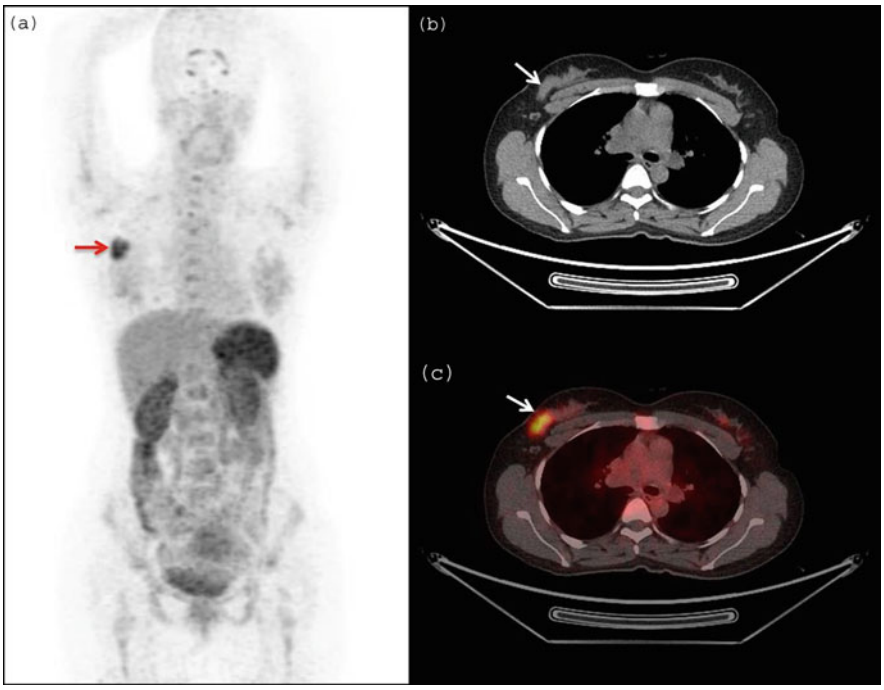

Fig. 7.2 MIP PET image of 57-year-old female patient with breast carcinoma, 45 min post i.v. injection of 3–5 mCi ^{68}Ga-DOTA-RGD$_2$. (**a**) Abnormal tracer uptake was seen in right hemithorax which on CT and fused PET/CT transaxial images (**b**) and (**c**) localized to a moderately tracer avid (SUVmax 8.5) lesion in the upper outer quadrant of right breast

2020a). Similar results have also been observed with 64-Cu-labeled RGD ligands in patients with gliomas (glioblastoma multiforme) (Zhang et al. 2020).

7.4 Radiomolecular Therapy

The introduction of radioiodine (^{131}I) for the treatment of Graves' disease in the early 1940s by Saul Hertz was a revolution in patients with hyperthyroidism. ^{131}I now forms the standard of care in most patients with hyperthyroidism or differentiated thyroid cancer. ^{123}I, ^{131}I formed one of the earliest theranostic pairs (Seidlin et al. 1949). Nuclear medicine theranostics in most cases involves the use of a common molecule that can be tagged with a radionuclide for SPECT/PET imaging and a therapeutic radionuclide for treatment. Examples of such theranostic pairs include ^{68}Ga-DOTATATE for PET/CT imaging of NENs and ^{177}Lu-DOTATATE for PRRT, ^{68}Ga-PSMA for PET/CT imaging of prostate cancer and ^{225}Ac or ^{177}Lu-PSMA for RLT, ^{123}I-mIBG for SPECT imaging of neuroblastomas, and ^{131}I-mIBG for therapy. The advantage of such theranostic pairs is the ability to visualize the disease prior to its treatment (Alzahrani et al. 2012).

Well-differentiated neuroendocrine neoplasms (NEN) have an overexpression of somatostatin receptors (SSTRs), especially the SSTR-2 subtype. Targeting of SSTRs is advantageous from a theranostic perspective with the use of ^{68}Ga-labeled peptides such as DOTANOC, DOTATATE, and DOTATOC that form theranostic pairs as ^{177}Lu-DOTATATE and ^{90}Y-DOTATATOC (Lamberts et al. 1990a, b; Singh et al. 2013; Prasad et al. 2016b). ^{68}Ga-labeled somatostatin analogs have high sensitivity (82–97%) and specificity (80–92%) in the detection of NENs, especially of the gastro-entero-pancreatic origin (Gabriel et al. 2007; Haug et al. 2009), and are useful in the detection of extra-GEP NENs (Parihar et al. 2018f) and non-NEN neoplasms such as meningiomas (Parihar et al. 2020b) as well. Further, a novel agent 68Ga-Exendin has shown to have superior imaging characteristics in the localization of insulinomas, a type of well-differentiated NEN, with high levels of GLP-1 expression on the tumor cells (Parihar et al. 2018g).

In patients with advanced metastatic NEN, PRRT is a targeted therapy that can provide high-dose radiation selectively at the disease sites, thereby minimizing adverse effects from systemic toxicity. The radionuclides used in PRRT have traditionally been 90Y or 177Lu, although alpha-emitters (such as ^{225}Ac) have been gaining traction recently (Sathekge et al. 2019). ^{177}Lu is less nephrotoxic than ^{90}Y (maximum tissue penetration—1.1 cm, high energy pure-beta emitter) because of the narrower range (0.2 cm) and lower energy (maximum—497 keV) of the former. Another advantage with ^{177}Lu- is its additional gamma emissions which facilitate obtaining a post-therapy scan highlighting the localization and distribution of the delivered radiopharmaceutical and any potential unexpected radiotracer uptake (Parihar et al. 2020c). The FDA approval of ^{177}Lu-DOTATATE-based PRRT was highly based on the evidence provided by the NETTER-1 trial that compared outcomes among patients treated with ^{177}Lu-DOTATATE and maintenance octreotide versus the standard octreotide therapy (existing standard of care). The authors showed a significant survival benefit for the ^{177}Lu-DOTATATE arm with a progression free survival at 20 months being 65.2% versus 10.8% in the control arm (Strosberg et al. 2017).

Breast cancer is the leading cause of cancer-related death in women. The requirement of newer therapies is paramount in patients who have failed to respond to chemotherapy, hormonal therapy, or external beam radiation. ^{99m}Tc-diphosphonates, ^{201}Tl-chloride, and ^{99m}Tc-methoxy-isobutyl isonitrile (MIBI) are the most widely used SPECT tracers for breast imaging, whereas ^{18}F-FDG forms the standard PET/CT imaging modality with further additional tracers (e.g., ^{18}F-FES, ^{68}Ga-PSMA) being studied in specific clinical indications (Vadi et al. 2019; Ulaner et al. 2021).

The human epidermal growth factor receptor-2 (HER2) targeting antibodies have been utilized for the detection and characterization of HER2-positive lesions by Baum et al. (2010) in patients with recurrent metastatic breast cancer. Another study by Sharma et al. (2014) reported the utility of ^{99m}Tc-DTPA-bis-methionine (DTPA-bis-MET) in the detection of breast cancer.

7.5 Emerging Therapies

The emergence of radiotheranostics is based on the use of a single radionuclide or a pair of radionuclides that can be labelled to a single targeting agent which can be used for imaging and therapy. Essentially, what you see is what you treat, achieving a personalized treatment approach.

Theranostics combines the practice of personalized and precision medicine and requires a multi-disciplinary team of experts, primarily from the fields of nuclear medicine, radiation oncology, medical and surgical oncology, and onco-pathology. Additionally, based on the particular cancer site being managed, experts from urology, gastroenterology, neurosurgery, pulmonology, etc. may be involved in the decision-making process.

Fibroblast activation protein (FAP) is a protein that is overexpressed on the cellular surface of the cancer-associated fibroblasts. FAP inhibitors (FAPI) have recently been used in PET imaging of multiple malignancies. ^{68}Ga-FAPI PET/CT was shown to have superior imaging characteristics with high tumor/background ratios in around 28 different malignancies (Kratochwil et al. 2019). They showed that primary malignancies of the esophagus, breast, lung, sarcomas, and cholangiocarcinomas showed the highest avidity on ^{68}Ga-FAPI PET/CT. One of the main advantages of the FAPI tracers is that they can be tagged with ^{177}Lu for subsequent treatment of these malignancies.

Alpha-therapy has the advantage of depositing a high dose of radiation within a short range, due to the high linear energy transfer of alpha particles in comparison to beta-particles. Even though most studies on theranostics have been performed with beta-emitters, commonly ^{177}Lu, ^{90}Y, and ^{131}I, the success of alpha-therapy with ^{223}Ra in mCRPC prompted exploration of alpha-agents in several other malignancies as well (Kratochwil et al. 2014; Parker et al. 2013; Morgenstern et al. 2018; Parihar et al. 2021). The main alpha-emitters including ^{225}Ac, ^{213}Bi, ^{227}Th, ^{211}At, and ^{212}Pb are being currently studied in hematologic and several solid malignancies (Haberkorn et al. 2017; Targeted Alpha Therapy Working Group 2018). Presently, the major hindrances to the growth of alpha-therapy include its high costs and availability that further limit the contribution to the scientific evidence by only select well-equipped and resource-plenty institutes and countries.

PSMA ligands have been increasingly utilized in prostate cancer, for both diagnosis and therapy. Another advantage of PSMA (Fig. 7.3) is that it can bind to endothelial cells in the neovasculature which has been used for targeting extra-prostatic malignancies (Parihar et al. 2018h, i, 2020b). This has both diagnostic as well as novel theranostic applications. This is of special significance in malignancies with extremely poor prognosis and limited overall survival with the current management algorithms, such as aggressive sarcomas, glioblastoma multiforme, and advanced pancreatic malignancies.

Melanocortin-1 receptor (MC1-R) is being studied as a theranostic target in patients with melanoma. Preclinical models have shown high, selective uptake of MC1-R targeting agents in metastatic melanoma disease sites (Miao and Quinn 2008). Further clinical studies can shed light on this interesting and potential

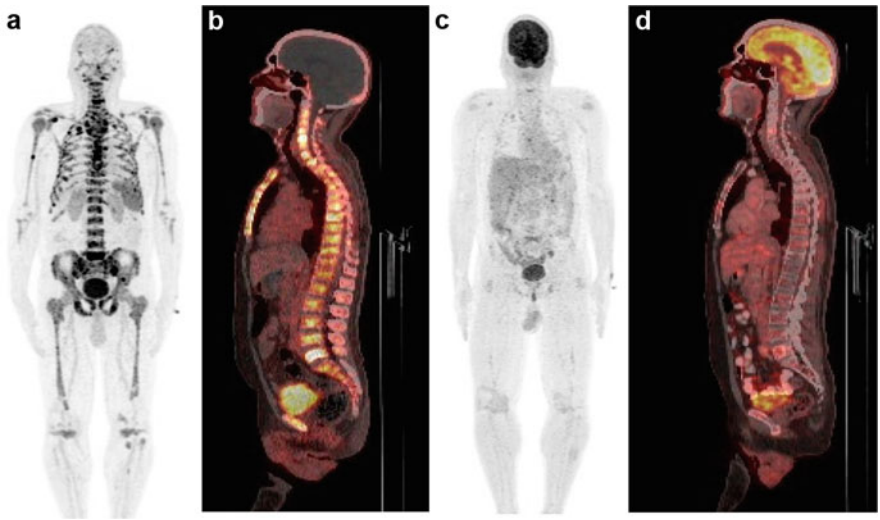

Fig. 7.3 ^{68}Ga-Pentixafor PET/CT in a 60-year-old male with multiple myeloma showing diffuse and focal tracer uptake in axial and appendicular skeleton (MIP image—**a** and sagittal fused PET/CT image—**b**), showing diffuse and focally increased tracer uptake in marrow and lytic bony lesions. The corresponding ^{18}F-FDG PET images (**c**, **d**) did not show any abnormal uptake in marrow and elsewhere in the skeleton

theranostic target that can add on to the armamentarium in the management of patients with advanced melanoma.

Radio-immunotherapy comprises tagging a radiolabel with a monoclonal antibody (or minibodies, haptens, etc.). The antibody is chosen according to the specific cancer antigen being targeted, e.g., CD20 in lymphomas. The antibody localizes to the disease sites and mediates antibody-induced cytotoxic effects. Additionally, the radiolabel irradiates the cell to which the antibody is bound and adjacent tumor cells to which the antibody might not be bound (bystander effect). Thus, the cell-killing efficacy is increased. ^{131}I-Tositumomab and ^{90}Y-Ibritumomab tiuxetan are the only FDA-approved radio-immunotherapies in lymphomatous diseases till date (Kraeber-Bodéré et al. 2016). Recently, a novel anti-CD37 targeting radioimmunotherapy, ^{177}Lu-Lilotomab satetraxetan, was given the fast-track designation by the FDA due to its encouraging results in Phase 1/2a studies (Kolstad et al. 2020).

Chemokine receptors play an important role in cancer progression, and among these chemokine receptors, CXCR4 is the most widely expressed receptor on malignant tumors, and its role in tumor biology has been studied extensively (Zlotnik et al. 2011). No in vivo method suitable for whole body CXCR4 disease quantification has been described, and this unmet clinical need has been reported recently. 68Ga-Pentixafor is a CXCR4 targeting high affinity PET imaging probe, and the tracer has been evaluated in multiple myeloma (Fig. 7.3), in lymphoproliferative disorders, and in lung carcinoma (Fig. 7.4), and the imaging results are extremely promising (Watts et al. 2017).

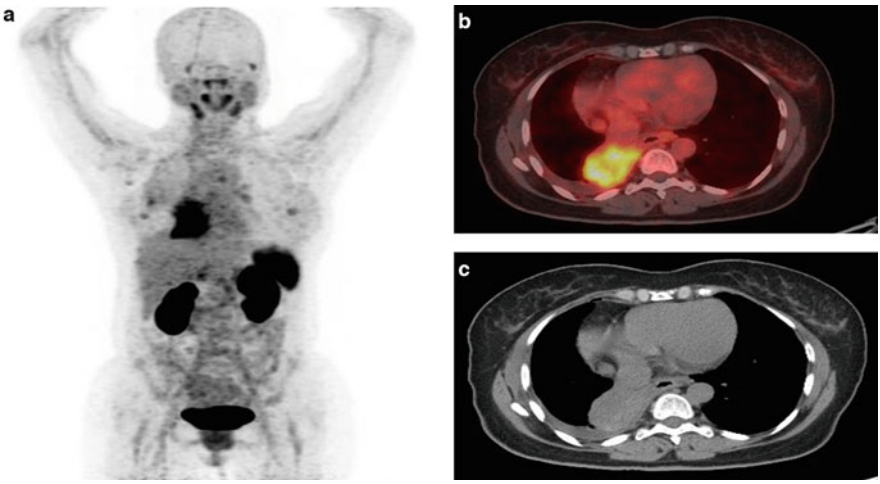

Fig. 7.4 ^{68}Ga-Pentixafor PET/CT in a patient with adenocarcinoma right lung (**a**) MIP image showing tracer uptake in bilateral thoracic region, (**b**) trans-axial fused PET/CT image showing tracer avid soft tissue mass ($SUV_{max} = 12.20$) in the lower lobe of the right lung and (**c**) is the corresponding CT image demonstrating the primary lung mass

The clinical applications of 68Ga-Pentixafor/177Lu/213Bi-Pentixather as a "theranostics pair" for the diagnosis and treatment of CXCR4 expressing cancers are emerging. CXCR4-based theranostics which had not been investigated in routine clinical practice till now (except few preliminary proof of concept studies), therefore, may be a potential game changer both in the diagnosis and treatment of CXCR4 overexpressing solid tumors and hematological malignancies especially with failure of conventional therapies. A pilot study in GBM patients using ^{68}Ga-Pentixafor PET/CT for quantitative imaging of CXCR4 expression (Fig. 7.5) demonstrated that ^{68}Ga-Pentixafor PET imaging in GBM (known to have high CXCR4 expression) is viewed to open up new theranostics applications (with beta and alpha radionuclides) for long-term survival benefits. However, the diagnostic utility of this tracer needs to be validated in large-scale prospective studies through multicenter trials.

Precision radiomolecular oncology is the future of evidence-based, personalized medicine and a step toward the optimal management of cancer. Through nuclear medicine, we can achieve the combined target of early and accurate assessment of malignant involvement as well as optimal and efficacious therapy with minimal and manageable adverse events.

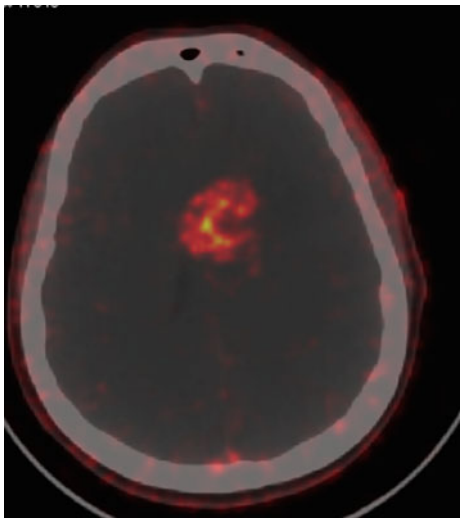

Fig. 7.5 68Ga-Pentixafor PET image in a 58-year-old female patient with intense tracer uptake (SUVmax = 7.9) demonstrating recurrence in the region of the central primary GBM tumor

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8 Drug Repositioning of the Phenylpiperazine Derivative Naftopidil in Prostate Cancer Treatment

Kenichiro Ishii, Yoshiki Sugimura, and Masatoshi Watanabe

Abstract

Naftopidil, a selective α_1-adrenoceptor antagonist, is commonly used for the treatment of benign prostatic hyperplasia, a prostatic disease occurring in elderly men. In drug repositioning studies conducted from our laboratory, we demonstrated that naftopidil has growth inhibitory effects by inducing G_1 cell cycle arrest in cancer cells, fibroblasts, and vascular endothelial cells. Moreover, naftopidil has been shown to bind directly to and inhibit the polymerization of tubulins; thus, naftopidil may exhibit general cytotoxicity in many types of cells. Recent evidence has supported that additive naftopidil treatment in combination with chemotherapy could be a new clinical application for the treatment of prostate cancer.

Keywords

Prostate cancer · Naftopidil · Drug repositioning · Cell cycle · Phenylpiperazine-based structure

K. Ishii (✉)
Department of Oncologic Pathology, Mie University Graduate School of Medicine, Tsu, Mie, Japan

Department of Nephro-Urologic Surgery and Andrology, Mie University Graduate School of Medicine, Tsu, Mie, Japan

Department of Nursing, Nagoya University of Arts and Sciences, Nagoya, Aichi, Japan
e-mail: kenishii@med.mie-u.ac.jp; kenishii74@gmail.com; kenishii@nuas.ac.jp

Y. Sugimura
Department of Nephro-Urologic Surgery and Andrology, Mie University Graduate School of Medicine, Tsu, Mie, Japan

M. Watanabe
Department of Oncologic Pathology, Mie University Graduate School of Medicine, Tsu, Mie, Japan

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8.1 Introduction

In the clinical setting, benign prostatic hyperplasia (BPH), a common prostatic disease in elderly men (Kawabe 2006), is generally treated using α_1-adrenoceptor (AR) antagonists. Indeed, in patients with BPH, α_1-AR antagonists have been shown to decrease prostatic smooth muscle tone and rapidly affect urinary flow.

Based on the selectivity for α_1-AR, α_1-AR antagonists can be classified as subtype-nonselective α_1-AR antagonists or subtype-selective α_1-AR antagonists. Importantly, research has demonstrated that cardiovascular side effects are less frequent in patients administered with subtype-selective α_1-AR antagonists than in those patients administered with subtype-nonselective α_1-AR antagonists (Roehrborn and Schwinn 2004). In Japan, only subtype-selective α_1-AR antagonists, which were developed in Japan, are prescribed for patients with BPH because these drugs exhibit high tolerability with fewer side effects (Yokoyama et al. 2006; Tsuritani et al. 2010).

α_1-ARs are divided into α_{1A}, α_{1B}, and α_{1D} subtypes (Bylund et al. 1994). α_{1A}-AR is the most abundant subtype in the prostate gland, followed by α_{1D}-AR (Walden et al. 1999). Tamsulosin is an α_{1A}-AR- and α_{1D}-AR-selective antagonist, and silodosin is a highly selective α_{1A}-AR antagonist. Naftopidil is also an α_{1A}-AR- and α_{1D}-AR-selective antagonist but has a comparatively higher selectivity for α_{1D}-AR than tamsulosin. Notably, naftopidil has been shown to enhance bladder capacity, promote voiding by blocking the activity of afferent nerves (Yokoyama et al. 2006), and clinically alleviate obstructive voiding and storage symptoms associated with BPH (Nishino et al. 2006; Takahashi et al. 2006).

8.2 History of α_1-AR Antagonists

The global incidence of prostate cancer (PCa) in men is increasing continuously (Gronberg 2003). The majority of PCa cases arise in the prostate, concomitant with BPH (Bostwick et al. 1992). The incidence of BPH has been shown to increase with age, to a greater extent than that of PCa (Alcaraz et al. 2009). Thus, generally, α_1-AR antagonists are often administered for the treatment of BPH before the diagnosis of PCa.

In two observational cohort epidemiological studies, a low prevalence of PCa has been reported in patients with BPH administered with α_1-AR antagonists. Indeed, the quinazoline-based, subtype-nonselective α_1-AR antagonists doxazosin and terazosin were shown to decrease PCa incidence (Harris et al. 2007). Additionally, alfuzosin, a subtype-nonselective α_1-AR antagonist, and tamsulosin, a subtype-selective α_1-AR antagonist, reduce the incidence of high-grade PCa in a manner related to the cumulative duration of α_1-AR antagonist administration (Murtola et al. 2009). These data strongly suggested that α_1-AR antagonists may have anticancer effects.

Drug repositioning (DR) is a strategy used to develop new applications for existing approved drugs by discovering novel therapeutic effects or drug targets

(Masuda et al. 2020). Based on epidemiological evidence, quinazoline-based, subtype-nonselective α_1-AR antagonists, such as doxazosin, prazosin, and terazosin, have been extensively investigated and have been shown to have growth inhibitory effects in PCa cells (Kyprianou 2000). In PCa cells, the growth inhibitory effects of quinazoline-based, subtype-nonselective α_1-AR antagonists have been shown to be involved with apoptosis induction (Kyprianou and Benning 2000; Lin et al. 2007). Kyprianou et al. evaluated the structures of quinazoline-based compounds and reported that quinazoline-based, subtype-nonselective α_1-AR antagonist-induced apoptosis may be independent of the α_1-AR signal and biological characteristics of PCa cells (Anglin et al. 2002; Benning and Kyprianou 2002). Additionally, Garrison et al. reported that doxazosin and the novel lead quinazoline-derived compound DZ-50 reduced the viability of vascular endothelial cells, leading to the suppression of tumor vascularity in PCa xenografts (Garrison et al. 2007). Although doxazosin and DZ-50 have quinazoline-based structures, these effects in vascular endothelial cells may not involve an apoptotic mechanism. Notably, several studies demonstrated that the subtype-selective α_1-AR antagonist tamsulosin has no growth inhibitory effects in PCa cells (Kyprianou and Benning 2000; Benning and Kyprianou 2002).

8.3 Important Observations

8.3.1 Anticancer Effects of Naftopidil in PCa Treatment

In DR studies from our laboratory, we demonstrated that naftopidil has growth inhibitory effects by inducing G_1 cell cycle arrest in PCa cells, renal cell carcinoma (RCC) cells, and colon adenocarcinoma cells, as well as in normal prostatic fibroblasts and vascular endothelial cells (Kanda et al. 2008; Hori et al. 2011; Iwamoto et al. 2013; Ishii and Sugimura 2015) (Fig. 8.1).

In PCa cells, naftopidil inhibits cell proliferation in human LNCaP cells, which are androgen sensitive and androgen receptor positive, as well as human PC-3 cells, which are androgen insensitive and androgen receptor negative, in a concentration-dependent manner (Kanda et al. 2008). The antiproliferative mechanisms of naftopidil involve the induction of G_1 cell cycle arrest linked to increased expression of p27 and p21 in LNCaP cells and p21 in PC-3 cells as well as the inhibition of AKT phosphorylation at Ser473, particularly in PC-3 cells. In vivo analyses have shown that oral administration of naftopidil suppresses PC-3 tumor growth by reducing microvessel density (MVD). Additionally, naftopidil-induced apoptosis was not detected by Hoechst 33258 staining, DNA ladder formation, or poly-(ADP ribose) polymerase cleavage.

Additionally, Hori et al. demonstrated that naftopidil exerts antiproliferative effects, which are independent of α_1-AR subtype (α_{1A}, α_{1B}, and α_{1D}) expression in PCa cells and normal prostatic fibroblasts; these findings supported that naftopidil is likely to promote G_1 cell cycle arrest in several types of cells (Hori et al. 2011). Accordingly, we hypothesized that the antiproliferative effects of naftopidil may be

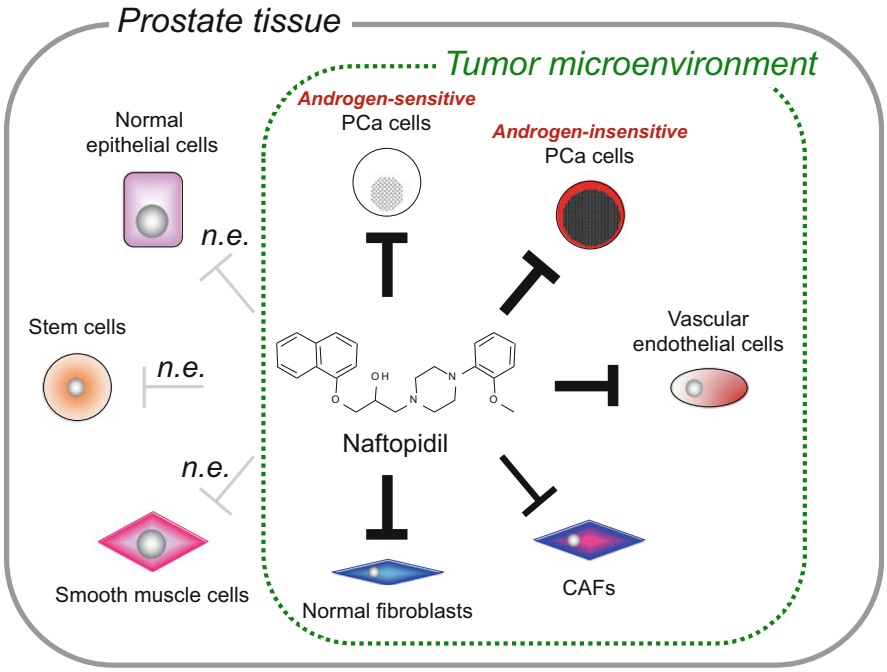

Fig. 8.1 Growth inhibitory effects of naftopidil in the tumor microenvironment. The tumor microenvironment in prostate cancer (PCa) includes a number of cells, such as androgen-sensitive PCa cells, androgen-insensitive PCa cells, normal fibroblasts, carcinoma-associated fibroblasts (CAFs), and vascular endothelial cells. Our studies of drug repositioning suggested that naftopidil may induce G_1 cell cycle arrest to block highly proliferative cell growth (Kanda et al. 2008; Hori et al. 2011; Iwamoto et al. 2013; unpublished data). n.e., not examined

related to its off-target effects. Interestingly, naftopidil strongly inhibits the proliferation of normal prostatic fibroblasts compared with that of PCa cells and decreases the secretion of the tumorigenic soluble factor interleukin-6 derived from normal prostatic fibroblasts, implying that stromal support of PCa cells may be suppressed by naftopidil in the tumor microenvironment. Importantly, no antiproliferative effects were observed following tamsulosin treatment in PCa cells or normal prostatic fibroblasts.

Similar to the results in PCa cells and normal prostatic fibroblasts, Iwamoto et al. demonstrated that naftopidil inhibits RCC cell and vascular endothelial cell proliferation via promotion of G_1 cell cycle arrest (Iwamoto et al. 2013). In an in vivo RCC xenograft model, oral administration of naftopidil was found to strongly decrease MVD in tissues, suggesting that naftopidil may have both direct effects in cancer cells and indirect effects in stromal cells, such as fibroblasts and vascular endothelial cells, in the tumor microenvironment. Additionally, tamsulosin did not show any antiproliferative effects in RCC cells or vascular endothelial cells. However, naftopidil has been shown to inhibit the proliferation of human lung fibroblasts

and bleomycin-induced lung fibrosis in mice (Urushiyama et al. 2019). Additionally, naftopidil also induces G_1 cell cycle arrest and decreases the mRNA expression of *COL4A1* (which encodes type IV collagen) and *ACTA2* (which encodes α smooth muscle actin) in human lung fibroblasts. These results suggested that naftopidil may have potent therapeutic effects on the tumor stroma of PCa, including fibroblasts and vascular endothelial cells.

Carcinoma-associated fibroblasts (CAFs) are present in the tumor microenvironment of PCa and are characterized as activated fibroblasts that promote PCa cell proliferation. In the PCa cell microenvironment, normal fibroblasts and CAFs secrete various growth factors, cytokines, extracellular matrix proteins, and microRNAs, which function to support PCa cell survival and proliferation in a paracrine manner (Ishii et al. 2018b). In our laboratory, we examined the effects of naftopidil on the proliferation of primary cultured CAFs derived from patients with PCa. Naftopidil weakly inhibited the proliferation of primary cultured CAFs compared with that of PCa cells, normal prostatic fibroblasts, and vascular endothelial cells (unpublished data; Fig. 8.1). This result may be explained by the slower proliferation of CAFs compared with that of other cells. Because naftopidil inhibits cell cycle progression, highly proliferative cells may be strongly affected by naftopidil in the tumor microenvironment of PCa. Additional work is needed to fully elucidate the roles of naftopidil in CAFs.

Clinical studies have shown that the incidence of PCa is reduced in patients with BPH administered with naftopidil for at least 3 months compared with that in patients administered with tamsulosin (Yamada et al. 2013). Moreover, our DR studies in patients with latent PCa concomitant with BPH also suggested that naftopidil may have applications in long-term prevention by blocking progression to clinical PCa. Thus, long-term naftopidil use for patients with BPH may have various clinical benefits, and naftopidil may have application in the chemoprevention of PCa in patients with BPH.

8.3.2 New Clinical Applications of Naftopidil in PCa Treatment

Recently, we proposed two possible clinical applications of naftopidil, i.e., in combination treatment with radiotherapy (RT) or as a chemotherapy for PCa treatment (Iwamoto et al. 2017; Ishii et al. 2018a).

Clinically, α_1-AR antagonists, including naftopidil, improve outcomes in patients with PCa and urinary morbidities related to brachytherapy (Merrick et al. 2005) and extra beam RT (Prosnitz et al. 1999) without impairing safety. Indeed, additive naftopidil treatment combined with RT has been shown to increase RT efficacy in PC-3 cells by directly suppressing growth and by blocking the RT-induced expression of the antioxidant enzyme manganese superoxide dismutase (Iwamoto et al. 2017). Conversely, additive tamsulosin treatment combined with RT did not exert these effects.

Additionally, additive naftopidil treatment combined with docetaxel (DTX) was shown to promote DTX efficacy in LNCaP cell-derived tumors (sub-renal capsule

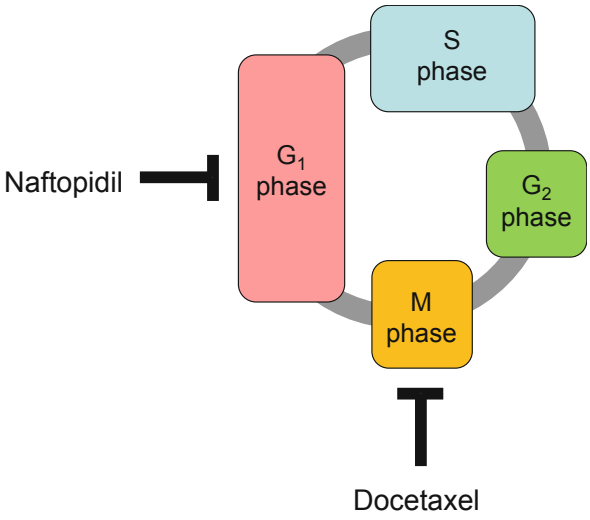

Fig. 8.2 The additive effects of naftopidil treatment combined with chemotherapy for prostate cancer. Naftopidil inhibits the proliferation of prostate cancer (PCa) cells by inducing G_1 cell cycle arrest. Docetaxel (DTX) induces G_2/M cell cycle arrest and apoptosis in PCa cells by inhibiting microtubule depolymerization. Our studies of drug repositioning strongly suggested that the combination of G_1 cell cycle arrest-inducing naftopidil and G_2/M cell cycle arrest-inducing DTX may inhibit cell cycle progression

grafting) and PC-3 cell-derived tumors (intratibial injection) in an in vivo analysis (Ishii et al. 2018a). Notably, additive naftopidil treatment showed synergistic effects on DTX-dependent apoptosis in PCa cells in both in vitro and in vivo analyses. Particularly in patients with castration-resistant PCa having bone metastases, this combined treatment strategy can result in enhanced clinical outcomes compared with DTX treatment alone. We suggest that a combination of G_1 cell cycle arrest-inducing naftopidil and G_2/M cell cycle arrest-inducing DTX may strongly inhibit cell cycle progression (Fig. 8.2).

8.3.3 Structure of Subtype-Selective α_1-AR Antagonists With Anticancer Effects

Among approved drugs and unapproved compounds, the five subtype-selective α_1-AR antagonists can be divided into two groups: the α_{1A}-AR highly selective antagonists tamsulosin, silodosin, and RS100329 and the α_{1D}-AR highly selective antagonists naftopidil and BMY7378. Importantly, Hori et al. demonstrated that naftopidil and RS100329 show antiproliferative effects in PCa cells and normal prostatic fibroblasts (Hori et al. 2011). Moreover, both naftopidil and RS100329 have a phenylpiperazine-based structure and have been shown to promote G_1 cell cycle arrest. Similarly, in small-cell lung carcinoma cells, the Ca^{2+}/calmodulin-dependent protein kinase inhibitor KN-62, which exhibits a phenylpiperazine-based structure, also induces G_1 cell cycle arrest (Williams et al. 1995, 1996). Conversely, BMY7378, which also has a phenylpiperazine-based structure, does not induce G_1 cell cycle arrest at low concentrations (10 μM) but weakly promotes G_1 cell cycle arrest in PCa cells when used at a fivefold higher concentration (Hori et al. 2011). Tamsulosin and silodosin, which do not have a phenylpiperazine-based

structure, did not induce G_1 cell cycle arrest. These reports led us to hypothesize that α_1-AR antagonists with a phenylpiperazine-based structure may suppress the proliferation of cancer cells and stromal cells by inducing G_1 cell cycle arrest.

In studies evaluating the mechanisms of growth inhibition by phenylpiperazine derivatives, including naftopidil, Ishii and Sugimura demonstrated that naftopidil can bind directly to tubulins and that three phenylpiperazine derivatives, i.e., naftopidil, RS100329, and BMY7378, inhibit the polymerization of tubulin; indeed, the phenylpiperazine-based structure of these derivatives shows tubulin polymerization-inhibitory activity (Ishii and Sugimura 2015). These findings suggest that the chemical structures of α_1-AR antagonists contribute to differences in the growth inhibitory mechanisms of these compounds.

In a comparison of the growth inhibitory effects of the three phenylpiperazine derivatives, researchers have shown that the characteristics of the compound strongly depend on the substituent group. Our studies of DR suggest that the existing tubulin-binding drug naftopidil may exert a broad-spectrum cellular cytotoxicity in various cell types. For example, naftopidil inhibits the proliferation of cancer cells, such as PCa cells, RCC cells, and colon adenocarcinoma cells, as well as stromal cells, such as fibroblasts, CAFs, and vascular endothelial cells. Therefore, modification of the substituent group on naftopidil may facilitate the design and synthesis of novel tubulin-binding drugs.

After we reported that the phenylpiperazine derivative naftopidil could act as a tubulin-binding drug (Ishii and Sugimura 2015), several groups designed and synthesized new phenylpiperazine derivatives having antiproliferative effects (Guo et al. 2015; Prinz et al. 2017; Demirci et al. 2019). Particularly, Prinz et al. focused on the phenylpiperazine-based structure and developed a new tubulin polymerization inhibitor (Prinz et al. 2017). Thus, developing potent naftopidil-based anticancer drugs without compromising safety in patients with PCa is possible.

8.4 Concluding Remarks

Clinically, naftopidil has high tolerability with fewer side effects in patients with BPH. Our studies of DR imply that naftopidil-inhibited cell cycle progression may block the progression of latent PCa concomitant with BPH to clinical PCa. We believe that long-term orally active naftopidil may have clinical benefits in patients with BPH as a chemopreventive agent for PCa during BPH treatment.

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Part II

Drug and Design Discovery

Biomarker-Based Drug Discovery with Reverse Translational Approach

9

Ramesh K. Goyal and Geeta Aggarwal

Abstract

The development of new drugs and their approval has become very low as only few molecular entities were approved between 2010 and 2019 with an average of 31 drugs/year between 2010 and 2014 and 44 drugs/year between 2015 and 2019. In 2018 and 2019, the US FDA has approved 59 and 48 new molecular entities (NMEs), respectively, based on their potential positive impact on quality medical care and public health. In conventional drug development, the promise of safer drugs with large quantity is approved notwithstanding that ended in a failure more than its success, and there is gap in the industry's ability to predict drug candidate's performance early. The attrition in clinical development as a consequence of late clinical trial failure has been very high. The concept of reverse translational research is one of the steps to overcome the attrition rate in drug development through the path 'bedside to bench/clinics to laboratories' instead of 'bench to bedside/laboratories to clinics'. It is a science of integrating and understanding the mechanism of action 'man to mice to molecule' through multiple levels of safety, efficacy and acceptability based on relevant science. The concept of reverse translation emerged with the advent of genomics and thereby the significance of cellular and molecular biomarkers. This is based on targeted therapies involving the development of safe, novel biomarkers, which have emerged on diagnosis, prognosis and theranostic role. Point-of-care and in-field advanced technologies for rapid, sensitive and selective detection of molecular biomarkers have attracted much interest in clinical development programs. Reverse translational research integrates biomarkers from different investigations like epigenetic, genomic, proteomics, miRNA and siRNA. The

R. K. Goyal (✉) · G. Aggarwal
Delhi Pharmaceutical Sciences and Research University (DPSRU), New Delhi, India
e-mail: goyalrk@gmail.com; geetaaggarwal17@gmail.com

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robustness of molecular biomarker development has the ability to conduct proof--of-concept studies for delineating the pathways involved in particular disease for targeted therapies and personalised medicine. The outcome has a narrow focus on the discovery of individual biomarkers to search for genetic variation in patients and development of personalised/precision medicines.

Keywords

Biomarkers · Precision medicine · Repurposed drugs · Drug discovery · Diagnostics · Targeted delivery

9.1 Introduction

The process of drug discovery and development continues to be slow, risk-laden, inefficient and costly and is delivering result products of questionable value in terms of efficacy and safety through translational research approach (benchtop to bedside) (Cook et al. 2014). The translational approach in research generally begins with the conception of ideas to solve the identified problems. It is the process of applying discoveries generated during research in the laboratory, through preclinical studies, to the clinical trials for providing new drugs in the market (Woolf 2008; Carini et al. 2019). It forms a connecting link between basic research/applied science and clinical research (Cohrs et al. 2015; Fyfe 2019). However, the outcome of translational research into a fruitful therapeutic entity is many a times insufficient when the results are interpreted in terms of the clinical impact (Ashburn and Thor 2004; Scannell et al. 2012).

The cumulative risk associated with translational drug discovery approach to bring a new drug to market lies in the failure rate of a drug candidate at each stage of clinical trial, i.e. 46% during Phase I, 66% in Phase II and 30% in Phase III (Paul et al. 2010). It is estimated that 12–15 years and capitalised cost of approximately $2.87 billion are required from drug discovery to product launch (DiMasi et al. 2016). It is pertinent to note that besides the high budget, it majorly suffers with the additional detrimental factors such as high attrition rates mostly during the middle of the project. The metrics associated with the drug discovery and development process are clearly of concern and eye-watering. The most feared outcome is the therapeutic failure and value proposition of marketed drug products produced through benchtop-to-bedside research (Carvalho et al. 2014; Waring et al. 2015; Waring and Naylor 2016).

Contrary to the conventional benchtop-to-bedside research being used for the drug discovery, the *reverse translational approach* (bedside-to-benchtop research), generally based on the data collection on a real-time basis, starts with the factual studies covered by the real-life patient experiences present in the clinic. The said mechanism of research usually works in an opposite direction to understand the mechanism and experiences observed by the patients. Here, the therapeutic failure represents an opportunity to identify new therapeutic targets and novel biomarkers of drug response. Thus, in the reverse translation paradigm, research becomes a

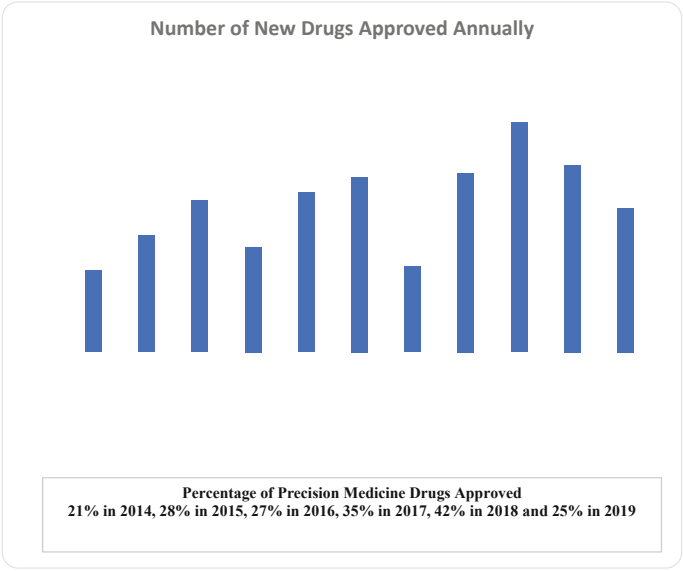

Fig. 9.1 Number of new molecular entities approved by CDER at FDA and percentage of precision medicine drugs

continuous, seamless and cyclical process. It observes and interprets studies of each of the patient by proposing a new hypothesis. This new hypothesis will eventually be helpful in directing the next reiteration of benchtop therapeutic research and ultimately to get the clinical trial studies to analyse the human experience (Shakhnovich 2018).

The advent of precision medicine with reverse translational research (bedside to benchtop) and its focus on the identification and grouping of subpopulations, as well as the tools to identify such populations, led to the concept of precision medicine drug discovery and development. Rapid progress in reverse translational approach through genomic sequencing, biomarker identification, data collection and analytics has accelerated the adoption of trials that test precision medicines, to identify patient groups who are likely to respond to a therapy by providing a more personalised approach to treatment. The examples of successful precision medicine drug discovery include Herceptin (trastuzumab) and *Gleevec* (imatinib), in which biomarker-based reverse translational approach was used for precision medicine development.

Precision medicines now represent a significant and growing proportion of drugs in the industry pipeline, particularly in oncology where the majority of therapies in development are personalised (Chan and Erikainen 2018; Waring and Naylor 2016; Naylor et al. 2015). The Center for Drug Evaluation and Research (CDER) at FDA has approved many new molecular entities from 2010 to 2020 (Fig. 9.1). In 2020, till August 2020, a total of 37 new molecular entities are approved. The maximum number (59) of new approved drugs was observed in 2018. It is important to note that in 2005, only 5% of the approved drugs were classified as precision medicines

drugs by CDER at FDA, but the percentage of precision medicine drugs approved annually was increased to 21%, 28%, 27%, 35%, 42% and 25% in 2014, 2015, 2016, 2017, 2018 and 2019, respectively. It is also important to observe that while the percentage of approved precision medicine drugs decreased to 25% in 2019 as compared to 42% in 2018, there were significant number of approvals of drugs for non-cancer diseases (7 out of 11) in 2019. In this year, new therapies are approved, where no prior treatment was available (FDA 2020).

Precision medicine development is increasing. These drugs, which target specific genetic, molecular and cellular markers and provide patients with personalised treatments, are highly attractive targets for drug developers. The precision medicine approach has rational design, higher chances of success and better impact on patient outcome. It is possible with next-generation sequencing and powerful molecular information of patients through reverse translational approach and biomarkers.

This chapter includes not only the relevance of the precision medicine, but it also explains the potential of novel biomarkers and reverse translational research for the development of personalised medicines from the conception of idea to further laboratory bench work based on experiments and outcomes of the clinical responses.

9.2 Reverse Translational Approach to Drug Discovery and Precision Medicine Development

The drug discovery and development landscape are inundated with potential drug candidates that have shown huge possible aptitude and effectiveness in preclinical models but failed when administered to clinical trial subjects. Although these failures are subjected to various reasons, one being the most pervasive causes wherein the inability of preclinical models fails to recapitulate the human physiology accurately and precisely. Owing to advances with both in vitro and in vivo models, it is imperative to improve those towards a more definite and clearer model. Additionally, it will be necessary to incorporate results obtained from human clinical trials, which can establish the reason of different responses by different patients when given a specific therapy. Such investigation and knowledge could guide researchers to develop more relevant animal models. Additionally, by reflecting human physiology, models can envisage clinical replies to drug treatments more exactly (Seyhan 2019).

It is a known fact that the cumulative knowledge acquired by studies conducted jointly on humans and animals is considered to provide the scientific evidences and technical capabilities for drug discovery. In addition to this, continuous progress in molecular biology, for decoding the complete human genome, has been effective to provide multiple opportunities for selecting new molecular targets (Day et al. 2009). The use of technique of reverse translational approach by choosing biomarkers can support conduct of mechanistic studies in cellular or animal models. Through this approach, the researchers and clinical pharmacologists are expected to be exposed not only to a new scientific evidence but also to novel opportunities for precision medicine drug development and individualised treatment (Carvalho et al. 2014).

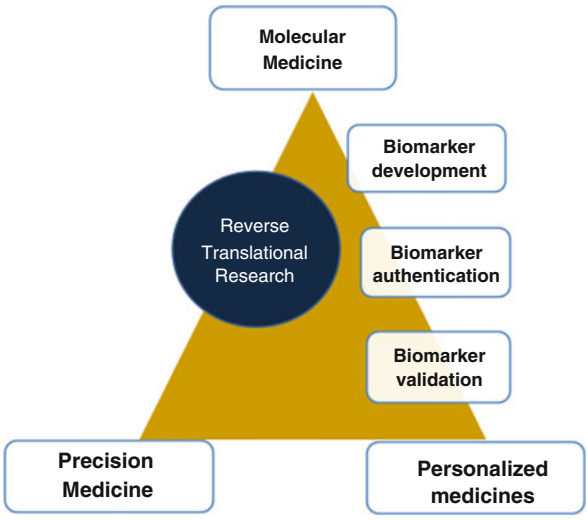

Fig. 9.2 Golden triangle of reverse translational research, molecular medicine targeting personalised and precision medicine, wherein all converge to form a real discovery that could result in providing safer, cheaper and effective therapy

The precision medicine has the potential to transform medical interventions by providing an effective, tailored therapeutic strategies based on the genomic, epigenomic and proteomic profile of an individual. Furthermore, increased utilisation of molecular stratification of patients, such as for assessing the mutations that give rise to resistance to certain treatments and disease conditions, will provide clear evidence for medical professionals to decide on treatment strategies for individual patients (Vogenberg et al. 2010). Reverse translational research to precision medicine is likely to offer improved medication selection and targeted therapy, to reduce adverse effects, to increase patient compliance, to shift the goal of medicine from reaction to early prevention, to improve cost-effectiveness and patient confidence to approve new therapeutics during post-marketing surveillance and to alter the perception of medicine in the healthcare system (Wan et al. 2017).

Currently, reverse translational approach has been known for employing the well-robust steps to study the safety and efficacy parameters during clinical trials and providing innovative medicines and treatment options. Interestingly, this setup may result in getting the valuable extensions of biomarker used in drug discovery and precision medicine development (Seyhan 2010).

The increasing availability of novel biomarkers, biobanks and databanks, high-throughput methods and computational tools may prove invaluable in facilitating drug discovery and precision medicine development through reverse translational research (Fig. 9.2).

9.3 History of Integration of Reverse Translational Research and Biomarkers in Drug Discovery

Reverse translational approach can be utilised to improve biomarker discovery, study of its validation parameters, utilisation and practical application. It is expected that the application of biomarkers would be helpful even for predicting the drug development in the early stages, which can further ensure that the drug candidates have requisite safety and toxicity profiles that can be considered effective before administering and starting the trials in humans (Strimbu and Tavel 2010).

A biomarker, as defined by *Wikipedia*, 'is an indicator of a biological state (i.e. cellular, biochemical, molecular, genetic, protein, metabolite, specific post-translational modification or physiological or physical sign) that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention' (Aronson and Ferner 2017).

Earlier in the fourteenth century, the clinicians used the techniques of uroscopy for identifying and diagnosing disease via examining the features such as colour and sediment of patient's urine, e.g. presence of sugar in urine for diagnosing diabetes, was a biomarker (Eknoyan 2007). The history of biomarker was observed and known to have an early intervention of Philadelphia chromosome in the year 1960 wherein a biomarker was used for indicating the right patient for a drug. In the experimental phase, the drug such as imatinib, 'Gleevec', has been observed to decrease the proliferation of Philadelphia chromosomal cells which ultimately slowed the disease progression. Considering the consequences of the landmark of the observed specific mutations, as in the case of BCR-ABL wherein genes had been identified as biomarker for predicting resistance to imatinib, further leads to the pathway for the development of novel tyrosine kinase inhibitors (Kang et al. 2016).

Similarly, human epidermal growth factor receptor (HER-2) gene and other receptors are known to be the most promising biomarkers during mid-1980s wherein it has been reported that about 20–30% of breast cancer patients showed an upregulation of HER-2 receptor in cancer cells by these drug discoveries. Besides being known for a high risk of adverse outcomes, the research provided a clear idea and suggested attention of researchers towards a targeted therapy (Reynolds et al. 2014). Subsequently, in late 1980s, further discovery of HIV viral load as a marker provided an indication of disease progression rate for effectively measuring the antiretroviral treatment efficacy. The method of assessing viral load in patients while receiving combination therapy had been observed along with a higher reduction compared to those patients who are on monotherapy and showed effectiveness in slowing the progression of the said disease (Mayeux 2004; Kagan et al. 2015).

Generally, two types of biomarkers are known to be effective as being the personalised medicine which includes the predictive and prognostic biomarkers. The predictive biomarker is considered as a pretreatment or provides baseline measurement, which in turn can result in predicting whether the benefit can be obtained from a specific treatment. A predictive biomarker is known to be designated

for the use of a new treatment or to develop a new treatment methodology. Considering a typical example in the field of oncology, wherein a biomarker that can capture overexpression of the growth factor protein HER-2, known to be transmitting growth signals to breast cancer cells, could be a potential predictive biomarker to provide treatment for breast cancer patients by using a biosimilar such as trastuzumab (Herceptin), by blocking the effects of HER-2 receptors. While, prognostic biomarkers can be considered as a pretreatment measurement for providing the accurate information and collecting the research data regarding the long-term expected results in case of untreated patients and those receiving the standard treatment (Matsui 2013).

To combat the burden of disease like cancer and achieve improved treatment outcomes, modern oncology is also shifting from empirical treatment strategies to biomarker-based treatment models based upon the molecular profile of the tumour. The novel biomarkers, like circulating tumour cells (CTCs) and/or circulating fragments of tumour DNA (ctDNA) present in the blood, can be identified through liquid biopsy (blood test). This approach holds the promise to guide treatment selection, facilitate accurate patient risk stratification, predict response and identify the failure of treatment early, thereby allowing a timely shift of therapeutic strategy along with new therapeutic development (Payne et al. 2019).

The use of biomarkers during drug discovery is associated with two- to threefold increase in gaining regulatory approval and less attrition rates. This helps to speed up the process for getting new medicines for diseases whose treatment is not available till yet. Ideally, the companies should use biomarker strategy during the initial stages of drug discovery, but at least, their strategy should include collecting samples during clinical trials/clinical research. The collected samples during clinical trials/clinical research again become a valuable tool for biomarker and drug discovery as a part of reverse translational research. Thus, it is expected that reverse translational research and integration of novel biomarkers into clinical development would facilitate new medical product that could promote personalised medicine. In a currently changing global environment and various new diseases, the world of biomarkers is considered as good diagnostic companion of an individual as well as in clinical development. Furthermore, reverse translational research can recognise novel biomarkers for identifying novel therapeutic targets, expediting rapid development of diagnostics and personalised medicines during drug discovery (Fig. 9.3). Although this seems to have a long way to develop improved drugs that work optimally in selecting patients with the concept of right patient, right drug and dose at the right time for a definite outcome in personalised medicine (Shakhnovich 2018).

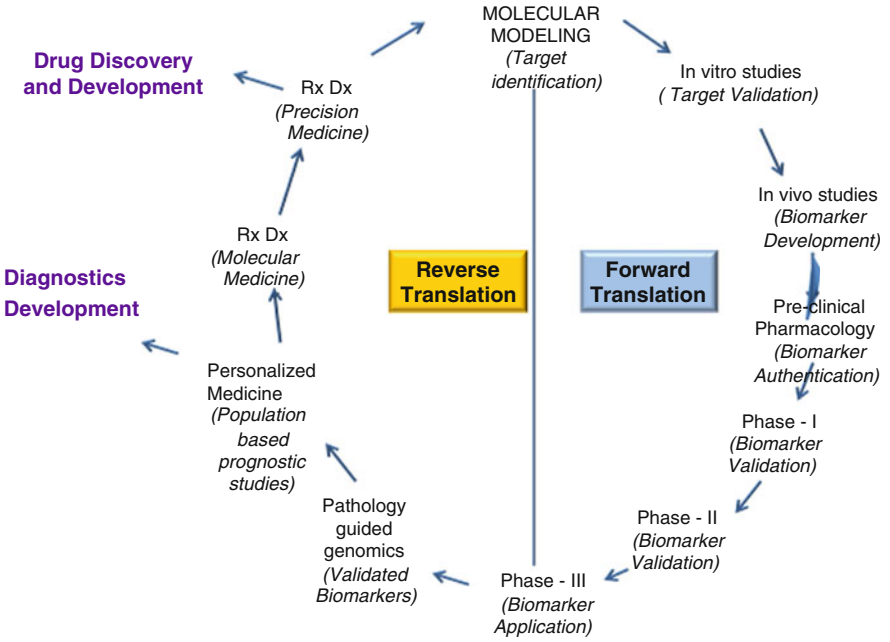

Fig. 9.3 Biomarker-based drug discovery and precision medicine development through reverse translational approach

9.4 Development of Novel Biomarkers Through Reverse Translational Research

Biomarkers are now considered as key to rational, precision medicine drug development and successful clinical results. During forward and reverse translational approach, the development process of biomarker can be divided into four stages, which includes discovery, qualification, authentication and validation of biomarkers. During the discovery and qualification stage, the biomarkers associated with interest are identified from cell lines and animal models. Meanwhile, during verification and validation stage, cell lines as well as primary human data are utilised to assess and establish the sensitivity and specificity of biomarkers. As the research progresses from discovery to validation of biomarkers, the validated biomarkers are utilised in reverse translational research for drug discovery and precision medicine development (Fall et al. 2014).

Novel biomarkers are beyond typical mutated genes (BRCA1/2) related to increased risk of breast cancer and overexpressed proteins including HER2 or programmed death receptor-1 (PD-1)/programmed death ligand-1 (PD-L1) in breast cancer, tumour mutational burden (TMB), minimal residual disease (MRD), microsatellite instability (MSI) and their spatiotemporal variations. Same biomarker cannot work for every tissue, and changes apply throughout the duration of the

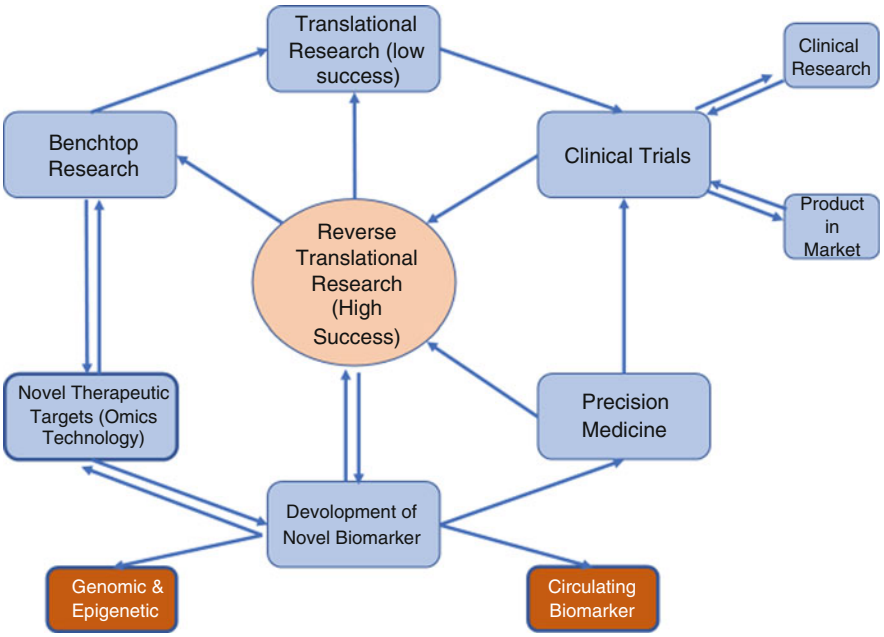

Fig. 9.4 Cycle showing interaction of reverse translational research with translational research for the development of biomarkers and precision medicine

disease and treatment. There is no universal biomarker yet; however, approaches such as methylscape (epigenome assays), protein phosphorylation and highly sensitive analysis on circulating tumour cells are exciting possibilities that are awaiting validation for novel biomarkers (Oz 2019).

Novel circulating biomarkers, i.e. biomarkers quantitated from biological fluids, are more advantageous over clinical and tissue-specific biomarkers due to its ease in measurement and analysis, lesser cost and lesser number of participants required to perform a clinical trial and to measure clinical end points. Various circulating biomarkers, including nucleic acid, proteins, metabolites, etc., can be developed by using high-throughput molecular techniques during reverse translational research (Fig. 9.4). Novel biomarkers take a central spot in drug discovery, patient stratification and predicting and overcoming resistance and recurrence (Hsueh et al. 2013; Ananthamohan et al. 2019).

The reverse translational research with the omics technology is a tool to develop non-invasive novel biomarkers of tissue damage called 'liquid biopsy' that will have a potential to revolutionise drug development and disease diagnosis. The availability of genomics and proteomics has increased the ability to identify novel epigenetic, genotypic and/or immunophenotypic biomarkers. It is expected to accelerate drug development by providing innovative drug development tools and also to significantly improve disease diagnosis by enabling non-invasive interrogation of disease mechanisms. The availability of novel and qualified biomarkers (genomic,

epigenetic and circulating biomarkers) as drug development tools will have a broad positive impact on patient safety in clinical trials as well.

9.4.1 Genomic Biomarkers in Drug Discovery

New drug discovery is time-consuming, mainly due to different types of testing and trials required, involving animal models and human volunteers. However, genomic information/genomic biomarkers can all change with testing made possible under laboratory conditions. New drugs can be tested directly on appropriate laboratory-grown specific tissues using cells, and the new procedure will drastically cut the cost and time of testing by avoiding or at least significantly reducing the number of trials done with animal models and human volunteers. Drugs developed in this way could have the potential to accelerate drug discovery by reducing the occurrence of unexpected safety concerns or difficulty determining efficacy in clinical trials.

It is well known that advances in genomics and biotechnology have outlined the disease pattern and subsequently the heterogenicity and biological processes of different diseases at the molecular level. It is usually expected and believed that having a clear understanding of the biology of the disease can facilitate the new drug discovery for providing effective treatments, while understanding of the heterogeneity of disease can further facilitate the development of novel biomarkers for diagnosis. The techniques such as approachability of high-throughput molecular assay technologies, including gene expression microarrays, single-nucleotide polymorphism arrays and protein arrays, facilitated the development of potential genomic biomarkers, which are the elementary condition for establishing the personalised medicine.

Genomic biomarkers are the variants in the DNA code that alone or in combination are associated with disease expression, disease susceptibility, disease outcome and therapeutic responses of existing and newer drugs. Genomic biomarkers are in various genes encoding transporters, drug-metabolising enzymes, human leucocyte antigen (HLA) alleles or drug targets, which are known to predict therapeutic efficacy and risk of developing adverse effects of newly discovered drug molecules (Lauschke et al. 2018). For example, in the field of oncology, a limited understanding of the facts associated with the oncogenesis of cancer is a major challenge for proposed planning before initiating the research (Simon 2011).

The genomic biomarkers developed through reverse translation can play an important role in the development of more effective treatments through personalised medicine, molecular medicine and precision medicine. These treatments in turn require the characterisation in certain steps such as the identification of factors which can drive the pathogenesis of the individual tumour, further understanding the networks in which these genes are primarily being involved and providing an early treatment with combinations of drugs to overcome resistant sub-clones. Additionally, the deep single-molecule sequencing techniques can be adopted for multiple samples from individual tumours which will enable to identify and characterise the clonal heterogeneity of each tumour (Navin 2015). It is further expected that with

the help of genomic biomarkers, sufficient sequencing power, the phylogenetical reconstruction and the evolution of individual tumours and further identification, mutations can be identified effectively (Campbell and Polyak 2007; Simon 2011).

9.4.2 Epigenetic Biomarkers in Drug Discovery

Conard Waddington in 1940s defined epigenetics as the modulation of expression of a genotype into phenotypes by environment. Subsequently, in 1948, DNA methylation was first explained in bacterial genomes. In 1975, it has been first reported that 5-methyl cytosines can be duplicated through cell division and gene regulation. Furthermore, epigenetic mechanisms were known to be flexible wherein it provides molecular substrate during research that allows for the stable propagation of gene expression states from one generation to the next cell generation. This epigenetic mark must also be stable to mitosis. These studies eventually proposed that there exists a relationship between epigenetic changes and disorders which includes, but are not limited to, various known cancers and other conditions such as mental retardation. The studies have demonstrated the effectiveness of histone acetylation and methylation which control the gene expression.

The relationship is based on the data collected between an individual's genetic background, environmental condition, aging and disease pattern usually associated with genetic disorders, immune disorders, neuropsychiatric disorders and paediatric disorders. It has been a great challenge in focusing on the epigenetic factor such as DNA methylation, histone modification, nucleosome positioning, non-coding RNA (ncRNA) and microRNA (miRNA) are essential in the regulation of gene expression. It is important to consider while initiating the research that autoimmune diseases are not known to have the same epidemiology, pathology and symptoms, but it does have a common mechanism and origin that can be explained by the sharing of immunogenetic mechanisms for understanding the disease pattern. However, identifying the cell-specific targets of epigenetic deregulation has been known to serve as a clinical marker for diagnosis, disease progression and targeted therapies. Biomarker is expected to be a growing sector wherein the primary focus is being on the development of biomarkers which can substantially affect the targeted drug development to modify the epigenome. Hence, the objective of a clinical biomarker is to provide with clinically relevant information for the presence or absence of disease, particularly in human diagnosis, wherein the patients' disease influences the treatment decisions such as in case of prognostic and therapy, providing optimisation biomarkers. Additionally, it provides an accurate measurement of epigenetic alterations in a patient either at single- or at multiple-genome locus.

Currently, many epigenetic biomarkers focused on DNA methylation techniques because of the practical considerations for being stable and easy to analyse, and it has been a well-established and proven role of DNA methylation in cancer. The said

concept of hypermethylation states that certain changes in chromatin structure, lower degree of condensed chromatin, and increased genomic instability and all such evident changes had led to tumour progression. Additionally, the biological process of tumour suppressor genes includes cell cycle, apoptosis, cell adhesion and invasion that are known to be inactivated by hypermethylation like cadherin 1 (CDH1). CDH1 is downregulated in many tumours either through loss of heterozygosity (LOH) and DNA hypermethylation to promote CpG islands. Accordingly, the researchers and clinicians following the traditional method of diagnosis could not effectively achieve sufficient results to predict the prognosis of cancer patients along with the probability of verifying for the high risk of re-occurrence which would benefit from chemotherapy. However, it is much expected that the value of biomarker will eventually be a guide to quantify wellness towards interpreting disease and physiological conditions. The effort on this stratification will be helpful to revolutionise drug discovery (Cheung et al. 2009).

9.5 Expanding Opportunities of Reverse Translational Research in Drug Discovery

The opportunities including the development of novel therapeutics, targeted therapies, repurposing of drugs, improved diagnostics and precision medicine drugs are expanding in drug discovery through reverse translational approach.

9.5.1 Drug Repurposing with Reverse Translational Approach

One of the extensions of reverse translational approach may be drug repurposing, alternatively known as 'drug repositioning', 'drug reprofiling', 'indication expansion' or 'indication shift', which usually involves the establishment of novel medical uses for already existing drugs which are regulatory approved, discontinued, abandoned or proposed experimental drugs. The drug discovery in terms of drug repurposing has gained considerable impetus in the last decade. It is expected that approximately about one-third of the approvals in recent years correspond to drug repurposing, and also these repurposed drugs are currently known to have contributed approximately about 25% of the annual revenue for the pharmaceutical industry (Naylor et al. 2015). Precisely, the advantages of planning and investing into research for repurposing existing drugs include considerably reduced time for research and development and regulatory approval and accessibility of safety and toxicity profiles that have been already tested and are in public domain with unmatched fiscal considerations in off-patent drugs. Generally, repurposing is known to have been conducted effectively in two steps. The first step involves the processing of shortlisted drug candidates for doing investigation in specific pathophysiological pathways of the disease of interest more precisely using in vitro and in vivo methodologies. The second stage is considered wherein the drug repurposing is intended for entering the clinical trial phases for respective approved and targeted

application and indication. The additional advantage of the strategy is that these drugs may be repurposed during any stage of their evolution right from their development stage or discovery.

A drug many a times is approved in spite of scientifically tested and known side effects in the desire of better therapeutic potential for the treatment of other diseases. Sildenafil is a classic example. It was originally tested and developed for treating patients having hypertension and angina pectoris, but because of its phosphodiesterase-5 inhibitory action, it has currently captured a huge market for effectively managing erectile dysfunction in patients (Boolell et al. 1996). Another example is itraconazole which was initially approved as an antifungal agent but then was found to have an excellent effect towards anti-angiogenesis property (Chong et al. 2007; Rudin et al. 2013). Saracatinib is yet another example. This molecule developed by AstraZeneca as an anticancer agent failed initially but exhibited substantial reversal and good effects of symptoms in Alzheimer's disease (AD) when tested in mice model (Nygaard et al. 2015).

One of the recent examples is remdesivir, which was initially developed by Gilead Sciences in 2009 for hepatitis C. It did not work for hepatitis C and was repurposed for Ebola virus and other viruses including coronavirus. In 2020, remdesivir is approved for use in the treatment of Covid-19. In Covid-19, the active metabolite of remdesivir interferes with the viral RNA-dependent RNA polymerase (RdRp), which evades proofreading by viral exoribonuclease (ExoN) in nanostructured protein (nsp 14), ultimately causing a decrease in viral RNA production (Yin et al. 2020). There are some relevant examples of existing repurposed drugs in the market described below (Table 9.1) along with the intended and new indication.

9.5.2 Development of Patient-Driven Diagnostics with Reverse Translational Approach

With the change in the technology and supporting techniques such as machine learning and the use of automated machines, the role of patients in health research is changing continuously and dramatically with positive results. Earlier the patients under testing were considered as study 'subjects'; however, today patients significantly play an important and critical role while initiating and collecting the data in every stage of the ongoing research activities. This is impactful when such involvement occurs, which further ensures that study results are more practical and useful to the researchers and patients and to others involved in making important healthcare decisions and commercialisation. It is imperative to note that active involvement of patients eventually bring issues with possible solutions that matter to them including the quality of life and the effectiveness of healthcare options. Furthermore, these steps are working alongside scientists as partners and are being tested and evaluated in designing and conducting research protocols and policy and are key measures for the successful dissemination of knowledge and implementation of the outcome and interpretation for further use. Considering patients' perspectives, the researchers will

Table 9.1 Examples of repurposed drugs in the market

Drug	Original indication	New indication	New target identified
Thalidomide	Morning sickness	Erythema nodosum leprosum, multiple myeloma	Cereblon (CRBN) (Takumi and Hiroshi 2020)
Sildenafil	Angina	Erectile dysfunction	Phosphodiesterase type 5 (PDE5)
Zidovudine	Cancer	HIV/AIDS	Reverse transcriptase enzyme
Celecoxib	Pain and inflammation	Familial adenomatous polyps	Cyclooxygenase-2 (COX-2)
Atomoxetine	Parkinson's disease	ADHD	Sodium-dependent norepinephrine transporter (NET), serotonin transporter and *N*-methyl-D-aspartate (NMDA) receptor
Aspirin	Analgesia	Colorectal cancer	COX-2
Ketoconazole	Fungal infections	Cushing syndrome	Steroidogenic enzyme
Dapoxetine	Analgesia and depression	Premature ejaculation	Postsynaptic 5-HT_{1C} receptors
Raloxifene	Osteoporosis	Breast cancer	Oestrogen receptor (ER)
Rituximab	Cancers	Rheumatoid arthritis	B-cell membrane proteins CD20 antigen
Duloxetine	Depression	Stress urinary incontinence	5-Hydroxytryptamine-2 (5HT2) and norepinephrine receptors
Fingolimod	Transplant rejection	Multiple sclerosis	Sphingosine-1-phosphate receptor
Bupropion	Depression	Smoking cessation	Nicotine acetylcholine receptor (nAChRs)
Lidocaine	Local anaesthetic	Arrhythmia	Sodium channels
Remdesivir	Ebola virus	Covid-19	RdRp in viral nsp12

ask an appropriate question for conducting the proposed studies. This can be further modified and designed in a better manner for more accurate results with more relevance for daily activities to be adopted precisely in practice.

One of the examples of using the reverse translation more effectively was seen in the case of quest for expedited Zika virus diagnostics during the 2015–2016 viral outbreak, wherein the outbreak resulted in a public health emergency declared by the World Health Organization and the United States Department of Health and Human Services. In this regard, it is important to understand that to combat the same, public need pushed it forward for developing an immediate requirement to develop the diagnostics and therapeutic measures necessary to effectively control the Zika virus.

It acknowledged the existing systems and pushed work towards further improvement that eventually helped the rapid translation of in vitro diagnostics and the positive outcome (Kurani et al. 2017; Shakhnovich 2018).

9.6 Challenges and Future Prospects of Reverse Translational Approach in Drug Discovery

The reverse translation is the technique of deep learning and large data mining, which is helpful for generating data and evaluating the ~~testing~~parameters for new drug development within all probable therapeutic areas. The large data collation and its availability may be a challenge at this time. The main challenge is the cost associated with data collection through genome sequencing and to adequately transform data to information to knowledge for researchers and clinicians.

Reverse translational approach is helpful in providing and collating the large available data either in the form of a published literature or study and analysis of patents and from the information available in the regulatory domain and clinical registry. This may be helpful in getting requisite information from multiple sources and compiling the required information with adoption of statistical measures. Moreover, the sophisticated algorithms along with the prediction of collated information lead to the probability of providing an accurate result with the help of multiple variances in treating and analysing a specific diseases or condition which is under question for evaluation. For example, a hypothetical drug that undergoes preclinical testing and experiments considering the potential of suitable candidate for pancreatic cancer, however, due to failure in clinical trials might be an alternative for selection of the said drug for treatment of brain tumour such as glioblastoma multiforme. This in turn is effective for many biotechnology and pharmaceutical companies which can utilise such data to reduce the cost and investment before initiating a new project for drug discovery.

Additionally, various methods have been earlier discussed for reverse translational research, which includes but are not limited to the molecular medicine approach. It is helpful in new drug development by understanding the mechanism of the pathophysiology of the disease, which can be identified and targeted with an expected outcome. The knowledge gained from the collected clinical data may be applied to both development of biomarkers and drug discovery processes. Therefore, shifting the conventional paradigm of drug screening from the existing rigorous methods to preclinical and clinical experiments could be helpful in identifying the successful targets and possible mechanisms to get the desired output. This should allow better predictive capability and decision-making on the part of scientists and managers in the drug discovery process.

For translational research, it is required to have more public–private partnerships, essential for providing the extension of the precompetitive space among the academia, industry and government to identify priority research areas and additional funding. This is required for the development of technologies such as artificial intelligence, machine learning and change in business model.

9.7 Concluding Remarks

It has been observed that robust and validated biomarkers are the need of the hour for improving and providing an effective diagnosis, monitoring desired drug activity and therapeutic response. Such an approach provides precise guidance for the development of safer and targeted therapies for various chronic diseases. While different types of biomarkers have been found to be useful in the field of drug discovery and development, it is imperative to note that the process of identifying, testing, analysing and validating disease-specific biomarkers needs specific attention and has been quite challenging. Reverse translational research integrated techniques, with recent advanced methodologies such as multiple 'omics' (multi-omics) approaches (genomics, transcriptomics, proteomics, metabolomics, cytometry and imaging), have made it quite possible in providing the acceleration to the research towards the discovery and development of specific biomarkers for complex and chronic diseases.

Although focused research in drug discovery has many challenges like high budget, personnel skills and the resource requirement, it is expected that reverse translational research is one of the important strategies to be considered. The novel biomarkers may eventually assist in providing an optimal decision-making during the course of drug development from initiation of conception to commercialisation. This is also likely to open the pathway to an effective implementation of personalised therapies for various complex and chronic diseases.

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Nanotechnology in Dentistry

10

Krishan Gauba, Arpit Gupta, and Shweta Sharda

Abstract

Nanotechnology has tremendously influenced the diagnostic and therapeutic sciences in medicine and dentistry. The development of nanomaterials has redefined dental practice to be able to efficiently and effectively achieve the goal of positive oral health. The nanomaterials' enhanced mechanical properties, increased durability in the oral environment, biocompatibility, better aesthetics and, above all, atraumatic experience to the patients make the use of various nanomaterials a promising tool for providing state-of-the-art dental services to the patients. Additionally, the concept of nanorobots for early diagnosis and prompt treatment of oral cancer has the potential to redefine the dental practice enormously. Future research needs to be focussed on addressing the production and social and biological challenges for adopting nanodentistry in clinical practice.

Keywords

Nanotechnology · Nanodentistry · Nanomaterials · Nanorobots · Nanocomposites · Nanodrugs

10.1 Introduction

Over the past few decades, medical and dental sciences have evolved tremendously. The massive advancements in science and technology have made the goal of achieving near-perfect health seem achievable. The idea and concepts of nanotechnology were

K. Gauba (✉) · A. Gupta · S. Sharda
Oral Health Sciences Centre, Postgraduate Institute of Medical Education & Research, Chandigarh, India
e-mail: gaubakrishan@gmail.com

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first highlighted by Richard Feynman in his talk on *There's Plenty of Room at the Bottom* in 1959. Since then, there has been extensive research on nanotechnology and its application in science, engineering, agriculture and medicine. One such revolutionary change in the dental practice is nanotechnology's inception into the early diagnosis, timely prevention and prompt treatment of oral diseases.

Richard Feynman
1918-1988
Image Source: Wikipedia

The word 'nano' is derived from a Greek word meaning 'dwarf'. It equals one thousand millionth of a meter (10^{-9} m) (Mansoori and Soelaiman 2005). Nanotechnology is defined as the science and technology of engineering matter at a nanoscale and using the modified properties in various fields of chemistry, physics, medicine and agriculture to obtain the most effective outcomes (National Nanotechnology Initiative 2005). This technology's founding principle is that the active molecule's shape and size are designed at a nanometre scale, which helps augment its properties. Nanotechnology has found profound usage in medical and dental sciences. Nanodentistry uses the materials of the nanoscale structure for the diagnosis, prevention and treatment of oral diseases. It has found tremendous scope in devising materials and drugs that exhibit superior benefits in relieving pain, tissue regeneration and improving oral health (Freitas 2000).

10.2 Approaches to Nanodentistry

Nanodentistry is an interdisciplinary science that relies on an amalgamation of biotechnology and nanomaterials to formulate devices and tools to provide effective and efficient oral health care. This novel science application is based on three major approaches: the bottom-up approach, the top-down approach and the functional approach (Chandki et al. 2012; Freitas 2000) (Fig. 10.1).

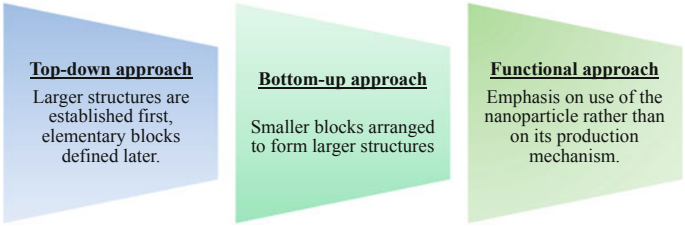

Fig. 10.1 Approaches to nanodentistry

10.2.1 The Bottom-Up Approach

When the smaller blocks are arranged atom by atom using physical forces and chemical linkages to produce larger structures, it is said to involve the bottom-up approach. The human body cells, for example, are made up of the agglomeration of enzymes, DNA and organelles that work in proper coordination with each other. In dentistry, this approach is used to formulate local anaesthesia, impression materials and tissue regeneration (Chandki et al. 2012; Khurshid et al. 2015).

10.2.2 The Top-Down Approach

In this approach, mechanisms like chemical vapour deposition (CVD), monolithic processing and plasma etching are used for microfabrication. In other words, the larger structure is established first, and its smaller components are studied in all possible minute aspects until the full specification is reached to the core elementary blocks. This approach is used in dentistry to synthesise nanocomposites, nano-needles and nano-impression materials (Chandki et al. 2012; Zhang and Webster 2009).

10.2.3 Functional Approach

This approach does not focus on the method of nanoparticle production; instead, the specific function or use of the nanoparticle is emphasised (Aeran et al. 2015; Chandki et al. 2012).

10.3 Nanomaterials

The past few decades have witnessed biomimetic approaches to develop nanomaterials to formulate various oral health-care products. The science of nano-technology is being explored to develop dental materials with superior mechanical properties, abrasion resistance, aesthetic properties and antimicrobial

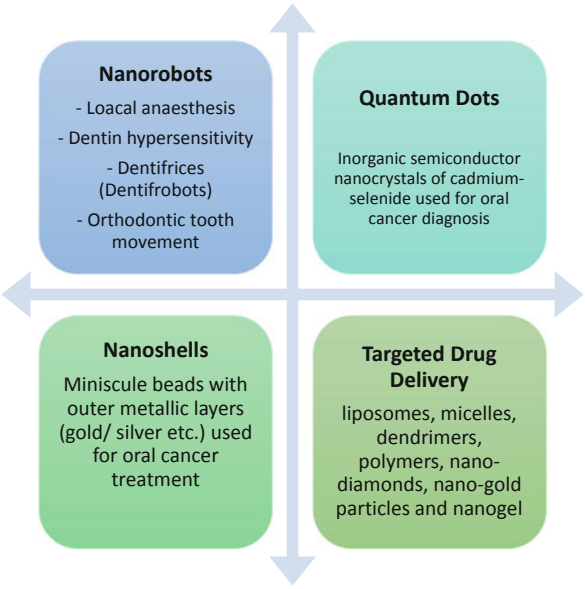

Fig. 10.2 Various nanoparticles added to dental materials for improved properties

properties (Fig. 10.2). Various nanomaterials are being used for the restoration of decayed, missing and fractured teeth using nanocomposites and nano-ceramics, local anaesthesia, desensitising agent for dentinal hypersensitivity, tooth and bone regeneration, nano-impression materials as well as diagnosis and treatment of oral cancer (Sharan et al. 2017).

Properties of an ideal nanomaterial in dentistry

- Superior mechanical strength
- Higher abrasion resistance
- Higher optical and aesthetic property
- Reduced shrinkage
- Better antimicrobial properties

10.3.1 Nanocomposites

High strength and good aesthetics are the two properties mainly desired in dental composites. These properties are primarily dependent on the filler particle size of the composite. The conventional composites, built up of micro-filler particles, exhibit enhanced aesthetics but not so good strength properties. To develop a composite restorative material with the required aesthetics and strength, nanofillers have been used (Lainović et al. 2013). Nanocomposites may contain either the nanosized filler particles or masses of these nanosized filler particles in the form of 'nanoclusters'. Various nanoparticles reported to significantly improve the hardness, flexure

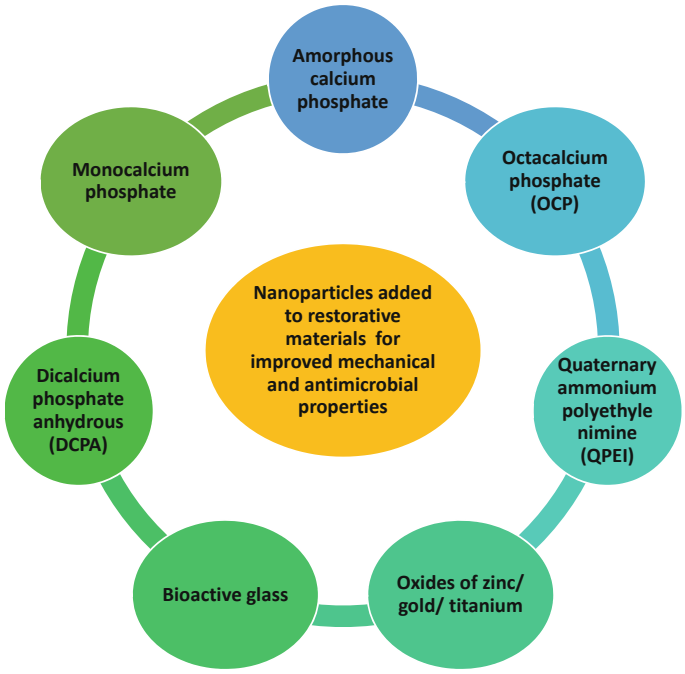

Fig. 10.3 Recent advancements in restorative materials

strength, modulus of elasticity, translucency and aesthetics of the restorative composite resins are shown in Fig. 10.3.

Nanofillers of metals (like silver, gold, titanium, zinc, etc.) and their metal oxides are known to exhibit antimicrobial properties due to their nano size and high surface area to volume ratio, allowing more significant contact with the bacteria. Shvero et al. (2015) reported quaternary ammonium polyethyleneimine (QPEI) to have an extended antibacterial effect against various oral pathogens, such as *Enterococcus faecalis*, *Streptococcus mutans* and *Actinomyces viscosus*. It is also found to be stable within the matrix and does not leach out into the surrounding environment. This added nanoparticle's bactericidal action is implicated in yielding restorative materials with significantly lower incidences of failure attributed to secondary caries (Bayda et al. 2019; Iftekhar 2019).

A recent modification of composite resins has been the addition of bioactive glass nanoparticles, which has been successfully used in bone regeneration for many years. The regenerative properties of bioactive glass are further enhanced by incorporating fluoride and silver elements, making it highly suitable for tooth restorations.

10.3.2 Local Anaesthesia

Incorporating nanoparticles in anaesthesia effectively draws the anaesthetic agent to the area, which is targeted by magnets, in order to block the nerves. The nanotechnology-based formulation of liposome has found wide clinical acceptance due to its capacity to encapsulate several drugs (Allen and Cullis 2013). Apart from liposomes, biopolymers, cyclodextrins, lipid nanoparticles and hydrogels are other formulations that exert an anaesthetic effect with an added benefit of lower toxicity of the local anaesthetic agent (de Paula et al. 2012). Even though in vitro studies on the effect of these formulations have demonstrated promising results, there is a paucity of clinical trials that can confirm their efficacy. The current research focuses on using computer-controlled nanorobots added to the colloidal suspension and applied to the patient's gingiva from where it can reach the periodontal ligament and dental pulp. The clinician shall have control over the nerve-impulse traffic and monitor the release of the anaesthetic agent until the completion of the treatment, after which the sensation can be immediately restored. Nanoanaesthesia is thus an emerging concept that is expected to make dental procedures painless and atraumatic in its real sense.

10.3.3 Dentin Hypersensitivity

Dentin hypersensitivity is caused by a larger number and increased diameter of the dentinal tubules. Thus, dentin tubule occlusion via precipitation or blocking into the tubules is the most commonly used mechanism to relieve dentin hypersensitivity. The use of nanorobots to block the exposed dentinal tubules is implicated in effectively preventing the stimuli from inducing a pain response (Zandparsa 2014). Moreover, the effectiveness of polyethylene-glycol-coated maghemite nanoparticles and nano-carbonate apatite containing dentifrices in dentin hypersensitivity treatment is also being explored.

10.3.4 Diagnosis and Treatment of Oral Cancer

Early detection of cancer is a critical factor that decides the prognosis of its treatment. Due to the nanoparticle's small size, the functional surface area that can bind to the cancer cells is vastly increased. The use of a wide range of nanomaterials like silver, gold and quantum dots is being explored for the early diagnosis and treatment of oral cancer. Quantum dots are inorganic semiconductor nanocrystals ($<$10 nm) of cadmium selenide. These are used as probes for the diagnosis of oral cancer. When used as an adjunct to MRI, these quantum dots that travel through the blood help improve the visualisation of tumour sites (Chen et al. 2018). Gold nanoparticles that can provide localised surface plasmon resonances at near-infrared wavelengths are promising contrast agents for optical coherence tomography (Chen

et al. 2018). Such technology efficiently diagnoses cell changes. Moreover, the false-negative rate of diagnosis is nearly zero (Oldenburg et al. 2006).

Nanoshells are a collection of minuscule beads with outer metallic layers (usually gold) that absorbs the infrared radiation and produces intense heat. This heat is used in the selective destruction of cancer cells without affecting the proximal healthy cells (Kanaparthy and Kanaparthy 2011). Recently, silver nanotechnology has also shown promising action against biofilms due to its high affinity towards the negatively charged microorganism and its ability to inactivate critical physiological functions within the microorganism (Guzman et al. 2012). The non-invasive real-time diagnosis and effective therapeutic action offered by nanoparticles thus result in timely detection and prompt treatment of oral cancer lowering the mortality rate caused by the disease.

10.3.5 Nanoparticles in Dentifrices

Incorporation of calcium carbonate nanoparticles in toothpaste has shown favourable results in enamel remineralisation. Dentifrobots are very small (1–10 micron) dentifrices delivered in the form of mouthwash or toothpaste. Using these dentifrobots at least once a day for cleaning the supragingival and subgingival surfaces leads to metabolisation of trapped organic matter into harmless and odourless vapours and continuous calculus debridement. These nanorobots incorporated in the dentifrices are minute mechanical devices that can safely deactivate themselves if swallowed. They are designed to precisely identify the oral pathogenic bacteria and allow the other harmless microflora to flourish in the oral cavity (Mehta and Subramani 2012).

10.3.6 Orthodontic Treatment

Nanotechnology has enormously influenced orthodontic therapy, making it painless, less traumatic to the periodontal structures, and reducing relapse chances. Nanoparticles of zinc oxide and chitosan are added to composite resins to improve the bond strength with added antibacterial effect. Such orthodontic nanocomposites overcome the common problem of bond failure and reduce the caries development risk due to its antibacterial action. An essential component upon which orthodontic therapy's success largely depends is the orthodontic bracket which is instrumental in carrying the archwire forces to the teeth. The addition of alumina nanoparticles augments the strength and corrosion resistance of these orthodontic brackets. One of the significant challenges in orthodontic therapy is overcoming the frictional force produced during tooth movement without excessive orthodontic forces that may cause loss of anchorage and root resorption. Orthodontic nanorobots are implicated in manipulating the periodontal tissues, allowing rapid and painless tooth straightening, rotating and vertical repositioning in lesser time than usual. Coating the stainless-steel wires with carbon nitride (Wei et al. 2010), zinc oxide (Kachoei

et al. 2013), fullerene-like molybdenum disulfide nanoparticles or tungsten disulphide nanoparticles (Redlich et al. 2008) and nitro carburising (Zhang et al. 2016) has been suggested providing excellent corrosion resistance and good elasticity. Thus, nanotechnology is implicated in redefining every element of orthodontic therapy, from the bonding nanocomposites to the orthodontic bracket and archwire, in bringing about the desired results.

10.3.7 Tooth Repair

Nanotechnology has found its application to simulate the remineralisation process to create the most hardened tissue in the human body, i.e. enamel, by using nano-rodlike calcium hydroxy-apatite crystals (Rathee and Bhoria 2014). Researchers are showing a keen interest in tooth remineralisation that would help regain the lost tooth structure without going for the conventional restorative procedures.

10.3.8 Bone Repair

Bone is a natural nanostructured composite composed of organic compounds (mainly collagen) reinforced with inorganic ones (HA). Nanotechnology aims to rebuild this natural nanostructure for clinical use. The desired features of nano-bone graft materials include good osteoinductivity, high porosity, ability to absorb natural proteins into the nanopores and degradable by osteoclasts as its natural counterpart (Mantri and Mantri 2013).

10.3.9 Nanotechnology in Endodontic Therapy

Nano-biomaterials (like quaternised polyethyleneimine, chitosan nanoparticle) that display better antimicrobial properties are being used to disinfect the root canal before its sealing for successful endodontic therapy. Additionally, incorporating nanoparticles such as bioglass, zirconia and glass ceramics to endodontic sealers enhances adaptation of the adhesive to nano-irregularities providing a better chemical bond to the tooth tissue (Utneja et al. 2015). Adding amorphous calcium phosphate nanoparticles (NACP) to an endodontic sealer is also found to exert enhanced antibacterial effects against endodontic biofilm and stronger dentinal bond strength (Wang et al. 2017). The effect of using silver nanoparticles with calcium hydroxide as an intracanal medicament against *Enterococcus faecalis* is found to be superior compared to calcium hydroxide alone (Afkhami et al. 2015). Thus, incorporating antibacterial nanoparticles in the conventional sealers promises improved sterile environments as desired for successful treatment outcomes. Overall, nanomaterials in endodontic therapy are expected to improve the mechanical strength and dimensional stability of the materials and make a profound improvement in their biological properties.

10.3.10 Denture Base and Liners

In prosthetic dentistry, acrylic polymers are used in the fabrication of artificial teeth, denture repair, crown and bridges, impression trays and obturators of cleft palates. Nanosized fillers are being used due to their superior dispersion properties, less aggregation potential, and biocompatibility with the organic polymer. Polymethyl methacrylate (PMMA) based denture materials with nano-hydroxyapatite (HAP) and nano-alumina (Al_2O_3) particles shows improved fracture resistance (Mousavi et al. 2020). The addition of zirconium oxide nanoparticles significantly improves hardness levels, flexural strength and fracture toughness of the heat-cured PMMA denture base (Gad and Abualsaud 2019).

10.3.11 Nano-Impressions

Good impression precedes precise construction of an appliance. Nanofillers are added to the impression material (vinyl polysiloxanes) to enhance its properties. Such nano-impression material has better flow, improved hydrophilic properties, fewer voids at the margin, better model pouring & enhanced detail precision (Verma et al. 2010).

10.3.12 Targeted Drug Delivery

The sub-micron size makes the nanoparticles a suitable candidate for targeted drug delivery. The prerequisites for an ideal drug delivery system are that they should be biocompatible, biodegradable, non-toxic and long-lasting enough to permit an effective dosage delivery and action. Additionally, such systems should be so designed to allow for a scheduled drug delivery (Izadi et al. 2020). Furthermore, such targeted drugs can be designed to avoid the first-pass metabolism. They can be fabricated with additional optical and electrical properties that can locate the drug intracellularly. Extensive research is being conducted to utilise liposomes, solid biodegradable nanoparticles, micelles, dendrimers, polymers, nano-diamonds, nano-gold particles and nanogel for the targeted drug delivery.

10.3.13 Nano-Needles and Nano-Tweezers

Contemporary medicine has unveiled the concept of cell surgery that involves modification of the subcellular components and/or organelles for disease treatment. To perform cell surgery, nano-needles (nanosized needles made up of stainless steel) are being designed and fabricated, wherein specific cell structure like nucleus could be reached with utmost precision. Furthermore, to make cell surgery possible, nano-tweezers—based on carbon nanotubes—have been developed. These tweezers are so

designed to create a strong electromagnetic field around themselves that helps capture nanoparticles in close vicinity.

10.4 Challenges

Although the conceptualisation of nanodentistry involves scientific thinking and engineering models, its implementation in a practical sense is limited by the following challenges:

1. Production challenges
   - Precision in positioning and assembly of nano-molecules
   - Coordinating the functioning of the individual nanoparticles simultaneously and effectively
2. Biological challenges
   - Biocompatibility of the nanomaterial
   - Safety assessment
3. Social challenges
   - Economic barrier
   - Acceptance by the patient

10.5 Conclusion

Nanotechnology in clinical dentistry has achieved milestones in the fabrication of nanomaterials, especially in restorative dentistry. Current research is focussed on the development of nanorobots in the diagnosis and treatment of oral cancer. More research related to dental health care should focus on drug delivery systems. The safety profile of nanomaterials should be strictly assessed to ascertain that their use causes no adverse effects. Overall, the application of nanotechnology in dental practice promises highly advanced diagnostic and therapeutic services for improved oral health care.

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11 Nanoparticles: A Potential Breakthrough in Counteracting Multidrug-Resistant Bacterial Infections—A Holistic View on Underlying Mechanisms and Antibacterial Properties

Ankush Parmar and Shweta Sharma

Abstract

In the present scenario, a serious predicament faced across the globe is the infection caused by bacteria. Bacterial infections rank higher among dreadful diseases and are considered to be the foremost leading causes of death worldwide. Although the recent decade has witnessed a notable development in the production of cogent antibiotics, still the efficacy of these remains questionable. Another major concern is the facile selection of antimicrobial therapy which in turn is totally empirical in nature and is often accompanied with numerous severe side effects, viz., systemic toxicity, hypersensitivity, teratogenicity, and mutagenicity. Additionally, the clinical application of antibiotics is hampered, owing to the multidrug resistance (MDR) evoked in bacteria. This further worsens up the situation and leads to a reduced therapeutic potential thereby ultimately leaving an innate effect on the public health. Apart from this, biofilm-associated infections have also significantly reduced the efficacy of currently imparted antibacterial remedial therapy, thus leaving no viable therapeutic option available. This alarming situation thus calls for the development of and designing novel alternate routes for eliminating the lacunas of the contemporary antibacterial therapeutic approach.

In this context, nanotechnology has appeared to be a pioneer, and the previous decade has seen a tremendous rise in the worldwide utilization of nanomedicines as inventive devices for battling the high rates of antibacterial resistance. Ongoing researches have demonstrated that consolidating nanoparticles with antibacterial agents additionally improves their bactericidal properties. Consolidating antibiotics with nanoparticles likewise reestablishes their capacity to kill

A. Parmar · S. Sharma (✉)
Institute of Forensic Science & Criminology, Panjab University, Chandigarh, India
e-mail: 25shweta@pu.ac.in

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microbes that have gained immunity toward them. Moreover, nanoparticles labeled with antibiotics have appeared to expand the co-localization of antibiotics at the site of the bacterium-antibiotic interaction and to encourage binding of antibiotics to bacteria. This review article will tend to highlight the physicochemical properties, mode of action, and bactericidal activity of nanoparticles in combating antibacterial resistance.

Keywords

Nanoparticles · Antibacterial · Multidrug resistance · Mechanisms · Physicochemical properties

11.1 Introduction

Recent era has witnessed a significant enhancement in the field of drug discovery and modern medicine, which has ultimately uplifted the health sector. Despite the tremendous strides being made, the researchers across the globe are facing momentous challenges when it comes to overcoming bacterial resistance (Allahverdiyev et al. 2011; Beyth et al. 2015; Wang et al. 2017). On a worldwide level, bacterial infections have been accounted for causing escalated mortality and morbidity and have proven to be a grave issue (Allahverdiyev et al. 2011; Beyth et al. 2015). Multidrug resistance (MDR) and biofilm-associated infections are some of the other factors, which have hampered the utility of present-day treatment therapies (Beyth et al. 2015).

Lately, broad-spectrum antibiotics were being employed as the first line of defense on a widespread scale for combating bacterial pathogen-based ailments. However, it became eminent that prolonged use of these antibiotics has proven to be ineffective (Wang et al. 2017). This can be ascribed to the fact that the genomic structure of these bacterial strains comprises of a super resistance gene called NDM-1 (Hsueh 2010) which facilitates them to develop an innate immunity toward active pharmaceutical formulations (Wang et al. 2017).

Antibiotics work via predominantly controlling three major mechanisms, viz., cessation of cell wall synthesis and translation and transcription (DNA replication) mechanisms (Wang et al. 2017). However, the bacteria are capable of developing resistance against any individual previously mentioned mechanisms. Apart from these, modification or degradation of antibiotic via cleaving enzymes (viz., β-lactamases and aminoglycosides) (Poole 2002) altered cellular compartmental structure (Jayaraman 2009), and evoked efflux pumps (Knetsch and Koole 2011) are some of the other prevalent factors which have resulted in the significant declination of the potency of the marketed antibiotics (Wang et al. 2017). This alarming situation, hence, calls for the development of novel alternative remedial therapies that can offer better patient compliance, reduced dosing, and effective killing of bacterial pathogens.

With the advent in science and technology, a recent field collectively coined as "nanotechnology" has emerged which has revolutionized the concept of modern-day medicine. It is by virtue of this that nanotechnology has become an imperative part of varied scientific paradigms. Nanoparticles (NPs) are sub-micron-sized colloidal suspensions having particles ranging between 1 and 100 nm in size (Farouk et al. 2018). These particles offer a narrow particle size distribution, which facilitates them with an innate ability to penetrate through the bacterial cells with certainty and ease (Farouk et al. 2018). Another illustrative property, which is of paramount importance, is their large surface to volume area ratio, which allows these particles to strongly and specifically interact with the bacterial cell wall even at smaller doses, hence resulting in an enhanced antibacterial activity (Farouk et al. 2018; Magiorakos et al. 2012). This escalated antibacterial activity can be justified based on the mode of action of NPs. As these particles tend to establish effective communication with the bacterial cell wall on one to one basis, the need for penetration is surpassed, thus viably circumventing the resistance mechanism offered by the bacteria (Farouk et al. 2018). This raises the expectation that nanoparticles would be less inclined than antibiotics to advance resistant bacteria (Beyth et al. 2015; Farouk et al. 2018). Consequently, it can be said that these nano-sized particles can act as a viable alternative to traditional antibiotic therapy for fighting bacterial afflictions (Farouk et al. 2018).

The following review article is precisely divided into four sections wherein the first section chiefly corresponds toward the introduction of the problem. In the latter part, the effect of physicochemical properties of nanoparticles on the antibacterial property has been comprehensively discussed. The present monologue also centers on defining the underlying mechanistic components of nanoparticles, which help in evading the resistance developed by bacterial pathogens. The last phase of the following manuscript pertains toward the application of the varied types of nanoparticles in mediating a theranostic approach for effective treatment of bacterial infections.

11.2 Physicochemical Properties and Invigorated Tool

In order to gain an insight into the antibacterial property of NPs, it becomes a prerequisite that the physicochemical properties of the NPs should be thoroughly investigated (Farouk et al. 2018). It has been deciphered that bactericidal properties by certain metals are possessed when they are present in their bulk forms, while other few depicts antibacterial property when they are exclusively present in nano form (Seil and Webster 2012). Thus, it can be precisely said that an individual nanoparticulate system will result in the generation of the varied types of bactericidal effect. Hence, the following section will shed some light on certain imperative and crucial factors, which tend to affect the antibacterial property of NPs (Fig. 11.1).

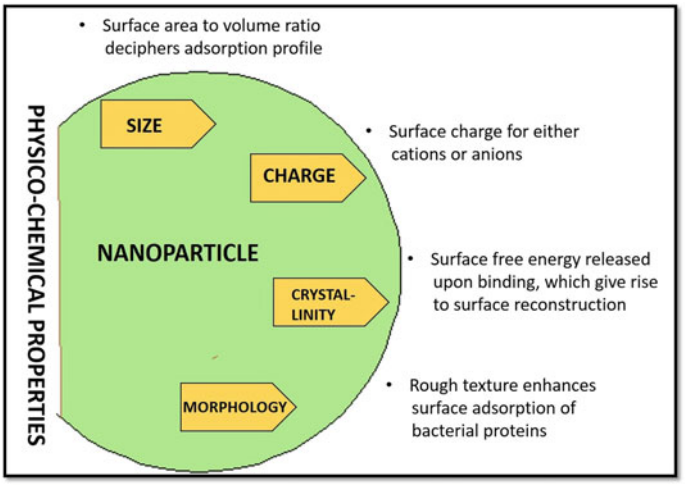

Fig. 11.1 Graphical representation showing varied physicochemical parameters of nanoparticle and their influence on the bacterial cell

11.2.1 Size/Diameter

Development of bacterial biofilms is a notable procedure, which makes these deleterious pathogens immune to traditional antibiotic treatment therapies. However, bacterial adhesion is the underlying phenomenon, which firmly substantiates the growth of these. Recent studies highlighted the fact that the size plays a notable effect on the therapeutic interventional property of the nanoparticulate system. Esfandiari et al. in a novel approach designed Ag-functionalized TiO_2 nanotubes, and the efficacy of this versatile nanostructured system was tested against *E. coli* (Esfandiari et al. 2014). The study clearly pointed toward a size-dependent bactericidal effect of the developed nanoparticulate system. It was found that the nanotubes having a smaller opening diameter (~100 nm) and AgNPs (~20 nm) produced a significantly pronounced effect than their larger counterparts.

In another study planned by Pan et al., three nano-Mg $(OH)_2$ slurries of different morphologies were utilized, and their antibacterial properties were tested on model *E. coli* bacteria (Pan et al. 2013). The fact, which came into light from this study, was the establishment of an inverse relationship between the NPs size and bactericidal effect. The smaller-sized slurries tend to have a comparatively higher antibacterial property, while a vice versa phenomenon was observed in case of larger-sized slurries. The TEM analysis showed no evidence of cellular co-localization of NPs; however, a breach in cell wall integrity was noticed (Pan et al. 2013). Both the studies clearly demarcated the importance of particle size in determining the extent and mechanism of antibacterial property. This size-dependent toxicity can be explained by the fact that a smaller-sized particle offers a greater surface area to the volume ratio. This aids in establishing an enhanced contact among the NPs and bacterial cell wall also as such smaller particles can swiftly translocate themselves

deeply into the cellular periphery of the target pathogens from the outside milieu (Deplanche et al. 2010; Gurunathan et al. 2014).

11.2.2 Morphology and Texture

Morphology or the shape of the NPs is another factor of paradigm importance, which plays an intricate role in deciding the fate of NP in inducing a bactericidal response. It became evident from the pertinent literature that NPs having a diverse spatial geometry/morphology interact with the periplasmic enzymes in a different manner. These vivid types of interactions can ultimately produce a slightly different level of damage in bacteria (Cha et al. 2015). In context to this, a study highlighted the effect of the variedly shaped nanoparticulate system on antibacterial properties (Yu et al. 2014). It was deciphered that the n-ZnO having a pyramidal geometry prevented the degradation of periplasmic enzymes. The outcomes also suggested that a photocatalytic activity was produced by these NPs and the underlying mechanism responsible for it was found to be the obstruction and reconstruction of these essential enzymes (Wang et al. 2017; Yu et al. 2014).

In a similar approach, Y_2O_3-based prismatic NPs were fabricated by Prasannakumar et al. (2015). The efficacy of these NPs in enticing a bactericidal activity was assessed in two bacterial strains, viz., *S. aureus* and *P. desmolyticum*. The study showed that the prismatic morphology of these NPs helped them to establish a strong and direct bridging with the bacterial cell wall. This interaction further resulted in the breakdown of the bacterial cell membrane, thus finally leading to cell lysis and apoptosis (Prasannakumar et al. 2015). Actis et al. studied the effect of AgNP geometry on the survival and growth rate of *S. aureus* (Actis et al. 2015). It was seen that among all the fabricated AgNPs, cubical-shaped NPs showed maximum bactericidal activity due to it its high surface area to the volume ratio and facade reactivity (Actis et al. 2015; Wang et al. 2017). Apart from the broad research in regard to the impacts of various NP attributes on bacterial cells, few investigations have highlighted the impact of texture. It has been witnessed that an increase in the roughness of the NPs surface leads to a significant enhancement in the adsorption of bacterial proteins. This escalation in the bacterial protein adsorption on the corona of NPs results in a diminished bacterial adhesion (Ben-Sasson et al. 2013; Sukhorukova et al. 2015).

11.2.3 Surface Charge Density

In recent studies, it has been repeatedly shown that the surface charge density also known as *zeta potential* has an adverse effect on the adhesive property of the NPs. The highly charged positive particles tend to attach them more firmly to the negatively charged bacterial cell wall. On the other hand, in case of negatively charged NPs, a complete paradoxical scenario is seen. This point was highlighted in a study where two types of Mg-based NPs, viz., Mg $(OH)_2$_MgCl and Mg

$(OH)_2$_$MgSO_4$, were tested for their antibacterial potential (Pan et al. 2013). The outcomes of the study demonstrated that the Mg $(OH)_2$_$MgSO_4$ NPs were readily absorbed and inbound onto the bacterial cell as compared to the Mg $(OH)_2$_MgCl NPs. This facile interaction can be ascribed based on charged moieties present on the surface of the NPs. The Mg $(OH)_2$_$MgSO_4$ had a positive charge on their corona hence were able to form ionic interactions with the charged bacterial cell wall. On the other hand, the Mg $(OH)_2$_MgCl NPs were negatively charged owing to which the electrostatic repulsive forces dominated and the NPs were unable to interact with the negatively charged bacterial cell (Pan et al. 2013).

It has also been portrayed that the accumulation of positively charged (cationic) NPs can lead to inhibited cell growth and colonization. Another factor, which came into a light, was that the abundant accumulation of cationic NPs resulted in a restricted bacterial adhesion. The abovementioned fact was corroborated by the findings of the study conducted by Fang et al. (2015). They elucidated the underlying mechanism behind the bactericidal effects produced by cationic NPs. The study pointed out that ion exchange resulted in deeper penetration of these NPs across the bacterial cell envelope, thus establishing direct communication with the cellular components. This interaction among the particles and cellular bodies was thought to be responsible for evoking a bactericidal response (Fang et al. 2015). Apart from this, it has also been hypothesized that the production of ROS entities is also significantly enhanced in the presence of positively charged NPs (Wang et al. 2017). This escalated level of ROS production finally allows the bacteria to meet their final fate, i.e., cell lysis and apoptosis.

11.3 Nanoparticles' Mode of Action for Combating Bacterial Resistance

A number of mechanisms have been proposed for elucidating the role of NPs in overcoming bacterial resistance. Among them, the first and foremost types of NPs are those which tend to display numerous modes of action in a simultaneous order (Pelgrift and Friedman 2013). The generation of these simultaneous mechanisms will prove to be highly beneficial as multiple gene mutations will be required in the same bacteria to evoke defense mechanism which is deemed to be highly unlikely possible (Blecher et al. 2011; Huh and Kwon 2011; Knetsch and Koole 2011; Schairer et al. 2012). Apart from this, another strategy, which has been seamlessly used, is the simultaneous entrapment of several antibiotics within the corona of nanoparticles and delivering the active payload cargo to the target bacterial site (Blecher et al. 2011; Zhang et al. 2010). It is a well-versed fact that a significant antibacterial activity can only be attained when direct contact between the NPs and the bacterial cell is maintained (Wang et al. 2017).

NPs possess several alluring physicochemical, biological, and mechanical properties of diverse nature, which provides them with an intrinsic ability to establish effective interaction with the target site (cell wall)/pathogens (Farouk et al. 2018). This specific interaction of NPs with the bacterial cell wall is facilitated by

electrostatic attraction, van der Waals forces, receptor ligand, and hydrophobic interactions (Armentano et al. 2014; Gao et al. 2014; Li et al. 2015; Luan et al. 2016; Wang et al. 2017). This direct interaction helps in lodging the NPs inside the cellular periphery of the bacteria where they disrupt the cellular morphology (cytosol shrinkage, cell wall rupturing, and membrane detachment) (Baranwal et al. 2018; Dakal et al. 2016). Further, they bind with the essential biomolecules (viz., DNA, RNA, protein, and lipids) and interfere with the underlying metabolic pathways ultimately resulting in altered cellular function and apoptosis (Baranwal et al. 2018; Dakal et al. 2016; Li et al. 2008; Wang et al. 2017).

The antimicrobial efficacy of nanoparticles can also be corroborated by the fact that they are capable of de-phosphorylating the tyrosine residues present in essential proteins hence modulating the signal transduction pathway (Baranwal et al. 2018; Dakal et al. 2016). Another vantage point, which came into light, was the enhancement in the permeability index of the bacterial cell, which resulted in an escalated delivery of active payload cargo. This might be ascribed to the sequence of irreversible changes occurring in the morphology of the cellular compartments owing to the interaction of NPs with a sulfur group present in cell wall proteins (Baranwal et al. 2018; Ghosh et al. 2012).

It has been deciphered that the surface charge (zeta potential) plays a key role in deciding the antibacterial efficacy of the nanoparticulate system, as it tends to govern the electrostatic interaction occurring between the NPs and bacterial cell (Farouk et al. 2018). A positive charge on the corona of the NPs allows them to interact strongly with the negatively charged cell membrane ultimately leading to disrupted cellular organelles, bacterial flocculation, and reduced survival rate (Farouk et al. 2018). Apart from these, there are certainly other mechanisms such as cessation of translation and transcription process, interrupted cell division, and cell lysis due to the production of toxic ions by NPs, which are found to be responsible for the generation of genotoxicity and cytotoxicity in bacteria (Farouk et al. 2018; Hajipour et al. 2012). The most eminent antibacterial mechanisms are as follows (Farouk et al. 2018; Hemeg 2017; Wang et al. 2017; Table 11.1).

11.3.1 Damage to the Cellular Membrane

A nonspecific mode of action is displayed by the NPs on the cellular membranes; however, an exact mechanism is yet to be discovered (Farouk et al. 2018). Nevertheless, it has been hypothesized that a certain class of cationic cyclic decapeptides commonly known as polymyxin are responsible for the antibacterial activity (Aruguete et al. 2013; Farouk et al. 2018), as they work in an orderly fashion and are responsible for disrupting the bacterial cell membrane. Another hypothesis, which has been formulated to substantiate the antibacterial efficacy of the NPs, relies upon the fact that maintenance of direct contact between NPs and bacterial cell results in an augmented cellular permeability. This further results in the formation of "voids" or hollow spaces, thus suggesting subsequent damage to the lipidic bilayer

Table 11.1 Bactericidal activity of nanoparticles

Type of NPs	Bacterial strains/cells	Proposed mode of action	Effect caused	Refs.
Interaction with cell barriers				
HAPw/n-ZnO	*S. mutans, Candida albicans, S. aureus, and E. coli*	–	Pronounced bactericidal effect in *S. mutans*, *Candida albicans*, *S. aureus* in contrast to *E. coli* Destruction of the bacterial cell membrane	Yu et al. (2014)
Nano-diamonds	*E. coli and B. subtilis*	Formation of covalent bonds with adjacent cellular matrix and intracellular proteins	Restricted enzymatic activity Disordered translation Metabolic pathways Apoptosis	Wehling et al. (2014)
TiO_2	–	Adhesion of NPs with bacterial cell wall resulted in increased membrane permeability, ROS, free hydroxyl radicals, and hydrogen peroxide production. Degradation of cell wall and cytoplasmic membrane	Intrinsic damage to the bacterial cell membrane Altered morphology Inhibited cell functions Leaked cellular (cytoplasmic) components (viz., ions and micronutrients) Apoptosis Cell lysis Complete mineralization of the organism Gradual degradation of cell wall	Foster et al. (2011) and Joost et al. (2015)
n-ZnO	*E. coli*	Adhesion of NPs with bacterial cell wall resulted in ROS production	Damaged cell membrane (honeycomb structure formation, pit, bit, or hole formation) Restricted enzymatic activity Leakage of intracellular protein	Padmavathy and Vijayaraghavan (2011)

(continued)

Table 11.1 (continued)

Type of NPs	Bacterial strains/cells	Proposed mode of action	Effect caused	Refs.
			Cell lysis and apoptosis	
Fe_2O_3 and TiO_2	*E. coli* and *S. aureus*	Inactivation of bacteria due to compression	Decomposition of bacterial cell Aggregation of bacterial cells	Zhukova (2015)
Graphene nanosheets	*E. coli*	Inactivation of bacteria due to the destructive extraction of molecular components of the cells (viz., phospholipids)	Degradation of cellular membrane	
Nano-TiO_2 (anatase)-based thin films	*E. coli*	Inactivation of bacterial cells	Enlargement in cellular structure Disruption of cellular membrane Leakage of cytoplasmic content Alteration in the chemical composition of cellular organelles Lipid peroxidation and decomposition of membrane fatty acids Cell death	Joost et al. (2015)
Al_2O_3	*E. coli*	NPs interact with cellular membrane LPS via hydrogen bonding and ligand exchange	Pits of irregular shape are formed Alteration in the level of cellular phospholipids contents Cellular membrane perforation Disruption of cellular membrane Leakage of cytoplasmic content	Ansari et al. (2014)
Diffusion				
Graphene/ iron oxide	MSRA	Diffusion of NPs inside the bacterial cell membrane resulted in the	Inactivation of bacterium due to localized heat and	Pan et al. (2016)

(continued)

Table 11.1 (continued)

Type of NPs	Bacterial strains/cells	Proposed mode of action	Effect caused	Refs.
		large-scale generation of ROS and hydroxyl radicals	oxidative stress Apoptosis	
Uncoated Ag, Au, Nic, and Si	*E. coli*	Diffusion leads to the production of superoxide (AgNPs), hydroxyl radicals (au and Ni), and singlet oxygen (Si NPs)	Altered cell membrane permeability and accumulation of generated toxic enmities resulted in bactericidal killing	Zhang et al. (2013)
Adsorption				
ZnO	*E. coli*	Zinc ion interaction with the -SH protein groups results in the generation of ROS	Disorganization of morphological symmetry of cell membrane Disruption of cellular functions Dispersed cell membranes Leakage of intra/ intercellular proteins Formation of "pits" Destroyed cellular membrane permeability Depresses enzymatic activity of cellular membrane Apoptosis	Padmavathy and Vijayaraghavan (2011)
Inhibited translation and transcription				
TiO_2	*E. coli* and K 12 cells	NPs integrated specifically with G-C rich DNA	Compression of bacterial DNA Degeneration and fragmentation of nucleic acids Diminished gene expression	Iram et al. (2015) and Zhukova (2015)
Ag	*E. coli, S. aureus*	Upregulation of antioxidant transporter and efflux pumps coding genes	Sterilization and inhibited growth Collapsed antioxidant potential	Nagy et al. (2011)

(continued)

Table 11.1 (continued)

Type of NPs	Bacterial strains/cells	Proposed mode of action	Effect caused	Refs.
Au-SPIONs	*P. aeruginosa*	NPs interact via establishing an S-S bonding with cellular membrane proteins	Disturbed cellular metabolism Inhibited redox systems	Niemirowicz et al. (2014)
SPIONs	–	ROS and superoxide production, hydroxyl radical formation, oxidative stress, catabolism of carbon source and generation of nicotinamide adenine dinucleotide (NAD)	Damaged cellular macromolecules (nucleic acids and proteins) Death of residual bacteria Lipid peroxidation	Bajpai and Gupta (2011), Durmus et al. (2012), Hajipour et al. (2012), and Leuba et al. (2013)
Suppressed metabolic gene expression				
TiO_2	C3H10T1/2 cells and *S. epidermidis*	Physicochemical properties (elevated coronal texture and subordinate water contact angle) and chemical constituents (presence of oxygen and fluorine in significantly higher levels)	Diminished adhesion and colonization (inhibited growth) of pathogens on NPs Escalated adhesion of C3H10T1/2 cells on NPs Decreased biofilm formation	Peng et al. (2013) and Roguska et al. (2015)
OSM-2	*Lactococcus*	–	Increased metabolic profiles Altered bacterial colonization Enhanced acetogenesis and methanogenesis due to an enhancement in the growth of acetogenic bacteria and archaebacteria Reduced biofilm formation	Pan et al. (2015)

cell membrane eventually leading to loss of plasma membrane (Farouk et al. 2018; Leroueil et al. 2007; Niskanen et al. 2010).

11.3.2 Production of Toxic Ions by Metallic NPs

Recently a tremendous zeal has been witnessed, where a large-scale development and application of metallic nanoparticles are taking place for eradicating the grave issue of bacterial infections. It has been deciphered that on coming in direct contact with the bacterial proteins the metallic NPs results in the formation of sparingly soluble metal ions, viz., Ag^{2+}, Zn^{2+}, and Cd^{2+} (Farouk et al. 2018). These metal ions are considered competent enough to evoke a toxic response in bacterial strains. This can be explained by taking an example of silver NPs where lodging of these NPs into the bacterial cellular periphery results in the precipitation of $AgCl^{-}$ in the cytoplasm, thus resulting in an inhibited respiration and ultimately apoptosis (Farouk et al. 2018; Niskanen et al. 2010). Degradation of the metallic NPs results in a gradual and consistent release of metal ions, which are readily absorbed by the bacterial cells. These absorbed ions establish a bridging with the functional groups (viz., amino (-NH), mercapto (-SH), carboxylic (-COOH)) of proteins and nucleic acids present in the cellular organelles (Wang et al. 2017). Disturbed enzymatic activity, altered cellular compartmental morphology, inhibited physiological and metabolic processes, and diminished survival rate are some of the utmost consequences, which are faced by the bacterial cell, which have encountered such metallic ions (Wang et al. 2017).

11.3.3 Oxidative Stress (ROS Generation)

Amid all known anti-oxidizing agents, oxygen is considered the most powerful one. Repeatedly, it has been demonstrated that during the process of respiration it acts as an efficient electron acceptor and hence can prove to be a critical factor in governing the survival rate of bacteria (Farouk et al. 2018). Oxygen can exist in varied states such as singlet, doublet, or triplet; however, it has been shown that both singlet (O_2) and triplet ($3O_2$) oxygen can prove to be toxic for cells and bacteria, respectively (Farouk et al. 2018). Peroxidation of lipidic bilayer membrane and precipitation of intra/intercellular proteins are one of the most significant effects that are produced on the generation of singlet oxygen. This finally results in the disruption of bacterial cellular compartments and ultimately killing of bacteria (Bronshtein et al. 2006; Farouk et al. 2018). Oxygen in the singlet state is the major deriving source for catalyzing several detrimental and unstructured oxidation processes taking place inside the bacterial cell.

However, during the respiratory cycle, consumption of singlet oxygen molecules by the bacterial cells results in the formation of free radicals (hydrogen peroxide activity). These generated free radicals exert oxidative stress on the nucleic acids, proteins, and lipidic bilayer membrane, thus making it difficult for the bacteria to

sustain (Bronshtein et al. 2006). It became apparent from studies that interaction taking place between DNA and bacterial cells is greatly affected by ROS production (Pramanik et al. 2012). Further, evidence corroborated the fact that ROS play an intricate role in deciding the fate of the bacteria's survival (Wang et al. 2017). This might be attributed to the fact that ROS tend to escalate the gene expression levels of oxidative proteins, which further governs the bacterial cell apoptosis mechanism (Wu et al. 2011).

11.3.4 Non-oxidative Mechanisms

In the course of time, researchers have utilized varied state of the art analytical techniques (viz., electron spin resonance (ESR), liquid chromatography-mass spectrometry (LC-MS)) in conjugation with imaging and spectroscopic analytical techniques (viz., transmission electron microscopy (TEM) and Fourier transform infrared (FT-IR)). They also utilized flat cultivation method in accordance with proteomic tools to decipher the antibacterial activity of metallic (MgO) NPs on *E. coli* (Leung et al. 2014; Wang et al. 2017). The experiment was conducted under three different light conditions, viz., UV, natural, and dark conditions. The outcome of the study clearly depicted that the incubation of NPs resulted in three vital phenomena (Leung et al. 2014; Wang et al. 2017):

1. The energy dispersive X-ray (EDX) spectra clearly outnumbered the presence of any MgO ion inside the periphery of the bacterial cell. Subsequently, TEM analysis revealed the emergence of "pores" on the palisade region along with disrupted and deformed cellular compartmental morphology.
2. A miniscule amount of generated ROS was detected by metallic NPs.
3. Negligible changes in the level of cell wall constituents (viz., lipopolysaccharide (LPS) and phosphatidylethanolamine (PE)) were observed on tearing the bacteria with the prepared NPs.

On the premises of these outrageous results, it can be summed up that MgO NP treatments did not result in any sort of escalation in the lipid peroxidation or ROS-associated protein production. However, these NPs resulted in a significant decline in the levels of several cellular metabolomic processes, which had an innate relationship with the essential regulatory processes of cell replication, lysis growth, and division (Leung et al. 2014; Wang et al. 2017).

11.3.5 Mutation in Bacterial DNA

It came into being from varied studies that metallic ions produced because of dissociation of NPs establish a facile interaction with the nucleic acid of microbes. This interaction results in the termination of transcription and cell division cycle (Dakal et al. 2016; Durán et al. 2016). This can be attributed to the fact that these

metallic ions so formed are intercalated between the DNA base pairs of the bacterial genome, thus resulting in a mutation and ultimately bacterial cell death (Hemeg 2017). In this context, studies were conducted where the antibacterial effect of metallic nanoparticles, viz., AgNPs and CuNPs, were assessed (Chatterjee et al. 2014; Dakal et al. 2016; Durán et al. 2016; Yoon et al. 2007). The study clearly depicted that AgNPs were capable of inhibiting the cell division and DNA replication, whereas CuNPs on coming in contact with the bacterial cell resulted in the degradation of bacterial DNA (Hemeg 2017). In a different experiment, a combinatorial approach employing both X-ray irradiations and BiNPs was used as potent vectors for antibacterial activity. The study pointed toward an efficient killing of the pathogen bacterial population. The exact mechanism behind this vicious killing was found to be the generation of free radicals, which brought intricate damage to the nucleic acid component (viz., DNA) of the bacterial genome (Hemeg 2017; Lellouche et al. 2012a).

11.3.6 Adsorption of Nanoparticles in Bacterial Cells (NP Interaction With Cell Barrier)

The level of toxicity of NPs is greatly governed by the electrostatic or charged interactions occurring between the NPs and the bacterial cell surface. It has been noted that a positively charged particle tends to establish much-enhanced cytotoxicity as compared to its negatively charged counterparts (Hemeg 2017). Keeping this point in consideration, surface-engineered TIO_2 NPs (AgNP-impregnated N-doped titania) were prepared by Wong et al. (2015). It was deciphered that the prepared NPs were able to establish an effective bridging, and they were readily absorbed into the bacterial cell surface. The study also pointed out that this swift translocation of NPs on the cellular surface resulted in enhanced cytotoxicity. The major mechanism behind this toxicity generation in the bacterial cell was found to be the initiation of redox reactions, which further lead to an escalation in the oxidative stress levels. Damage to bilayer lipidic membrane and intracellular proteins were some other detrimental effects, which originated due to the adhesion of these metallic particles (Hemeg 2017; Wong et al. 2015).

11.3.7 Altered Bacterial Membrane Permeability

In a series of studies, researchers prepared polyvinyl alcohol (PVA)-stabilized AgNPs (Dakal et al. 2016; Durán et al. 2016; Hemeg 2017; Sirelkhatim et al. 2015). The outcomes of the study clearly demarcated that the metallic ions thus formed adhere to the charged lipopolysaccharide membrane. This results in altered cellular membrane permeability and a subsequently enhanced ROS level production. Further, it was noticed that these factors lead to an alteration in the viscosity of the cellular membranes, thereby inhibiting and disrupting the respiratory transport mechanisms (electron transport chain, ETC) as well as electrochemical proton

gradient pump (viz., homeostatic imbalance), respectively, being operated across the bacterial cells. Disturbed physiochemical mechanisms ultimately lead to cell lysis and triggered apoptosis (Hemeg 2017). Similar results were obtained with other metallic nanoparticles as well (Chatterjee et al. 2014; Huo et al. 2016; Khashan et al. 2016; Sirelkhatim et al. 2015).

11.3.8 Cellular Envelope Permeation and Destabilization of Cellular Organelles

An effective translocation and subcellular co-localization of NPs become a prerequisite for attaining a significantly high level of cytotoxicity. However, the level of cell lysis acts as a function of zeta potential (surface charge foliage) of the NPs (Hemeg 2017). A study conducted by Lellouche et al. showed a promising application of metallic nanoparticles in apprehending the biofilm formation around catheters due to two bacterial strains, viz., *E. coli* and *S. aureus* (Lellouche et al. 2012b). In their study, they engineered the surface of catheters with MgF-NPs. The results displayed the significant antibacterial efficacy of the designed system. The surface grafted NPs were able to restrict the bacterial colonization in a comprehensive manner and offered long-lasting sterilization ability to the catheters (Lellouche et al. 2012b). The charge foliage imparted on the corona of these particles allowed them to permeate readily through the highly inaccessible cellular envelope of the bacteria. Once the NPs are lodged inside the periphery of the cell, a sudden decrease in cytoplasmic pH is observed. This drop in pH results in an escalation of the cellular membrane permeability. Owing to this peroxidation of the lipidic bilayer, membrane takes place, thus killing the bacterial colony (Hemeg 2017; Lellouche et al. 2012b).

In another study, Shamaila et al. synthesized gold nanoparticles, and the bacterial killing propensity of these NPs was tested in enteric bacterial human pathogens, viz., *E. coli*, *S. aureus*, *B. subtilis*, and *K. pneumoniae* (Hemeg 2017; Shamaila et al. 2016). It was deciphered that the proposed nanoparticulate system was capable of producing antibactericidal effects. It also came to light that the size and dose of the NPs had an innate relationship with the cellular toxicity. The mode of action of these particles was found to be the effective and deep-seated colocalization of these moieties inside the cellular organelle, viz., ribosome. This translocation facilitated the disorientation of the 30S ribosomal subunit because of which translation phenomenon was interrupted and cell lysis took place (Hemeg 2017; Shamaila et al. 2016).

11.3.9 Bacterial Film Disruption

Certain biological entities generally called as *quorum sensing molecules* are produced during the maturation phase of bacterial biofilm growth. These molecules chiefly comprise two major components, viz., matrix and carbohydrates (extracellular), which aids in establishing direct communication between the adjacent/

neighboring cells (Hemeg 2017; Neethirajan et al. 2014). The spread of bacterial infection takes place when these gradually growing bacterial cells are detached (Hemeg 2017). In lieu of this, a strategic solution has been provided by the NPs. Lellouche et al. prepared crystalline yttrium fluoride (YF_2) nanoparticles and assessed their characteristic antibacterial and anti-biofilm property (Lellouche et al. 2012a). The study pointed to size-dependent toxicity of prepared nanosystems. It was noted that smaller NPs exhibited more enhanced cytotoxicity in contrast to their bigger counterparts. The outcomes of the study revealed that an infinitesimally low concentration (mM) of NPs was able to produce a prominent cytotoxic effect in bacteria, thus retarding the growth of bacterial biofilm (Lellouche et al. 2012a). Several other research groups assessed the bactericidal effect of varied NPs (viz., Se, TiO_2, CdS, ZnO, Bi, and Ag) (Dakal et al. 2016; Dhanabalan and Gurunathan 2015; Durán et al. 2016; Guisbiers et al. 2016; Hernandez-Delgadillo et al. 2012; Lee et al. 2014; Wong et al. 2015). The outcome of the study clearly corroborated the abovementioned findings, and similar results highlighting the antibacterial targeting propensity of NPs were reported.

11.4 Bactericidal Activity of Nanoparticles

In response to the harsh environmental milieu, cell wall and membrane are the two most vital defensive parameters, which offer a protective niche to the bacteria. In other words, it can be precisely said that the exact morphology of these pathogens remains intact due to the protective coating offered by the bacterial cell wall (Wang et al. 2017). Owing to a complex physicochemical composition of the cellular membrane components, the intake of NPs generally takes place through diverse adsorption pathways in both Gram-positive (+ve) and Gram-negative (−ve) bacteria, respectively (Lesniak et al. 2013; Wang et al. 2017). In case of Gram-negative strains, the NPs are highly derived toward the bacterium, and a strong interaction among them is established.

This can be explained on the basis that numerous LPS units are exposed on the outer periphery of the cell wall, which imparts a significantly high negative charge. This in turn offers direct communication between the NPs and the host based on charge-charge interactions (Lesniak et al. 2013; Sarwar et al. 2015). On the other hand, the presence of teichoic acid on the outer corona of Gram-positive bacteria aided in widely distributing the NPs in accordance with the molecular phosphate chains across the bacterial cell wall, thereby preventing the aggregation of functional particles (Sarwar et al. 2015; Wang et al. 2017).

It became apparent from varied scientific studies that the NPs possess enhanced bactericidal effect in case of Gram-positive bacteria while in their counterpart's, viz., Gram-negative bacteria, showed comparatively lesser bacterial cell lysis/killing (Wang et al. 2017). The presence of LPS, phospholipids, and proteins across the cell wall of Gram-negative bacteria results in an altered cell morphology, thus creating a shielding barrier across the bacteria. This penetration barrier allows only

a certain group of entities to surpass through them and retards the movement of any other molecule other than macromolecules.

On the other hand, a thin layer comprising of both peptidoglycan and teichoic acid along with numerous miniscule pores is present on the cell wall of the Gram-negative bacteria. This allows the transverse passage of any foreign material swiftly through the bacterial cell, hence ultimately leading to disrupted cellular membranes and apparent cell lysis/apoptosis (Sarwar et al. 2015; Wang et al. 2017). Thus, it can be collectively said that the unique cellular composition of the vivid bacteria provides a strategic opportunity for the NPs to invade and attack the target pathogens in an efficient and comprehensive manner. Some of the studies depicting the antibacterial activity of nanoparticles are summarized in Table 11.1.

Cell membrane plays a prominent role in controlling the respiratory function of the bacteria. However, it has been depicted by ongoing studies that the respiratory mechanism of the bacterial cell membrane is highly influenced by the activity of nanoparticles (Erdem et al. 2015; Wang et al. 2017). Erdem et al. in their study evaluated the cytotoxic potential of TiO_2 NPs against two bacterial strains, viz., Gram-positive (*B. subtilis*) and Gram-negative (*E. coli*), respectively (Erdem et al. 2015). The study demonstrated an inhibited growth of bacterium due to the production of ROS entities. On the other hand, it was also deciphered that lipid peroxidation and disruption of the cellular respiratory pathway were induced owing to the presence of these NPs.

In a different study, Sondi et al. investigated the bactericidal efficacy of silver nanoparticles on Gram-negative *E. coli* (Sondi and Salopek-Sondi 2004). The treated bacterial plates were further visualized under TEM to observe the bactericidal effect of developed NPs. TEM analysis revealed the evident presence of circular pits, which signify innate damage to the bacterial cell wall, by the NP activity. Further, this resulted in escalated cellular membrane permeability and efflux of the NPs inside the cellular periphery. This resulted in an inactivated respiratory electron transport chain and lately apoptosis (Sondi and Salopek-Sondi 2004).

With recent strides in nanoparticulate therapy, another point, which came into consideration, was the bacterial cell potential. This tends to play a pivotal role in establishing direct communication between the NPs and bacterial cell, hence governing the phenomenon of apoptosis (Wang et al. 2017). A perfect example corroborating this hypothesis was demonstrated in a study conducted by Nataraj et al. (2014). They utilized fluorescence microscopy as an invigorated tool for assessing the detrimental bactericidal potential of TiO_2-based NPs on the bacterial cell membrane. It was observed that NP treatment resulted in an altered cell membrane potential which became quite apparent from the marked changes taking place in the fluoresce intensity of the cytoplasm (Nataraj et al. 2014; Wang et al. 2017).

The NPs tend to penetrate the bacterial cell wall by employing two varied penetration mechanisms, viz.:

1. *Diffusion*: The first and foremost type of penetration strategy used by the NPs is diffusion. The diffusion of nanoparticles in the bacterial cell wall or membrane is

responsible for the generation of ROS. These ROS so generated results in the inactivation of bacteria (Table 11.1).

2. *Adsorption*: Adsorption also plays an intrinsic role in controlling the bactericidal potential of NPs. The interaction of NPs with the bacterial milieu results in the fragmentation of these particles into their native ionic forms. These ions so formed tend to establish a bridging with the charged functional moieties (viz., COOH, PO_4^{3-}) situated on the palisade region of the bacterial cell. This phenomenon of adsorbing the NPs in bacterial vasculature is called *bio-sorption* (Nataraj et al. 2014). The bio-sorption process results in the lysis of cell wall and its congruent membranes, thus creating a detrimental effect on the bacterial population (Table 11.1).

Recently gradual interests of researchers have been focused on the ability of NPs to interfere with the translation and transcription mechanism, thereby altering the protein and nucleic acid synthesis phenomenology (Table 11.1). Su et al. in a study investigated the chief key mechanism responsible for the bacterial denitrification by CuO NPs (Su et al. 2015b). The detailed proteomic bio-informatic analysis revealed an alteration in the intracellular protein expressions due to the interaction of these metallic NPs with bacterial cellular components. The alteration in translational machinery resulted in the disruption of nitrogen metabolism cycle along with the inhibition of two other major cellular phenomena, viz., respiratory cycle (electron transport chain) and substance transport (Su et al. 2015b).

In an incessant attempt, Su et al. utilized varied state of the art techniques to investigate the effect of AgNPs on the translational and metabolomic profile of *E. coli*. The outcome of the study revealed that the Ag ions released from the NPs resulted in depressed enzymatic activity and inhibition of ribosomal subunit protein expression and activity of certain other proteins (Su et al. 2015b). In a similar study, Cui et al. utilized proteomic and metabolomic assays to ascertain the potency of Au NPs in evoking an antibacterial activity in Gram-negative *E. coli* bacteria (Cui et al. 2012). The study demonstrated two facile modes of action by which the NPs were able to incite a bactericidal activity in the model organism;

(a) Inhibition of bridging between ribosomal subunit and transfer ribose nucleic acid (viz., tRNA) results in disturbed protein synthesis.
(b) Alteration in the cellular membrane potential leads to depressed ATPase enzymatic activity and reduced ATP production. This ultimately results in the gross cessation of cellular activity.

The knowledge of the exact mechanism responsible for the bactericidal efficacy of NPs becomes prerequisite. Whole genome analysis is one such technique, which has equipped the present-day researcher with an ability to elucidate the antibacterial efficacy (apoptosis) in real time. A perfect example of this approach has been depicted in a study conducted by Su et al. (2015a). In their study, they utilized this

technique of paradigm importance to elucidate the mode of action of ZnO NPs on Gram-negative *E. coli*.

In addition to this, they further utilized genome-wide toxico-genomic approach on a comprehensive level to draw a comparison between the molecular response profiles of ZnO NPs and free Zn ions. The outcomes of the study indicated a wide-scale alteration in the bacterial genome, thus hindering the expression of ~387 genes. Apart from this, a significant inhibition in translation, gene expression, and RNA modification along with a demarcating alteration in the structural physiology of ribosomes was observed (Cui et al. 2012).

The normal physiological processes such as metabolism generally maintain the growth and multiplication of bacteria. A slight alteration in the metabolic processes can induce a high level of damage to the membrane and cell wall components of the bacteria. This produces a state of oxidative stress in bacteria and ultimately leads to cell lysis/apoptosis (Wang et al. 2017). It is not so that these metabolomic cycles take place individually in an isolated manner; rather, they formulate an integral part of the diverse activities taking place in a living cell. It is by virtue of this property that metabolic alterations can be used as a viable alternative to inhibit and control the growth of these deleterious microorganisms. In this context, ROS production and metal ion dissolution are the two highly claimed key mechanisms found to be responsible for the generation of an altered metabolomic process in bacteria (Table 11.1).

Leung et al. in a study utilized liquid hue spectrum analysis to probe the probable mechanism responsible for producing bactericidal effects in *E. coli* by MgO NPs (Leung et al. 2014). It was observed that the interaction of NPs with the bacterium resulted in unregulated metabolic protein expression along with the upregulated activity of both weak thiamine ester binding and riboflavin metabolic proteins. The study also pointed toward a significant downregulation of the essential mapping proteins. Owing to which, a reduction in the metabolomic activity of bacterial cells takes place, thus substantiating the hypothesis that targeting of protein by NPs can result in changed bacterial cellular metabolic profiles (Leung et al. 2014; Wang et al. 2017).

Another study reported an inhibition in the expression of a model de-nitrifier protein present in *P. denitrificans* by CuO NPs (Su et al. 2015b). An increase in the concentration of CuO NPs from 0.05 to 0.25 mg/L resulted in a diminished nitrogen removal efficiency from 98.3% to 62.1%, respectively. On further evaluation, it came to light that the facile communication of the NPs resulted in compromised surface morphology and integrity of the bacterial cells. This alteration in the cell membrane permeability allowed the swift translocation of these particles inside the vicinity of the bacterial cells. Proteomic analysis in concordance with the bioinformatics analysis further revealed unregulated expression and suppression of proteins responsible for carrying out nitrogen metabolism, electron (viz., NADH dehydrogenase and cytochrome), and substance (viz., GtsB (glucose transport)) transport. Catalytic potential and expression of nitrate and nitrite reductase enzymes were suppressed by the activity of nanoparticles (Su et al. 2015b).

The morphology of the biofilm provides an innate immunity to the bacteria making them resistant toward most of the chemical moieties (Wang et al. 2017). It has been demonstrated that the interaction of NPs with the extracellular polymeric substances (EPSs) results in altered integrity of the biofilm (Su et al. 2009). The outcome of the study conducted by Ansari et al. strongly supported the abovementioned fact (Ansari et al. 2012). In their study, it was deciphered that ZnO NPs inhibited the production of EPSs. This further amounted in generating a bactericidal activity against the biofilm of drug-resistant Gram-negative bacteria, viz., *E. coli* and *K. pneumoniae*, respectively (Ansari et al. 2012).

Another point, which came to a light, is the conduction of electrical signals by potassium ion channels across the bacterial biofilm (Lundberg et al. 2013). These ionic pumps are in turn also found to be responsible for coordinating the inter−/intracellular metabolic pathways in the bacterial biofilm. However, it was deciphered that Mg NPs can effectively and swiftly adhere and permeate through the perineum of the biofilm (Lundberg et al. 2013). This leads to a disruption in the cell membrane potential along with escalated lipid peroxidation levels and intercalation with the nucleic acid such as DNA (Lellouche et al. 2012c). Consequently, these changes in the physicochemical parameters of the bacterial cells ultimately amount to an inhibited bacterial biofilm growth and colonization (Lellouche et al. 2012c).

Salem et al. in an elaborative study deciphered the potential toxic effect of Ag and ZnO NPs on two Gram-negative bacterial strains, viz., *E. coli* and *V. cholerae* (Salem et al. 2015). The minimum inhibitory concentration (MIC) and inhibition of metabolic activity (INT) assays pointed out that a univocal amount of NPs resulted in the generation of similar bactericidal activity. It was also highlighted in the study that the NPs specifically targeted the metabolic pathways of the bacterium, which resulted in efficient apoptosis and cell lysis (Salem et al. 2015).

11.5 Conclusion

Bacterial strains impervious to the antimicrobial now being used has to turn into a genuine general medical issue that expands the need to grow new bactericidal materials. Thus, solid interest in creating novel systems or new systems can adapt to these significant issues. The rise of nanotechnology has made some new antimicrobial alternatives. Nanoparticles having varied parent compositions have exhibited gigantic potential as bactericidal agents, showing their potential as proficient antitoxin reagents in bacterial infections, wounds, and related medical issues. The adequacy of these nanoparticles changes with their physicochemical characteristics, viz., particle size, surface charge, morphology, and texture. Different nanoparticles depict bactericidal effect against various pathogenic bacterial species. Similarly, NPs have indicated adequate biocompatibility when fused in framework materials. Nanoparticles today are a promising platform for elective measures to control bacterial infections.

Antimicrobial nanoparticles offer a diversified array of classes and applications. These antimicrobial nano-sized particles offer sustained bactericidal activity with

reduced side effects, in contrast to other miniscule-sized antibacterial agents, which depicts a short-term effect and enhanced ecological toxicity. The upsurge in the number of drug-resistant bacterial strains is one of the significant issues with nanoparticulate anti-infection agents because of their particular targeting ability, though these particles physically pulverize cell films which circumvent the growth and development of these deleterious microorganisms. Because of these points of interest given by nanoparticles, endeavors have been made to utilize them in varied biomedical fields. Propelled qualitative research and development, committed endeavors, fruitful application, and commercialization of antibacterial nanoparticles will help in elevating the standard of living.

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Emerging Role of Cannabinoid System Modulators in Treatment of Cancer

12

Sheetal Singh, Smita Pattanaik, Ravimohan S. Mavuduru, and Shrawan Kumar Singh

Abstract

Endocannabinoid system (ECS) and its agonist/antagonists are emerging drug target in different pathophysiological conditions, and its role as an anticancer agent has been extensively explored recently. Psychotropic effects of cannabinoid, a century-old ingredient of *Cannabis sativa*, are widely used as a palliative care for cancer patients apart from its recreational uses. This chapter outlines briefly the overall ECS system which is further extended to exploration of this system in various diseases and cancer. Recent reports have suggested implications of ECS system components as an anticancer agent through different signaling pathways. Important pathways like MAPK and mTOR-AKT contribute to tumor development, angiogenesis, metastasis, and chemotherapy resistance in different cancer types. Interestingly, cannabinoids are found to reverse their effects, through the induction of apoptosis, autophagy, and immune system modulating pathways. We have elaborated the pathways through which ECS system mediates cancer cell death, inhibits the angiogenesis pathway, and negates the chemotherapy resistance in different cancer types. We have also explored how ECS system modulators may regulate diverse signaling mechanisms in tumor microenvironment and whether they impart a therapeutic effect. Finally, we highlighted recent and ongoing clinical trials that include cannabinoids as a therapeutic strategy and several combined approaches toward novel therapeutic avenues in treatment of cancer.

S. Singh · S. Pattanaik (✉) · R. S. Mavuduru · S. K. Singh
Department of Pharmacology, Post Graduate Institute of Medical Education and Research, Chandigarh, India
e-mail: pattanaik.smita@pgimer.edu.in; shrawanksingh2002@yahoo.com

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Keywords

Cannabinoids · G-protein-coupled receptors · Cannabinoid 1 receptors · Cannabinoid 2 receptors · GRP55 · Palliative care · Signaling pathways · Apoptosis · Chemoresistance

12.1 Introduction

Cannabinoids are a group of alkaloids which are derived from the plant *Cannabis sativa*. Their existence was first confirmed by China. Archaeological studies suggest that *C. sativa* is cultivated for fibers since 4000 BC. The medical use of cannabis was first mentioned in the Pen Ts'ao, a Chinese pharmacopeia (Read 1936). Later, Hua T'o (AD 110–207), founder of Chinese surgery, used cannabis compound during surgical procedures to anesthetize patients (Li and Lin 1974). Moreover, countries like Nepal and India used the seeds of cannabis as spice and also for making cooking oil (Touwn 1981). In India, cannabis seeds have been used for medicinal and recreational purpose. Atharvaveda (Indian holy book) also mentioned cannabis use against various diseases (Bowker 1997). In Indian traditional medicine, innumerable functions of cannabis were identified such as analgesic, anticonvulsant, hypnotic, tranquilizer, anesthetic, anti-inflammatory, antibiotic, antiparasitic, antispasmodic, appetite stimulant, digestive, aphrodisiac, diuretic, and expectorant (Mikuriya 1969; Aldrich 1997). Cannabis was considered sacred in Tibet and used to facilitate meditation. However, over time, cannabis extracts have been explored worldwide. In 1854, cannabis was included in the US dispensatory and was available in pharmacies of western countries (Robson 2001). Meanwhile, at the same time, recreational use of cannabis rapidly started spreading among the adult population apart from its medicinal uses. Subsequently, possession or trading of cannabis became an offense by law throughout the USA. As a result, cannabis extract and its constituents were removed from the US pharmacopeia in 1942 and lost its therapeutic legitimacy (Kandel 1984). In 1996, California was the first state to pass Medical Marijuana Law (MML). As of January 2016, majority of US states lifted the prohibition barriers and started considering outright legalization. As a result, 25 additional states have legalized marijuana for medicinal use and 5 states legalized for recreational use (Pacula and Smart 2017).

12.2 Discovery

A renewed interest in the studies about cannabis began in the late twentieth century. Two researchers in 1964, Gaoni and Mechoulam, identified Δ^9 THC as the major psychoactive ingredient of *C. sativa* (Mechoulam and Gaoni 1965). This discovery became the milestone toward identifying other compounds of cannabis. In 1987, two new potent cannabinoid agonists HU-210 and desacetyl-L-nantradol were developed (Melvin and Johnson 1987). The receptors on which they act are called the

cannabinoid receptors. It was hypothesized that the receptors of cannabinoids may be intracellular due to their lipophilic nature. Later in 1988, cannabinoid receptor was found to be on the plasma membrane of the neuronal tissue in the brain (Devane et al. 1988). In 1990, cannabinoid receptor 1 (CB1) was cloned by Matsuda. After 3 years, in 1993 another receptor CB2 was identified by Munro (Matsuda et al. 1990; Munro et al. 1993). Further research revealed that both the cannabinoid receptors belong to the G-protein-coupled receptor (GPCR) superfamily.

12.3 Distribution of the Receptors

12.3.1 Cannabinoid Receptor 1 (CB1)

CB1 receptors are found primarily in the central nervous system (CNS). However, these receptors are not exclusive to CNS and also expressed in immune cells, pituitary gland, blood vessels, lung, bladder, adrenal gland, liver, adipose tissue, and reproductive and gastrointestinal tissues (Cacciola et al. 2010).

12.3.2 Cannabinoid Receptor 2 (CB2)

CB2 receptors are located predominantly in immune cells (B and natural killer cells) and also in the spleen, thymus, tonsils, splenic macrophage-monocyte preparations, peripheral blood leukocytes, and mast cells. Activated receptors can regulate immune cell migration as well as cytokine release in the brain. Later, its expression was also reported in neuronal microglia cells, cerebellum, brain stem cells, mid-brain, striatum, and hippocampus (Cacciola et al. 2010).

CB1 and CB2 receptors share 44% overall identity with each other. They are distinct in their distribution across the tissue and their sensitivity toward various agonists and antagonists.

12.3.3 GPR55 Receptors

GPR55 (ligand-gated ion channel) receptors are non-CB1/CB2 G-protein-coupled receptor protein which interacts with cannabinoid receptor ligands. In 1999, GPR55 was first identified via in silico studies and subsequently cloned (Sawzdargo et al. 1999). GPR55 receptors are distributed both in CNS and in periphery. Ryberg et al. found out that endocannabinoids can activate these receptors by using [^{35}S] GTPγS assay in HEK293 cell line (Ryberg et al. 2007).

Over the years, the role of GPR55 has been extensively studied by the researchers in various pathological conditions. From those studies, it becomes evident that these receptors play pivotal roles in cancer (breast and brain) (Andradas et al. 2011) immune regulation (Balenga et al. 2011), pain modulation (Schuelert and McDougall 2011), diabetes, obesity (Moreno-Navarrete et al. 2012), and

osteoarthritis (Whyte et al. 2009). In prostate cancer cells, increase in the phosphorylation of AKT and ERK1/2 and mobilization of calcium ions are reduced by GPR55 silencing (Pineiro et al. 2011). Thus, this shows that GPR55 provides additional benefit to CB1 and CB2 receptor functioning.

This chapter reviews the current knowledge of ECS and elucidate the physiological and pathophysiological roles of cannabinoid receptors. We will further discuss about various cannabinoid receptor agonists and antagonists and their putative use as adjunctive anticancer agents.

12.4 Cannabinoid Receptor Ligands

12.4.1 Classification of Ligands and Their Affinities Toward Receptors

In the classification according to the chemical structures, ligands fall into four major classes: classical, nonclassical, aminoalkylindole, and eicosanoid (Howlett et al. 2002) (Fig. 12.1).

1. Classical cannabinoids: Classical group of cannabinoids consist of ABC tricyclic dibenzopyran derivatives. The most investigated among classical cannabinoids have been Δ^9THC, Δ^8THC, HU-210, and desacetyl-L-nantradol. These cannabinoids are not selective and can bind both the receptors. Δ^9-THC has notably lower CB1 and CB2 affinity as compared to HU-210.
2. Non-classical group: This group contains analogs of Δ^9THC and is mostly found to have bicyclic and tricycling structures that lack a pyran ring. The most extensively studied member belonging to this group is CP55940. It has slightly lower affinities for both the cannabinoid receptors (CB1 and CB2) than HU-210 but does have the same intrinsic activity.

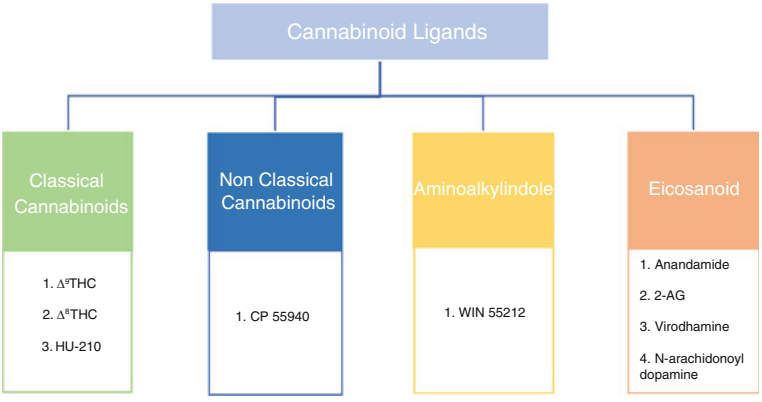

Fig. 12.1 Classifications of the cannabinoid ligands

Table 12.1 Ligands and their interaction with the receptors

S. no.	Ligand	CB1 receptor	CB2 receptor	Agonist	Antagonist	GPR55 receptor agonist
1	Tetrahydrocannabinol (THC)	✓	✓	✓	–	–
2	WIN 55,212-2	✓	✓	–	–	–
3	Cannabidiol (CBD)	✓	✓	–	✓	–
4	Arachidonyl-2′-chloroethylamide (ACEA)	✓	–	✓	–	–
5	Anandamide (AEA)	✓	✓	✓	–	✓
6	JWH015	–	✓	✓	–	–
7	AM251	✓	–	–	✓	✓
8	GW405,833 (GW)	–			–	–
9	JWH-133	–	✓	✓	–	✓
10	2-Arachidonoylgylcerol (2-AG)	✓	✓	✓	–	✓
11	CP 55940	✓	✓	✓	–	✓

✓ (tick mark) represents the binding affinity of ligand toward the particular receptor

3. Aminoalkylindole: Members of aminoalkylindole group have different structures from both the classical and non-classical cannabinoids. R-(+)-WIN 55,212-2 is the most studied member of this group which has the similar intrinsic activity as CP55940 and HU-210. However, it possesses higher CB2 affinity than CB1.
4. Eicosanoids: They are endogenous fatty acid amides. Endocannabinoids like anandamide and 2-AG are classified as the members of this group. They have lower affinity and relative intrinsic activity for CB2 receptor in comparison to CB1 receptor. Two other compounds included in this group are virodhamine and *N*-arachidonoyl dopamine (Table 12.1).

12.5 Pathophysiological Role of Cannabinoid System in Human Diseases

Over the years, various preclinical researches have confirmed the involvement of endocannabinoid system (ECS) components in different pathophysiological conditions and diseases. In this section, we will discuss briefly the implications of ECS components in different human diseases (Fig. 12.2).

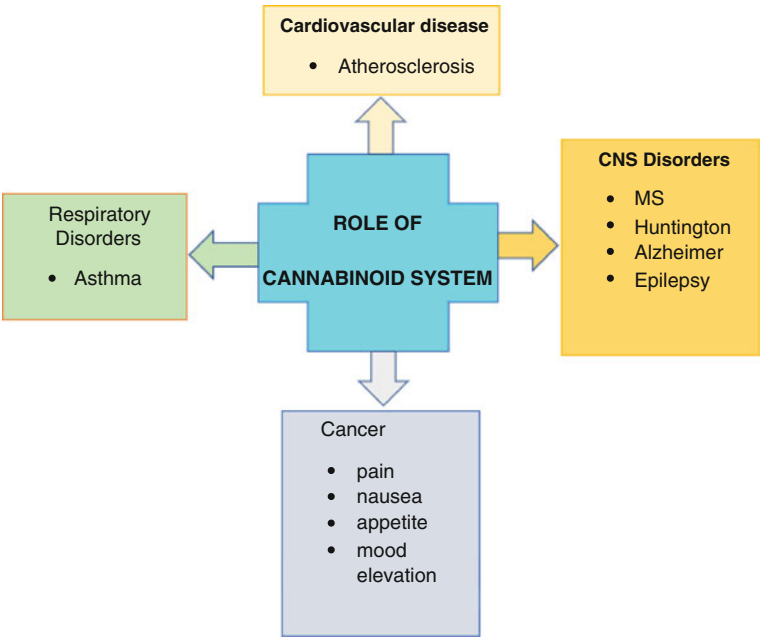

Fig. 12.2 Overview of the role of cannabinoid system

12.5.1 Cardiovascular Disease

12.5.1.1 Atherosclerosis

Atherosclerosis is a chronic inflammatory disease. It is characterized by accumulation of cholesterol and other substances under the intima of the vessels leading to proliferation of the vascular smooth muscles and recruitment of inflammatory cells which finally form atheromatous plaque that causes stiffening and narrowing of the vessels. Atherosclerosis compromises the circulation to vital end organs like the heart, kidney, and brain resulting in myocardial infarction, chronic kidney disease, and strokes. Cannabinoids affect atherogenesis via modulation of immune system response. CB1 and CB2 receptors coexist on the human macrophages. Thus, the role of both the receptors is considered in the regulation of atherosclerosis. CB1 receptors on activation switch on the MAPK signaling which causes the production of ROS (reactive oxygen species) and subsequently causes the pro-inflammatory response, whereas, CB2 receptors suppress these inflammatory pathways stimulated by CB1 receptors (Libby 2002). Oral administration of low dose of THC inhibits the progression of atherosclerosis in a mouse model. THC does so by decreasing monocyte adhesion and infiltrating the subendothelial region via CB2 receptor (Steffens et al. 2005). It is demonstrated in a study by Yuan et al. that THC downregulated the T_H1 immune response (Yuan et al. 2002). Apart from THC, WIN 55,212-2 can also activate the CB2 receptors which in response de-escalate the pro-inflammatory cytokine gene expression and cause attenuation of the downstream

NF-κb proteins (Sugamura et al. 2009). However, CB1 receptor blocking is a requisite in order to modulate inflammation. CB1 blockade is associated with the decreased expression of matrix metalloproteinase-9 (MMP-9) and inflammatory cytokines (Han et al. 2009). All these studies indicate that cannabinoids have anti-atherosclerotic activities. Therefore, they can be considered as potential treatment options for atherosclerosis.

12.5.2 CNS Disorders

12.5.2.1 Multiple Sclerosis (MS)

Multiple sclerosis is an autoimmune inflammatory disease of the central nervous system. Symptoms of MS include painful muscle spasms, tremor, paralysis, bladder control loss, and difficulty in speaking. Due to loss of myelin, there is impairment in conduction of impulse signals. MS is characterized by heightened T_h1-mediated immune response leading to increase in cytokines like tumor necrosis factor-α (THF-α), interferon-gamma (IFN-γ), interleukin-12, lymphotoxin-α, etc. Upregulation of pro-inflammatory cytokines causes neurological disabilities like MS. Thus, suppressing the pro-inflammatory cytokines might be the appropriate target for the treatment of MS. First-line treatment for spasticity includes gamma aminobutyric acid (GABA) B agonist like baclofen. They bind to the GABA receptors and cause membrane hyperpolarization. This as a result modulates calcium influx which further restricts the release of endogenous excitatory neurotransmitters. Cannabinoids target the same as it has been observed that the expression of CB1 receptors is high in cerebellum. Administration cannabinoid agonist WIN55,212-22 can increase the effect of GABA signaling and inhibit the release of glutamate (neurotransmitter) (Shen et al. 1996; Szabo et al. 2000; Garcia-Gil et al. 1999). Cannabinoids also reduce the expression of Th1 cytokines by suppressing IFN-γ and IL-12 (Klein et al. 1995, 1998, 2000). Some studies supported the beneficial effect of dronabinol and cannador (THC+ CBD) in improving the spasticity (Petro and Ellenberger 1981; Ungerleider et al. 1987; Vaney et al. 2004). Additionally, cannabinoids are found to be effective against pain in MS (Consroe et al. 1997). Another finding revealed that administration of Arvanil (capsaicin + anandamide) can inhibit both spasticity and pain in animal model of experimental autoimmune encephalomyelitis (EAE) (Brooks et al. 2002). In the study by Novotna et al., Sativex has been used as add-on therapy for patients who have failed to respond adequately to the medication. Results in this study have favored the role of Sativex as an effective anti-spasticity treatment (Novota et al. 2011). After this study in 2011 cannabis extract has received approval in Germany. Sativex has also secured FDA approval for its analgesic effects.

12.5.2.2 Huntington's Disease (HD)

Huntington's disease is an autosomal dominant neurodegenerative disease. HD is caused by mutation in htt encoding gene on chromosome number 4 leading to excessive repeats of CAG triplet (>39 repeats) (Roos 2010; Myers 2004).

Huntington's disease is characterized by choreatic movement, dementia, and progressive motor disturbance. Low expression of CB1 receptors has been demonstrated in patients diagnosed with HD (Blázquez et al. 2011). Therefore, activation of CB1 receptor can slow down the progression of this disease. It has also been shown that CB2 receptors are overexpressed in striatal parenchyma in diseased individuals of HD (Palazuelos et al. 2009). CB2 receptor activation can ameliorate the inflammation. Nabilone, a synthetic cannabinoid similar to Δ^9-THC (FDA approved), has also showed the sign of improvement in the study by Curtis and Rickards (2006) and Müller-Vahl et al. (1999). Along with THC, CBD is also investigated to have positive effects in HD. To summarize all the studies, cannabinoids may be useful strategies for HD treatment.

12.5.2.3 Alzheimer's Disease (AD)

Alzheimer's disease is a chronic progressive neurodegenerative disorder characterized by accumulation of neurotic plaques, which is rich in beta amyloid (aβ) peptides and hyperphosphorylation of tau protein. During the last few years, ECS has emerged as a potential therapeutic target to treat Alzheimer's disease. Several findings indicate the involvement of CB1 and CB2 receptors as there has been an increase in production of endocannabinoids after neuronal damage. Interestingly, three major observations after analysis of human post-mortem samples revealed (1) overexpression of CB2 receptors in microglial activation (Ramírez et al. 2005), (2) elevation in the 2-AG levels, and (3) reduction in CB1 receptors found to be associated with the neuronal loss (Benito et al. 2003). A cannabinoid agonist, WIN 55,212-2, and anandamide have been shown to prevent aβ-induced neurotoxicity mediated via the CB1 receptors (Ramírez et al. 2005). Similar results are observed in a study by Iuvone et al. on PC12 cells (Iuvone et al. 2004). It has also been observed that administration of HU210 and JWH-133 blocked the aβ-induced activation in cultured microglial cells. The studies conducted on mice also demonstrated that treatment with CBD decreases the level of ROS, prevents glutamate-induced toxicity, as well as inhibits the tau protein hyperphosphorylation (Esposito et al. 2006, 2007). Thus, the available evidences from various findings suggested that cannabinoids could be potential target in treatment for Alzheimer's disease due to its immunosuppressive, anti-inflammatory, and neuroprotective properties.

12.5.2.4 Epilepsy

Epilepsy is a neurological disorder caused by an imbalance between inhibitory and excitatory communication among neurons. The mechanism by which a normal brain becomes epileptic is quite diverse, ranging from (1) neural circuit level (abnormal synaptic connectivity) to (2) receptor level (abnormal GABA receptors) to (3) membrane level (abnormal ionic channel function). Cannabinoids protect the excitotoxicity of neuron by inhibiting the calcium channels and/or stimulating the potassium channel. Cannabis has been used in treatment of epilepsy for centuries. Researchers identified the role of THC and CBD as effective anticonvulsants in epilepsy treatment (Consroe and Wolkin 1977a, b; Gordon and Devinsky 2001).

CBD has been suggested as a beneficial drug for treating drug-resistant epilepsy in pediatric population (Cortesi and Fusar-Poli 2007). Study by Luszczki et al. demonstrated the synergistic effect of CB1 receptor agonist ACEA (arachidonyl-2′ chloroethylamide) on valproate by increasing its anticonvulsant action (Luszczki et al. 2006). Despite these hints from various studies, use of cannabinoids has not gained therapeutic status in the treatment of epilepsy (Kogan and Mechoulam 2007).

12.5.3 Respiratory Disorders

12.5.3.1 Asthma

Asthma is a chronic inflammatory lung disease, characterized by elevation of IgE and various cytokines (Interleukins 4, 5, 9, and 13) locally in the airway or systemically in blood. In this disease there is reversible constriction of the small bronchioles with excessive mucus production. Inhaled corticosteroids and bronchodilators represent the mainstay treatments for asthma (Global Initiative for Asthma 2020; Colodenco et al. 2018). Most patients with asthma, however, can overcome limitations in daily activities and impairments in overall quality of life by using appropriate pharmacological interventions. Still, there is a need for therapies that target IgE and cytokine production. Asthmatic patients have shown increased expression of CB1 receptor mRNA and elevated levels of AEA (Martin-Fontecha et al. 2014; Zoerner et al. 2011). The first evidence of cannabinoids as a therapeutic agent in airway was reported in the 1970s. The use of THC as a bronchodilator has been identified in a study by Vachon et al. (1973). THC attenuated allergic inflammation by decreasing the cytokine production, mucus secretion, and IgE levels in CB1- and CB2-independent manner (Braun et al. 2011; Jan et al. 2003). Additionally, it was investigated that CBD activates both CB1 and CB2 receptors. These receptors modulate the release of chemical messengers and inflammatory cytokines. Thus, cannabinoids may be beneficial agents for the treatment of asthma according to the available literature.

12.5.4 Cancer

12.5.4.1 Antiemetic

Patients undergoing chemotherapy experience acute nausea and vomiting. Emesis is a devastating side effect of chemotherapeutic drugs. It occurs due to the production of serotonin (5-hydroxytryptaminergic; 5-HT) through the enterochromaffin cells, which are found to be distributed across the GI tract. Standard treatment of chemotherapy-induced nausea and vomiting (CINV) includes 5-HT_3 receptor antagonists and neurokinin 1 (NK_1) receptor antagonists. Cannabinoid system interacts with 5-HT system to control CINV. Use of cannabinoids cause inhibition of nausea and emesis related to chemotherapy or radiotherapy. CB1 receptors are localized in this region of the brain (Cannabinoids 2003). Thus, administration of CB1 agonist can reduce the release of 5 HT (Hu et al. 2007). The US FDA approved

dronabinol and nabilone (1985) for their use to control nausea in cancer patients undergoing chemotherapy (Walsh et al. 2003). Cannabinoids are antiemetics that act via activation of CB1 receptors in both CNS and ENS and can improve the quality of life for cancer patients with CINV.

12.5.4.2 Appetite Stimulation

Majority of cancer patients experience loss of appetite and weight loss. The reason behind this is accumulation of pro-inflammatory cytokines and neuro-hormonal alterations. Appetite is regulated by hypothalamic region of the brain through production of peptides (orexigenic and anorectic peptides). Studies have provided the better understanding regarding the involvement of endocannabinoid system that might modulate the food uptake via CB1 receptors, which are present in the hypothalamus. It has been reported that cannabinoids can stimulate appetite in animal models. The role of THC as appetite stimulant was demonstrated in a study by Vincent et al. (1983). Dronabinol is an FDA-approved drug for treatment of appetite and weight loss in patients with HIV/AIDS.

12.5.4.3 Inhibition of Pain

Almost all the cancer patients deal with chronic pain varying from moderate to severe. This pain originates from the inflammatory response, occurring at the site of tumor which leads to the induction of nociceptive ligands (Cannabinoids 2003). Then these nociceptive ligands interact with nociceptors (receptors) due to which release of neurotransmitters occurs and thus pain arises. Administration of cannabinoids can suppress pain by inhibiting nociceptive neurotransmission via CB1 receptor activation (Starowicz et al. 2013). In European countries, Sativex is prescribed by physicians for its analgesic affects (Bifulco and Pisanti 2015). Pain has negative impact on the quality of life of cancer patients, which could be improved by cannabinoids.

12.5.4.4 Psychological Changes

Cannabinoids at low dose can produce anti-anxiety effects. They can contribute in improving patient's quality of life by uplifting the mood. THC and nabilone help in reducing depression and anxiety and improving sleep (Cannabinoids 2003).

The investigations on cannabinoid receptors are primarily focused toward their use in the treatment of chemotherapy-related adverse effects. However, there are many preclinical studies that are focused on anticancer role of cannabinoids in different cancers. It becomes a pre-requisite to understand the mechanism of action of ECS modulators with respect to a specific pathological condition.

12.5.5 Evidences Supporting Cannabinoid System Modulators as Effective Anticancer Agents (In Vitro and In Vivo Studies)

12.5.5.1 Prostate Cancer

Prostate cancer is one of the most common malignancies in men and also the second leading cause of cancer-related death. Various studies had been conducted focusing on the role of cannabinoid receptors in prostate cancer in vitro. Effect of WIN 55,212-2 has been described in various studies of prostate cancer. WIN 55,212-2 causes cell mortality, decreases the expression of proapoptotic protein, and upregulates anti-apoptotic. LNCaP (prostate cancer) cell line has high expression of CB1 and CB2 receptors, when subjected to WIN 55,212-2 resulting in a decrease of cell viability. Decrease in the expression of Bcl-2 protein has also been observed after the treatment with this agonist. Additionally, 24 hours post-treatment upregulation of Bax protein expression has been found in these cells. Therefore, the ratio of Bax to Bcl-2 sighted in this study deduced that apoptosis has been favored by the activation of caspase 9 and 3 with concomitant cleavage of PARP (poly ADP-ribose polymerase). This study also indicates that cannabinoid receptor agonist (WIN 55,212-2) induces activation of ERK1/2, which leads to induction of cyclin kinase inhibitor p27/KIP1. Sequentially, it inhibits the cell cycle regulatory molecules (cyclin D1, D2, E) and results in cell cycle arrest at G_1 phase and causes apoptosis (Sarfaraz et al. 2006).

The effect of WIN 55,212-2 is also demonstrated in an in vivo investigation by Roberto et al. on PC3 xenograft model. Results in this study revealed that intraperitoneal administration of WIN 55,212-2 thrice weekly over a 3-week period significantly reduces the tumor growth rate and no significant associated toxicities were found (Roberto et al. 2019).

In another study, the androgen-sensitive prostate cancer cell CWR22R ν1 xenograft model has demonstrated the similar results where the intraperitoneal administration of WIN 55,212-2 significantly suppressed the tumor growth along with reduction in PSA secretions in the serum (Sarfaraz et al. 2007).

All these studies delineate the role of WIN 55,212-2 as an anticancer agent which hinders the growth, migration, and invasion of prostate cancer cells, in addition to cell cycle arrest in G_0/G_1 phase and triggered apoptosis pathways.

12.5.5.2 Bladder Cancer

Bladder cancer is the ninth most common cancer in the world. Approximately 90% tumors in urinary bladder are of epithelial origin. Bladder carcinomas comprise of two different categories: (a) superficial non-muscle invasive papillary lesions, which are indolent lesion, and (b) muscle invasive bladder cancer (MIBC), which is an aggressive cancer with much poorer prognosis. Available literature on bladder cancer study supported the specific use of CB2 receptors as antitumor targets. Activation of CB2 receptor has been demonstrated to produce anticancer effect by inactivation of two mechanisms mTORC1 and FAK-Src pathways. During mTOR

inactivation, ceramide, a proapoptotic lipid, acts as a second messenger for cannabinoids. Thus, JWH015 (CB2 agonist) induces cell apoptosis through the stimulation of de novo synthesis of ceramide and inhibits the pro-survival signal Akt. This study has also suggested the impact of CB2 receptor activation in bladder cancer cell migration and aggressiveness via the inactivation of the FAK-Src pathway (Bettiga et al. 2017). Hence, this finding establishes the role of cannabinoid as anti-metastatic and antiproliferative agent.

12.5.5.3 Pancreatic Cancer

Pancreatic cancer is one of the leading causes of cancer death and is categorized among the most aggressive cancer. The use of cannabinoids was earlier restricted to palliative care for cancer patients. Thus, researchers started exploring anticancer effects of cannabinoids in various cancer types. Cannabinoid receptors are found to be upregulated in pancreatic cancer. Studies showed that that activation of CB2 receptors and inactivation of CB1 receptors may induce cell apoptosis. MiaPaCa-2 (pancreatic cancer cell line) manifested substantial cell death after treatment with CB receptor synthetic agonist (WIN-55,212-2 and JWH-015) (Fogli et al. 2006). Additionally, it has been demonstrated in a study that the inverse agonist of CB1 receptor (AM251) induced apoptosis in MiaPaCa2 cells and also affected transcriptional genes via the JAK/STAT and MAPK signaling (through CB1 receptor-independent pathways) (Fogli et al. 2006).

The study by Michalski et al. revealed the inverse correlation between CB1 receptor and patient survival. During this investigation, the cannabinoid receptors were evaluated along with the endocannabinoid metabolizing enzymes; fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MGLL) in both normal and pancreatic cancer cell from patients. This study showed the correlation between high FAAH/MGLL levels and survival of cancer patient (Michalski et al. 2008).

Additional studies on other pancreatic cancer cell line Panc1 cells supported the evidence of cannabinoids as antitumor agents. Treatment of cells with CB2 agonist, GW405,833 hydrochloride (GW), has been demonstrated to inhibit the proliferation and invasion. GW activates AMPK and autophagy in pancreatic adenocarcinoma cells by increasing AMP/ATP ratio. Autophagy induction evaluated to be related to inhibition of energy metabolism which is dependent on ROS production (Dando et al. 2013). Treatment of Panc1 cells with THC led to caspase 3 activation. This study explored the role of THC on the xenografted MiaPaca2 pancreatic mice model. Inhibitory effect of cannabinoids was established in the pancreatic tumor growth post-treatment. Cannabinoid agonist WIN 55-212,2 also decreases the cell growth in pancreatic cancer cells through possibly the activation of TRB3, a proapoptotic protein (downstream protein of p8 and ATF-4) responsible for the apoptosis induced by ER stress (Carracedo et al. 2006). Thus, induction of apoptosis by CB2 agonist is hypothesized to slow the progression of pancreatic cancer in patients.

12.5.5.4 Glioma

Glioma represents 40% of all brain tumors with less survival rate. Many studies have demonstrated the potential of cannabinoids as antiproliferative agents in vitro and in vivo.

Glioma cell lines SF126, U87-MG, U251, SF188, and U373-MG after administration of THC and WIN 55,212-2 decrease the survival of these cells (McAllister et al. 2005). Treatment with synthetic cannabinoid WIN 55,212-2 causes downregulation of Akt and Erk1/2 kinase signaling, inhibits proliferation, and induces apoptosis. A decrease of mitogenic/pro-survival signaling precedes reduction of phosphorylation of the proapoptotic protein BAD which then translocates to the mitochondrial membrane initiating apoptosis.

In vivo study on U87 and U373 cell lines induced animal models after treatment with CBD exerts antiproliferative effects (Massi et al. 2004). Another study that supported the role of cannabinoids in apoptosis was conducted by Sanchez et al. In this study JWH-133 initiates apoptosis via ceramide synthesis de novo and ERK activation (Sánchez et al. 2001).

Therefore, it can be concluded from the available literature that cannabinoids demonstrate potent antiproliferative activity in glioma and which can be evaluated further in other solid cancers as well.

12.5.5.5 Lung Cancer

Non-small cell lung cancer (NSCLC) is considered as one of the most aggressive solid tumor types. Patients with lung cancer still have an unfavorable prognosis with $<$15% 5-year survival rate. Many recent studies have showed that cannabinoids can act as an effective anticancer agent against NSCLC. In established NSCLC cell lines H358, A549, and H460, cannabinoid (CBD) exerts anti-invasive property through CB1 and CB2 receptors and TRPV1 (transient receptor potential cation channel subfamily member 1/vanilloid) receptor. Studies have also elucidated the role of TIMP-1 (tissue inhibitor of metalloproteinases)-dependent apoptotic death of NSCLC cells which is mediated through CB1 and CB2 receptors and TRPV1-dependent ICAM-1 (Intercellular Adhesion Molecule 1) (Ramer et al. 2012).

Another study showed that the downregulation of CB2 receptors in A549 and H1299 (lung cancer cell lines) decreased the proliferation, invasion, and migration and also induced apoptotic cell death. Collectively, these reports have suggested pro-oncogenic role of CB2 receptors in the progression of NSCLC by regulation of Bcl-2/Bax axis and caspase cascade activation. THC inactivated the phosphorylation of ERK1/2, AKT, and JNK1/2. CBD also increased susceptibility toward lymphokine-activated killer (LAK) cell-mediated cancer cell lysis (Preet et al. 2008; Haustein et al. 2014).

Further in the same study, when xenograft model of metastatic lung cancer was treated with CBD, the authors observed significant decrease in the lung cancer nodules. Interestingly, effect of CBD was found to be completely reversed by a neutralizing antibody to ICAM-1. These observations were enough to suggest that

cannabinoid-mediated apoptotic cell death was mediated at least partly by ICAM-1 (Haustein et al. 2014).

All the abovementioned studies favor the role of cannabinoids in inhibiting tumor growth via activation of apoptotic pathways. In summary, cannabinoid agents may be considered as potential anti-metastatic drugs.

12.5.5.6 Renal Cell Carcinoma (RCC)

Renal cell carcinoma accounts for 90% of all renal malignancies. RCC treatment is less effective due to lack of response to conventional therapies. This study shed light on the use of cannabinoids in treating cancer. Inhibition of proliferation in RCC is mediated via CB2 receptors. The study conducted on RCC cell lines 786-O (primary) and ACHN (metastatic) revealed distinct role of cannabinoid receptors in amelioration of cancer cell progression. In this study, treatment of RCC cell lines with a nonselective CB1/CB2 receptor agonist (WIN 55,212-2) inhibited cell proliferation significantly. WIN 55,212-2 induced cancer cell death by arresting the cell cycle in G_0/G_1 phase. In the same study, no cell death was observed when normal epithelial cells such as ASE-5063 were exposed to cannabinoid agonists (Khan et al. 2018). This further confirmed that the antiproliferative role of cannabinoids is confined only to the cancer cells.

12.5.5.7 Gastric Cancer

Effect of cannabinoids has also been observed in gastric cancer. It is suggested that both the receptors are involved in the inhibition of invasion and metastasis in cancer cells. In in vitro study on AGS and MKN-1 (gastric cancer cell lines), administration of WIN 55,212-2 demonstrated the decrease in the expression of MMP-2 and VEGF-A (Xian et al. 2010). In the study by Oh et al., administration of WIN 55,212-2 (cannabinoid agonist) indicated the antineoplastic effect in an in vivo model of gastric cancer. Animal model after treatment with WIN 55,212-2- demonstrated the increase in apoptosis. Downregulation in the expression of MMPs was observed by the treatment of the cannabinoid agonist. To be specific this study showed that WIN 55,212-2 downregulated the expression of MMP-2, MMP-7, and MMP-9, which suggests inhibition in metastasis in gastric cancer (Oh et al. 2013). Evidences suggested that cannabinoids, in addition to palliative care in oncology, can inhibit proliferation, angiogenesis, and metastasis.

12.5.6 Signaling Pathways Associated With Cannabinoid Receptor Activation (Fig. 12.3)

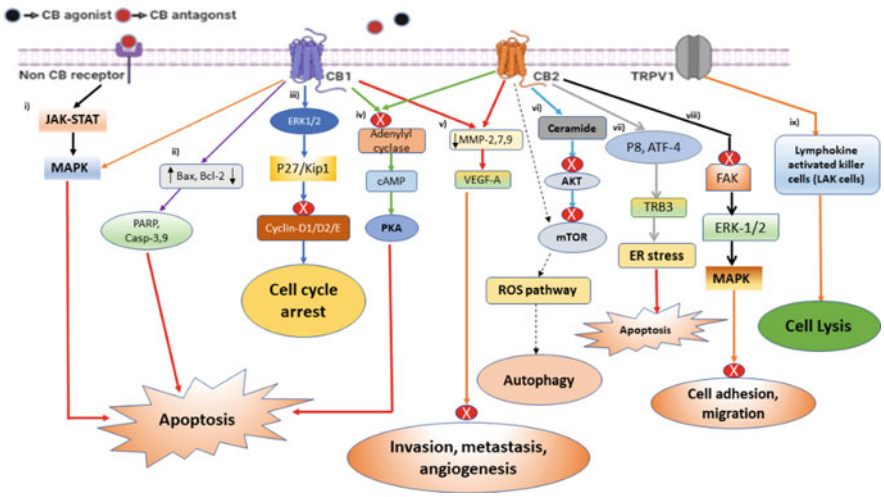

Fig. 12.3 Schematic representation of cannabinoid receptor-mediated downstream signaling cascade in various cancers: (i) non-cannabinoid receptors after interaction with cannabinoid ligands activate the JAK/STAT pathway. (ii) Apoptosis is induced by activation of CB1 receptor and increase in the ratio of Bax/bcl-2 occurs, which in response upregulates the expression of PARP and caspases 3 and 9. (iii) CB1 receptors act through Gi/o protein and activate ERK1/2 which in response activate p27/kip1 protein and bring induced cell cycle arrest via inactivation of cyclins. (iv) CB1 and CB2 receptors interact with Gi/o protein which inhibits adenylyl cyclase and subsequently elicits the cAMP accumulation, which ultimately leads to apoptosis. (v) Downregulation of MMP-2, MMP-7, MMP-9, and VEGF-A leads to inhibition of angiogenesis, metastasis, and invasion of cells. (vi) CB2 receptors stimulate the release of ceramide (second messenger) which inhibits AKT signaling following mTOR pathway causing programmed cell death. (vii) Additionally, proapoptotic protein TRB3 is activated which in response induces ER stress in a cell and ultimately causes apoptosis. (viii) CB2 receptor also leads to inhibition of FAK-src pathway which eventually induces apoptosis. (ix) Apart from cannabinoid receptors, non-CB receptors are involved in cell lysis via activation of lymphokine-activated killer

12.5.7 Clinical Trials

Preclinical models proposed the anticancer role of cannabinoids, notably by inhibiting cell proliferation, angiogenesis, metastasis, and invasiveness. Several cell signaling pathways that are involved in the antitumor activity of cannabinoids implicated the connection between ESC system and cancer. There are eight ongoing/completed clinical trials representing the antitumor effect of cannabinoids in various cancer types. Apart from their antitumor activities, these trials also aimed to assess

Table 12.2 List of clinical trials investigating the role of cannabinoid receptors in various cancers

S. no.	Title	Trial number	Status
1	Study on cannabinoid receptor expression in gastrointestinal disease (ClinicalTrials.gov n.d.-g)	NCT02735941	Completed
2	A study of dexanabinol in combination with chemotherapy in patients with advanced tumors (ClinicalTrials.gov n.d.-b)	NCT02423239	Unknown
3	A study: Pure CBD as single agent for solid tumor (ClinicalTrials.gov n.d.-c)	NCT02255292	Unknown
4	Efficacy and safety of dronabinol in the improvement of chemotherapy-induced and tumor-related symptoms in advanced pancreatic cancer (ClinicalTrials.gov n.d.-e)	NCT03984214	Recruiting
5	Phase 1 study of dexanabinol in patients with advanced solid tumors (ClinicalTrials.gov n.d.-a)	NCT01489826	Completed
6	TN-TC11G (THC + CBD) combination with temozolomide and radiotherapy in patients with newly diagnosed glioblastoma (ClinicalTrials.gov n.d.-h)	NCT03529448	Not yet recruiting
7	Assessment of single doses of oral dexanabinol in healthy subjects (ClinicalTrials.gov n.d.-d)	NCT02054754	Completed
8	Phase 1 study to evaluate safety and usefulness of an Ayurvedic cannabis preparation in the perioperative period in breast and oral cavity squamous cell cancer (ClinicalTrials.gov n.d.-f)	CTRI/2020/06/026049	Not recruiting

the safety in administration of cannabinoids/cannabinoid-based preparations on cancer patients (Table 12.2).

12.5.8 Endocannabinoid System, Chemoresistance, and Cancer

Resistance toward chemotherapy is one major limitation in the treatment outcomes and is a challenge for clinicians in cancer treatment. There are multiple mechanisms of chemotherapy resistance in cancer which include increased activity of efflux pumps (such as adenosine-triphosphate dependent transporters) or reduced drug influx, disruptions in apoptotic signaling pathways, etc. Sensitivity of tumor cells is altered due to mutations of *ABCB1*, *ABCC1*, *ABCC2*, *ABCC3*, and ABCG2 (Mansoori et al. 2017). Furthermore, multidrug resistance (MDR) proteins play pivotal role in chemotherapy resistance in cancer and cause major obstacles in cancer treatment. For instance, in breast cancer, multidrug resistance protein 1 (MDR1) and breast cancer resistance protein (ABCP OR ABCG2) are the efflux pumps which are responsible for the resistance against established chemotherapeutic regimens like paclitaxel and docetaxel (An et al. 2017; Chen et al. 2010). However, in some cases, autophagy-mediated pathways have been observed as additional mechanism of acquired resistance toward established chemotherapeutic drugs (Pan

et al. 2016). Thus, this section focuses on the role of cannabinoids in evading chemoresistance in cancer patients.

12.5.8.1 Cannabinoid Modulators Against Chemotherapy Resistance in Cancer

In addition to antiproliferative action of endocannabinoid system, studies have also showed that cannabinoid modulators are equally effective against resistant cancer cells. The role of THC and CBD has been seen in modulating the expression of MDR1 in leukemia cells (CEM/VLB100). THC acts through CB1 receptor, while CBD targets both the receptors and through TRPV-1 channels (Arnold et al. 2012). Another in vitro study by Holland et al. on leukemic cells showed that cannabinoid treatment reduces the expression of MDR1 in these cells. It is also illustrated in the studies that efflux activity does not get altered during this process (Holland et al. 2007, 2008).

Additionally, in glioma cells, THC and CBD synergize the cytotoxicity of vinblastine and temozolomide (Holland et al. 2006). This finding was also replicated in vivo (T98G glioma xenograft) when cannabinoids was co-administrated with temozolomide (TMZ). It was hypothesized that cannabinoids when administered in combination help in overcoming resistance by stimulating autophagy and thereby promoting cell death (Torres et al. 2011). Likewise, apoptosis-inducing potential of cannabinoid agonists was observed to be significantly enhanced by pharmacological inhibition of well-known cancer-causing oncogenes like EGFR, ERK79, or AKT in glioma cells.

Xian et al. found out in an in vitro study that treatment of 5-fluorouracil-resistant gastric cancer cells (SNU-620-5FU/1000) by cannabinoid agonist (WIN 55,212-2) can induce cytotoxicity. Furthermore, upregulation of cleaved caspase-3 and cleaved PARP and downmodulation of phospho-ERK1/2, phospho-AKT, BCL2, and BAX are some observations from this study that are adding to the anticancer effect of cannabinoids in resistant cancers too (Xian et al. 2013).

Cannabinoid agonists CBD and O-1602 were studied extensively in the amelioration of chemotherapy resistance in paclitaxel-resistant breast cancer cell lines and also in zebrafish xenograft model. In these models, single-agent treatment with paclitaxel was not enough in killing the chemo-resistant cancer cells. However, combination treatment with cannabinoids and paclitaxel was found to be effective in inducing cell death. Cannabinoids are shown to reduce the viability and metastasis in MDA-MB-231 cells via apoptosis possibly which is mediated through ROS activation. Additionally, other GPCRs have also been found to play significant role in tumor progression and metastasis. In that context, GPR55 contributed in inducing apoptosis in cells that were unresponsive toward paclitaxel (Tomko et al. 2019).

Anandamide induces a non-apoptotic form of cell death in bax−/− apoptosis resistance colorectal tumor cells (HCT116). It eliminates the resistant cells via CB receptor-independent manner. It is observed that colorectal cancer cells have increased expression of COX-2. Anandamide promotes cell death in apoptotic defective cells that express COX-2 (Patsos et al. 2010). Furthermore, COX-2 is

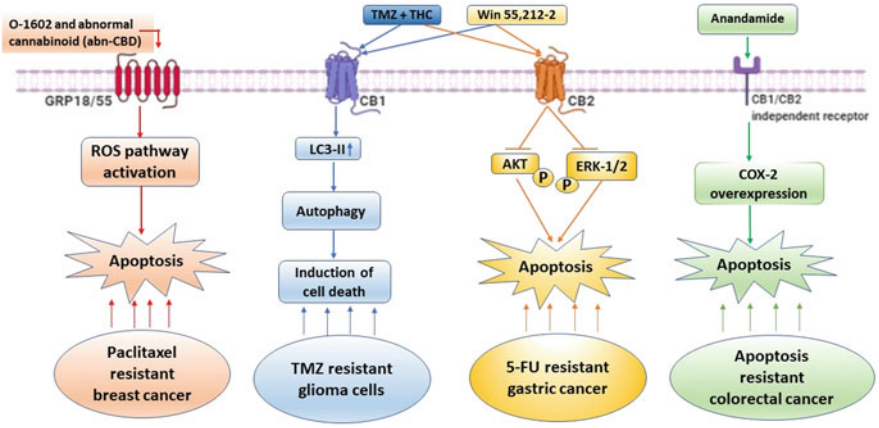

Fig. 12.4 Cannabinoid-mediated inhibition of chemotherapy resistance in different cancers

also overexpressed in a number of other tumor types including prostate, breast, and oral cancer; anandamide might also be effective in other cancers.

All these findings suggest that cannabinoids can increase the sensitivity of cancer cells to chemotherapy. Studies on glioma, leukemia, and gastric, breast, and colorectal cancer provide supporting evidences in favor of cannabinoids and their benefits in overcoming resistance. Additionally, co-administration of cannabinoid-based drugs could be a great strategy in order to enhance the sensitivity of cancer cells toward anticancer drugs. These results demonstrated that there is an association between cannabinoids and resistance toward anticancer drugs which are required to be explored more (Fig. 12.4).

12.6 Concluding Remarks

Considering the multifaceted action of cannabinoid receptors in different pathophysiological conditions, it is not surprising that endocannabinoid signaling plays an important role in cancer-causing pathways. In this chapter, we have tried to summarized the role of ECS in various cancers. Recent literature implicated immense potential of cannabinoid system modulators in many diseases. In cancer, cannabinoid receptor ligands may emerge as a prospective clinical regimen having already proven efficacy in the inhibition of cancer cell proliferation. Interestingly, cannabinoids have the potential to be an effective therapeutic in chemotherapy-resistant cancers. Therefore, these agents may serve as an excellent add-on therapy for the treatment of drug-resistant cancers. In view of these points, necessity of alternative drug might be fulfilled by cannabinoids simultaneously overcoming limitations of conventional therapies.

Currently, many pharmaceutical companies are favoring more extensive research on cannabinoids and related pathways by developing novel potent synthetic

cannabinoid analogs. Further investigations on endocannabinoid system as potential anticancer regimen is the need of the hour which may transform the area of anticancer therapeutic drug development.

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Nanodrugs: A Futuristic Approach for Treating Nephrolithiasis

13

Gupta Shruti and Kanwar Shamsher Singh

Abstract

Kidney stones or nephrolithiasis is a frequent condition resulting from a succession of several physiochemical events causing a huge burden on the health and economic status globally. Although they vary widely in their composition, calcium oxalate stones form the predominant kidney stones. The degradation of calcium oxalate stones using enzymes such as oxalate decarboxylase and oxalate oxidase has been a potential therapeutic approach for treatment of nephrolithiasis; however due to their reduced half-life under in vivo conditions as well as difficulty in delivery of these drugs to target site, their use as a therapeutic for nephrolithiasis is limited. In the recent years, the potential impact of nanoparticles in the diagnosis and treatment of nephrolithiasis has been widely recognized. One of the most important uses of nanomedicines is in the delivery and controlled release of drugs. Several nanoparticles such as those made up of metals or metal oxides besides lipid and polymer encapsulated nanoparticles have been reported lately for targeting kidney stones. The present article discusses the current challenges and futuristic approaches for the development of nanoparticles of oxalate-degrading enzymes for treating nephrolithiasis, and it highlights the recent developments in this area.

Keywords

Nanomedicine · Nephrolithiasis · Enzyme therapeutics · Calcium oxalate stones

G. Shruti · K. S. Singh (✉)
Department of Biotechnology, Himachal Pradesh University, Shimla, India
e-mail: kanwarss2000@yahoo.com

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13.1 Introduction

Nanotechnology is an innovative approach with potential utilization in drug delivery system. Using nanotechnology to produce nanodrugs is a revolutionary strategy that amends the delivery of compounds for improved medical diagnosis and curing diseases (Maurya et al. 2019). Nanodrugs improve the therapeutic index of the drugs by improving their efficacy through targeted delivery and triggered release. The in vivo fate of the drug is ameliorated as efficient delivery to the target site that is achieved with low accumulation at other sites in the body. The nanodrugs also have the ability to stimulate cellular uptake and improve intracellular trafficking (Hua et al. 2018).

Kidneys perform several vital functions such as maintaining homeostasis, urine formation, etc., and any impairment in its usual operation would lead to several issues like hypertension, inflammation, urinary tract infections, renal calculi and various others. Therefore, drug targeting to kidneys has remained a major area of research (Kandav et al. 2019). Use of nanomedicines has comprehensive role in treating renal diseases. Today it has become possible to synthesize nanomedicines in terms of kidney retention and those that can bind to key membranes and cell populations associated with renal diseases. Evolution with respect to three-level fabrication of new nanomaterial, coating and discovery of novel drug delivery agents for biodistribution of therapeutic molecules deep into the kidney tissues has been observed through the past several years (Upadhyay 2017). Nephrolithiasis or kidney stones is a renal disease whose increasing prevalence has received considerable attention from researchers all over the world. It is mainly related to elevated oxalate levels in the blood and urine which lead to accumulation of calculi in the renal pelvis, ureters and collecting duct, eventually resulting in renal pathological problems (Kandav et al. 2019). Current treatment options in patients with nephrolithiasis are limited and do not always lead to effective cure of renal stones. Hence rational medical management with new therapeutic strategies aiming to prevent kidney stone formation has become the need of the hour. Since a decade, attempts have been made to use plants and oxalate-degrading microbial enzymes to solubilize oxalate kidney stones and some success has been achieved in the same. In particular, this article highlights how oxalate-degrading enzymes could be developed in the form of nanodrugs for the treatment and management of kidney stones. It also discusses the various challenges in the development of such nanodrugs as well as reviews the current research that has been conducted in this aspect.

13.2 Nephrolithiasis and Its Management

Nephrolithiasis, i.e., kidney stone formation, is a common and painful disorder, the prevalence and frequency of which are surging at an astonishing rate (Gupta and Kanwar 2020a). It is a multifactorial process evolving from a chain of physiochemical reactions such as super-saturation, nucleation, growth aggregation and retention inside the renal tubes (Das et al. 2017). Calcium oxalate forms the chief

type of urinary calculi constituting about 70–80% of the urinary or kidney stones (Lin et al. 2017). Increased urinary super-saturation, predominantly of calcium phosphate and calcium oxalate is responsible for the enhanced accumulation of these salts in the renal tubules which ultimately lead to formation of kidney stones. Apart from the formation of stones in the kidney, oxalate crystals can destruct epithelium in the oral cavity and gastrointestinal tract, causing inflammation, diarrhea and gastric hemorrhage which indirectly becomes a cause of death (Gupta and Kanwar 2020b). Although the introduction of modern techniques such as nephrectomy, extracorporeal shock wave lithotripsy (ESWL) and drug treatment has provided significant thrust toward the treatment of nephrolithiasis, but these treatment options possess severe side effects such as traumatic effects, leading to acute renal injury, hypertension, hemorrhage, a decrease in renal function and increased chances of infection (Das et al. 2017). To effectively treat hyperoxaluria, oxalate accumulation needs to be reversed, potentially by the systemic clearance of oxalate (Zhao et al. 2017). Although the use of phytomolecules in the treatment and management of kidney stones has been proposed by several in vivo and in vitro studies and clinical trials, interestingly, this has emerged as a novel option, but much research is still needed to effectively curb the disease (Gupta and Kanwar 2018).

Enzymatic dissolution of oxalate stones may provide a potential therapeutic alternative, and much work has been done in this sphere. Till date three major oxalate-degrading enzymes have been reported, namely, oxalate decarboxylase (ODC, oxalate carboxylyase, EC 4.1.1.2), oxalate oxidase (OXO, oxalate:oxygen oxidoreductase, EC 1.2.3.4) and oxalyl-CoA decarboxylase (oxalyl-CoA carboxylyase, EC 4.1.1.8) (Gupta and Kanwar 2020b). Oxalate decarboxylase, specifically acting on oxalate, degrades it into most soluble products, CO_2 and formate (Pierzynowska et al. 2017). The enzyme is a homogenous polymerase with manganese ion and belongs to the cupin protein superfamily (Dunwell and Purvis 2004). Since its discovery the enzyme has been found in a number of fungal and bacterial species and can be an extremely assuring therapeutic for the clearance of oxalate and hence the treatment of hyperoxaluria and nephrolithiasis. Although these enzymes possess strong therapeutic abilities against nephrolithiasis, with respect to kidneys, most therapeutic molecules exhibit poor pharmacokinetics, and their persistence in the kidneys is too brief to display a therapeutic effect (Williams et al. 2016). This limitation can be overcome by development of enzyme nanoparticles (NPs).

13.3 Nanodrugs for Nephrolithiasis

Nanoparticles (NPs) are nanosized colloidal particles, in which a drug is encapsulated, entrapped or attached to the NP matrix (Maurya et al. 2019). Along with the speculations that NPs may prevent the unenviable side effects and surpass various physiological and physical barriers usually experienced during systemic drug administration, these are becoming critically significant as an applicable tool

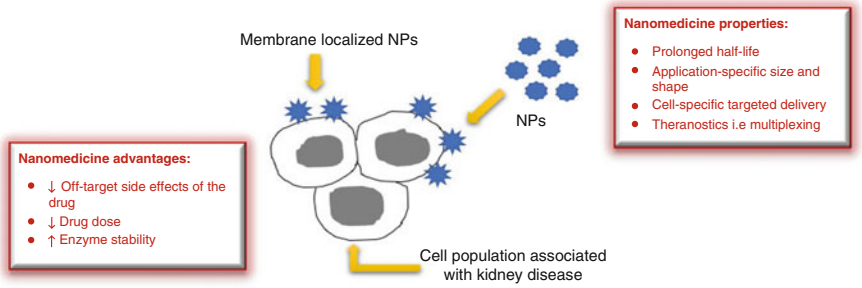

Fig. 13.1 Advantages of NP-associated anti-nephrolithiasis drugs

(s) in medicine, serving as effective diagnostic and therapeutic agents (Lee et al. 2015).

NPs can be made to shield the degradative effects before the drug reaches the target site and escape the body's defense system when coated with biocompatible and biodegradable polymers (Kandav et al. 2019). In drug delivery, the most significant potential of nanomedicine is its ability to control drug pharmacokinetics (Duncan and Gaspar 2011). This is a major advantage especially with respect to kidneys where most therapeutic small molecules exhibit poor pharmacokinetics as their persistence in the kidneys is for a very small period to provide any therapeutic effect. It is now possible to amplify the potency, acceptability and localization of nanomedicines by molding them in terms of kidney retention and binding to key membranes and cell populations associated with renal diseases (Kamaly et al. 2016). When administered conventionally, the nanomedicines are targeted to specific tissues, cells and organs in the body which would prevent the off-target side effects of the drug (Fig. 13.1). Specific targeting of the drug also reduces the amount of the dose to be administered as most of the drug is available at the disease site rather than at healthy organs (Wang et al. 2012). Numerous chemistries, materials and fabrication methods can be employed to design and produce NPs with optimal functions and characteristics such as application-specific NP size and shape, prolonged half-lives in circulation, targeting to specific cell types and multiplexing of functions (i.e., theranostics; Lee et al. 2012).

Several researchers all over the globe have reported that immobilization of enzymes on nanomaterials could considerably improve the enzymatic properties such as their stability, reusability and most importantly their targeting/localization to specific cell and tissues (Verma et al. 2020). Over the past few decades, ample amount of nanomaterials has been examined for their therapeutic potential. Technical approaches that combine different functionalities bring together liposomes, dendrimers, polymer-drug conjugates, and other NPs (Fig. 13.2) into the province of nanotechnology as opposed to conventional pharmacology and have been formulated. There are prominent varieties of organic and inorganic nanocarriers that can be employed in the development of nanodrugs for kidney stones (Table 13.1).

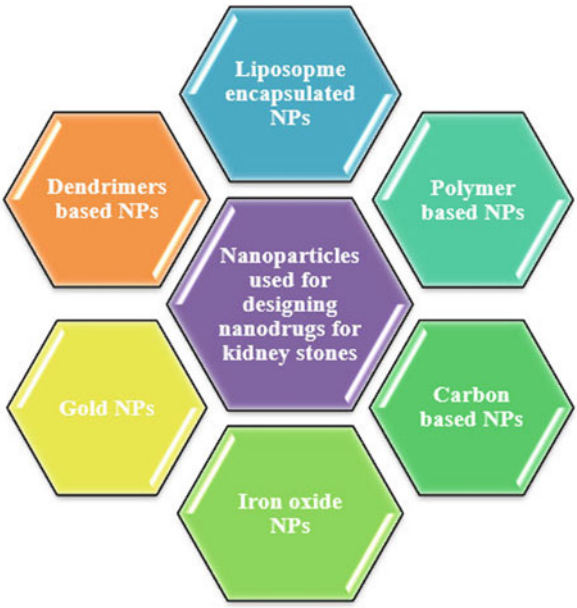

Fig. 13.2 Nanoparticles used to design nanodrugs for treating kidney stones

13.4 Challenges in the Development of Nanodrugs for Nephrolithiasis

The adult human kidney contains several nephrons which consist of two filtration units, the glomerulus filtration and hairpin-shaped tubule composed of a proximal tubule, the loop of Henle, a distal tubule and collecting duct (Kamaly et al. 2016). Under normal conditions, the glomerulus barrier filters blood on the basis of size and charge and ensures that only water and small solutes (urea, glucose, amino acids, and mineral ions) pass into the urine from the plasma and all the high molecular-weight plasma components such as erythrocytes and negatively charged components like albumin are retained in the blood (Eaton and Pooler 2013). In order to be delivered in the kidneys, NPs must be tiny enough to cross the glomerulus filtration barrier. Several organizational attributes of the glomerulus filtration unit such as the enlarged endothelial gaps can be utilized for transportation of NPs into kidney cells and various components. The size of the NPs remains among the major challenges while designing nanomedicines for kidney stones. Usually most of the NPs fall in the range of 30–150 nm and are not liable for filtration through the kidneys into the urine unless degraded into a size less than 10 nm. Colloids and particles in the range of 5–7 nm of hydrodynamic diameter can pass through the glomerulus and can be excreted, i.e., they fall below the kidney filtration threshold in healthy adults (Rahman et al. 2017). For inorganic NPs, a deviation from this range to 12–16 nm has been observed. This proposes an individual investigation of the renal clearance

Table 13.1 Diverse types of NPs used in the development of nanodrugs for treating kidney stones

Type	Composition	Properties	References
Organic NPs			
PVP NPs	Polyvinylpyrrolidone (PVP), poly[*N*-(2-hydroxypropyl) methacrylamide] (pHPMA), copolymers with PVP or pHPMA as major components	Water and organic solvent soluble, excellent biocompatibility and complexation capabilities Due to their innate retention in the kidneys and low level of accumulation in other organs, PVP NPs and their anionic derivatives might prove excellent drug carriers for kidney stones.	Lee et al. (2015)
Liposomal NPs	Spherical lipid bilayer with an aqueous core	Provides an effective route for delivery of enzymatic drugs for treatment of hyperoxaluria Enhances enzymatic activity and increases its stability, reduces phagocytosis leading to prolonged circulation half-life, and reduces immunogenicity.	Kamaly et al. (2016) Zhao et al. (2017)
Dendrimer-based NPs	Symmetrically branched polymeric macromolecules synthesized using polyamides with a size <10 nm	Globular macromolecules with three distinct domains, i.e., a central core, a hyperbranched mantle, and a corona with a peripheral reactive functional group. Possess uniform size distribution, solubility in water, multivalency, high drug/gene-loading ability, predictable release profile and favorable pharmacokinetics, which makes them favorable for use in drug delivery.	Lee et al. (2015) Longmire et al. (2014) Kamaly et al. (2016)
Inorganic NPs			
Gold NPs	Spherical-, rod-, or shell-based colloids made from the reduction of $HAuCl_4$	Display a very short acting time and reduced risk of adverse side effects.	Kamaly et al. (2016)
Iron oxide NP	Crystalline NPs of Fe_3O_4/γ-Fe_2O_3 Superparamagnetic iron oxide stabilized with dextran-induced iron oxide NPs	Exhibit theranostic potential due to a high surface to volume ratio and surface stemmed chemical residues that allow for chemical drug loading. Also possess intrinsic superparamagnetic properties that permit magnetic targeting. Display no toxic effect on renal and liver functions.	Kamaly et al. (2016) Upadhyay (2017) Mulens-Arias et al. (2020)

(continued)

Table 13.1 (continued)

Type	Composition	Properties	References
Carbon-based NPs	Polycyclic aromatic hydrocarbon	Targeted or non-targeted NPs, mainly used in the form of nanotubes, nanospheres, or nanosheets for drug delivery as well as diagnosis. Both single- and multi-walled carbon nanotubes induce platelet aggregation and hence display some degree of toxicity. Need further chemical modification to improve its targeting abilities and solubility in water and hence reduce toxicity.	Lee et al. (2015) Kamaly et al. (2016)

of each small-sized particle as different NPs have a distinct chemical composition and size-distribution profile (Chen et al. 2014).

In order to ensure reproducible synthesis of NP libraries, screening of NPs is required in a systemic and parallel fashion since NPs normally comprise diverse components and consist of a wide range of biophysical and chemical properties such as drug pay load, surface targeting ligands, release kinetics, etc. Production of uniform NPs is still quite an unrealized goal, even though multi-kilogram batches of NPs suitable for clinical development and commercialization have been produced through scale up of bulk techniques. Techniques such as microfluidics system and PRINT have been employed which would help in streamline a controlled and high-throughput investigation of kidney NP structure-activity relationships so as to produce uniform NPs with precise control of size, shape, chemical composition, drug loading and surface properties (Rolland et al. 2005; Xu et al. 2013; Kamaly et al. 2016).

13.5 Current Research in the Development of Enzyme-Based NPs for Treatment of Kidney Diseases

Although several therapies are available for the dissolution of kidney stones, due to difference in the size, hardness, chemical composition and position of the stones, none of them is effective in all cases. In spite of the tremendous development in nanomedicines, till date no nanodrug has been created for treating nephrolithiasis or kidney stones. Hence development of oxalate-degrading enzymes as nanomedicines with prolonged circulation and reduced immunogenicity is of great importance for the treatment of hyperoxaluria and therefore kidney stones (Fig. 13.3). In the past few years, some research papers regarding the development of enzymatic nanodrugs for treatment of nephrolithiasis have been published.

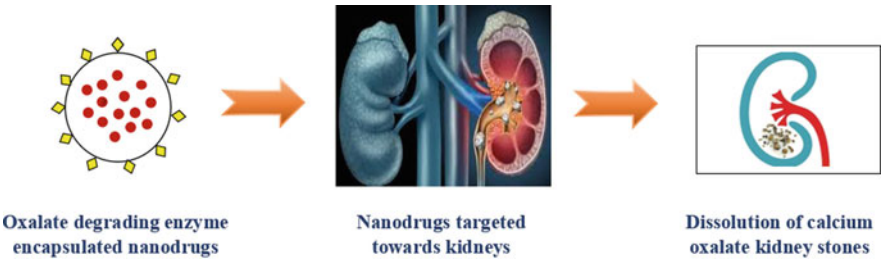

Fig. 13.3 Dissolution of calcium oxalate stones by oxalate-degrading enzyme encapsulated nanodrugs

The in vitro effects of immobilization of barley root oxalate oxidase onto three metal oxide NPs were studied by Chauhan and coworkers. Barley root oxalate oxidase was immobilized onto three NPs, i.e., zinc oxide (ZnO), copper oxide (CuO) and manganese oxide (MnO_2). As observed by X-ray diffraction and TEM studies, the NPs with very fine crystalline structure and with a diameter in the range of 30–70, 50–60, and 20–60 nm for ZnO-NPs, CuO-NPs and MnO_2-NPs, respectively, were produced. The immobilization improved the thermal and storage stability as well as the activity of the enzyme. The maximum improvement was observed in the case of MnO_2-NPs, while ZnO-NPs and CuO-NPs displayed no substantial improvement in their activities. The NPs displayed an increase in the optimum pH value, while a decrease in the K_m and optimum temperature was observed. The immobilized oxalate oxidase enzyme would act as a promising agent in the medical improvement of hyperoxaluria as well as in enzyme supplementation therapy for calcium oxalate nephrolithiasis (Chauhan et al. 2013). In another study conducted by Zhao and colleagues, a novel therapeutic for nephrolithiasis was developed by encapsulating oxalate oxidase in a thin layer of zwitterionic polymer. The nanocapsules produced enhanced the stability as well as the activity of oxalate oxidase and reduced phagocytosis leading to a prolonged circulation half-life due to reduced phagocytosis and also reduced immunogenicity. This nanocapsule design provided an effective route for systemic delivery of oxalate oxidase for treatment of calcium oxalate nephrolithiasis and hyperoxaluria (Zhao et al. 2017).

The investigation conducted by Lin and coworkers reported the immobilization of oxalate decarboxylase enzyme on Eupergit C, a copolymer of *N*,*N*-o-methylene-bis-(methacrylamide), glycidyl methacrylate, allyl glycidyl ether and methacrylamide. Although this approach led to the development of microporous beads rather than nanocapsules, the immobilized oxalate decarboxylase displayed improved resistance against both thermal and pH denaturation (Lin et al. 2011). Hence this analysis could be extended toward the development of NPs of oxalate decarboxylase that could be employed as a therapeutic for nephrolithiasis and hyperoxaluria.

13.6 Conclusion

The field of nanodrugs hold immense potential for treating diseases. Nanotechnology confers significant benefits to medicine, targeted delivery of drugs to specific tissues being the most important one. Nanodrugs not only improve the therapeutic index of the drugs by increasing solubility but also reduce their off-target side effects. Several preclinical researches have demonstrated the inherent prospects of nanomedicines in treating renal diseases. Nephrolithiasis is among the chronic kidney diseases which is a painful disorder and needs to be addressed with more effective therapeutic options. An expanding group of nanomaterials are present that can find implications in treatment of nephrolithiasis. In this review, we highlighted various types of NPs and their properties that could be applied for the treatment of kidney stones. We have also discussed the various challenges in the development of nanodrugs for nephrolithiasis and the current research that has been conducted in this regard.

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Conflict of Interest Further, the authors have no conflict of interest among themselves at their place of work or with the institution.

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Lipodermaceuticals: Technological Transformations

14

Rakesh Kumar Paul, Gajanand Sharma, Kaisar Raza, and Om Prakash Katare

Abstract

Skin diseases are one of the major global burdens and constitute the 18th most common noncommunicable diseases with affecting around 36.4 million people. Several skin disorders like dermatitis, psoriasis, scabies, acne vulgaris, alopecia areata, urticaria, and keratinocyte carcinoma have created a challenging task the drugs to penetrate and reach to the target sites. Lipid-based delivery systems are preferred more over conventional methods (chemical and physical) due to the several complications like patient compliances, pain at the site of application, and skin irritation. Currently, few lipodermal cosmeceutical products such as Revitalift, Ameliox, SkinGenuity, Celltight EF, Noicellex, Regenerationscreme Intensiv, NanoLipid Restore CLR, Psorisome, and Eye Essence have been developed using approaches like liposomes, ethosomes, and lipid nanoparticles. Application of these lipid-based technologies could be promising for the delivery of the drug into the deeper tissues. The present book chapter deals with various aspects which are pertinent for the development and translation of such carrier systems.

Keywords

Clinical trials · Dermatokinetics · Lipid-based nanoformulations · Marketed products · Skin diseases

R. K. Paul · K. Raza
Department of Pharmacy, School of Chemical Sciences and Pharmacy, Central University of Rajasthan, Ajmer, Rajasthan, India

G. Sharma · O. P. Katare (✉)
Division of Pharmaceutics, University Institute of Pharmaceutical Sciences, UGC-Centre of Advanced Study, Panjab University, Chandigarh, India
e-mail: drkatare@yahoo.com

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14.1 Introduction

As per the World Health Organization (WHO), the prevalence of skin diseases is increasing in hot and humid countries like India. The resistant property of the skin protects the body from several environments such as physical (ultraviolet radiations) and chemical and microorganisms (Kaur et al. 2020). Global Burden of Disease also reported that skin disease is leading to be the 18th most common noncommunicable diseases with around 36.4 million peoples suffered. The major skins diseases are dermatitis, psoriasis, scabies, fungal skin diseases, acne vulgaris, alopecia areata, urticaria, keratinocyte carcinoma, etc. (Karimkhani et al. 2017). It is expected that there will be an increase in the global market for dermaceuticals by 2020, which will be estimated at around USD 91.40 billion (Kaur et al. 2020). The application of dermal drug delivery process has also gained particular interest due to its direct delivery system to the site (skin surfaces), incidences of chronic skin diseases, and patient compliance. The delivery of the drug into the skin can be broadly classified as dermal (topically) or transdermal approach (Jain et al. 2017). But it's a great challenge for the researchers to overcome the limitations of drug absorption by the outermost layer of the skin. However, several techniques have been applied to improve the penetration of the drug into the skin either by disruption or by weakening the stratum corneum. Techniques such as chemical agents (glycols, ethanol, terpenes) and physical methods (electroporation, microneedles, microdermabrasion, and iontophoresis) were used for the successful delivery of the drug into the skin (Barry 2002; Jain et al. 2015). These two techniques are used lesser due to the several complications like patient compliances, pain at the site of application, and skin irritation. So, to overcome these techniques, some formulation-based skin delivery were nanoparticles, microparticles, and lipid delivery systems. These formulations can improve the solubilization of drug and can penetrate into the skin either by sweat gland, transepidermal, or hair follicle (Jain et al. 2015). Among these, lipid-based nanoformulations (liposomes, solid lipid nanoparticles, nanostructured lipid carriers) show the capability to challenge the other nanocarriers. These lipid nanoformulations increase the bioavailability, maintain the physical stability, and have the controlled release property of the active agents (Puglia and Bonina 2012).

On the other side, these lipid nanoparticles can improve the chemical stability of the active drugs which are prone to oxidation and hydrolysis or sensitive in the presence of light (Puglia and Bonina 2012). Scientific evidence related to lipid-based nanoparticle has revealed that the release rate can be influenced by the type of lipid used, vehicle, or the concentration of the surfactant used. Other parameters, such as the method for the preparation of lipid nanoparticles or the concentration of active drugs in the lipid matrix, also influenced the release rates (Jain et al. 2005). So, the lipodermal formulations create a lot of opportunities and can minimize the challenges for the other formulation.

14.2 Skin: As a Target Organ

Human skin is composed of dermis and epidermis layers. The outermost layer of the skin, stratum corneum, is responsible for protection from the hazardous environment by forming a barrier. The mechanism for penetration of actives could be by the sweat duct, by the hair follicles, or in between the appendages of the stratum corneum (Barry 2001). The follicles of the skin occupy around 0.1% of the surface area (Scheuplein 1967). But the number of follicles, opening diameter, and the volume of the follicle are the most important factors for the permeability (Otberg et al. 2004). Most of the actives enter through the intracellular micro-route mechanism, so it is required to enhance the permeation or disrupt the architecture of the surface layer (Barry 2001). Parameters such as diffusibility, structure, geometry of the skin, and the microcirculatory system are responsible for the penetration (Scheuplein 1967). However, the actives or payloads should be ionic or polar in nature if the diffusion is carried out by follicles. The diffusion mechanism can be well explained by Fick's first law. The concentration gradient is one of the major driving forces for the absorption of the drug. The concentration is high at the outer membrane, while deeper layers have low concentration. The chemical structure and the charge on the molecule are also important for the percutaneous absorption. Electrolytes and ions have a slow rate of absorption. The major challenging task for polar compounds is the low partitioning into the stratum corneum. This problem can be minimized by increasing the lipophilicity or by increasing the chain length of the compounds. Interestingly, it is also to be noted that too much lipophilic drug can tend to form a reservoir in the stratum corneum (Wiechers 1989). Other challenges such as disease, age, skin first-pass metabolism, reservoir capacity of the skin, irritation, and toxicity can also decrease the penetration of the drug (Katare et al. 2010).

The critical requirements for the dermal delivery of therapeutic agents are as follows: (1) should have low volume and molecular weight, (2) should possess good solubility in lipoidal and aqueous phase, (3) should have high diffusivity rate in the outermost layer of the skin (stratum corneum), and (4) should have less accumulation in the stratum corneum (Wiechers 1989). Some realistic promises of delivering the drug or payloads by the topical route are high surface area, low toxicity, no stability-related problems (flocculation, creaming, sedimentation), protection from enzymatic and hydrolytic degradation, reduction of water in transepidermal, and have a high capacity to entrap the lipophilic drugs (Ghasemiyeh and Mohammadi-Samani 2020). Several target locations of various skin diseases have been listed in Table 14.1.

Table 14.1 Target locations of the skin diseases

Disease	Target locations	References
Melanoma	Dermis or deeper subcutaneous layers	Dewar and Powell (2002)
Dermatitis	Epidermis and papillary dermis	Dainichi et al. (2018)
Keratinocyte carcinoma	Epidermal	Nehal and Bichakjian (2018)
Pyoderma	Infiltration of the dermis	Bhat (2012)
Psoriasis	Epidermis and papillary dermis	Dainichi et al. (2018)
Scabies	Lower stratum corneum	Arlian (1989)
Cellulitis	Cutaneous	Rossi and Vergnanini (2000)
Alopecia areata	Subcutaneous tissue	Wasserman et al. (2007)
Acne vulgaris	Epidermis and stratum corneum	Thiboutot and Del Rosso (2013)
Pruritus	Epidermis and upper dermis around the basal membrane	Ikoma et al. (2011)
Urticaria	Superficial portion of the dermis	Cooper (1991)
Asteatotic eczema	Stratum corneum	Brown (2017)

14.3 Lipid-Based Carriers for Topical Delivery

14.3.1 Liposomes

Intensive research in the field of lipodermal has emerged from the 1980s, which was published by Mezei and Gulasekharam, where studies were reported for the effectiveness of liposomes (Mezei and Gulasekharam 1980). But in the 1990s, econazole, an anti-mycotic agent, was commercialized as lipid vesicles (Cevc 2004). These lipid vesicles were composed of cholesterol, phospholipid, and aqueous. These vesicles could be prepared by the application of biodegradable lipids. The hydrophilic region of lipid when comes in contact with an aqueous medium, and the lipophilic tails remain in the external medium. Naturally, occurring phosphatidylcholine can deliver the payloads by permeation through the skin (Kulkarni et al. 1995). The rigid structure of the skin is disturbed by the fluidic behavior of phospholipid, leading to an increase in drug partitioning. So, the use of cholesterol helps to maintain the rigidity and stability of the gel-liquid crystalline phase (Jain et al. 2015). Techniques such as thin-film hydration, solvent injection techniques, and reversed-phase evaporation were used to prepare the liposomes. But thin-film hydration is the most suitable technique for topical delivery (Jain et al. 2017). Some theories behind the delivery of liposomes into the skin are by either adsorption mechanism or penetration through the transappendageal route (Hofland et al. 1995).

Drugs such as curcumin, siRNA, clotrimazole, resveratrol, and many more have been formulated in the form of liposomes for the topical delivery (Chen et al. 2012; Dorrani et al. 2016; Ning et al. 2005; Isailović et al. 2013).

14.3.2 Ethosomes

A new generation of lipid carriers, ethosomes, has the ability to deliver the hydrophilic and lipophilic payloads into the skin. The preparation of ethosomes is similar to the liposomes and composed of phosphatidylcholine, cholesterol, ethanol, and water. These ethosomes are prepared by thin-film hydration techniques because of the high entrapment efficiency (Maestrelli et al. 2009). Ethanol prevents the aggregation of the vesicles by forming a negative charge on the surface to develop an electrostatic repulsion. Likewise, liposomes and ethosomes also provide excellent physical stability with no sedimentation, flocculation, and coalescence property (Celia et al. 2009). Ethosomes of psoralen, apigenin, 5-aminolevulinic acid, and many drugs have been prepared for the delivery of the payloads into the dermal layer (Zhang et al. 2014; Shen et al. 2014; Fang et al. 2008a, b).

14.3.3 Lipid Particulate Systems

Lipid particulate systems were preferred for the biocompatible and nontoxic nature of the lipid excipients. This particulate system includes lipospheres and lipid nanoparticles (Jain et al. 2017).

14.3.3.1 Lipospheres

Lipospheres are composed of a solid lipid core in which phospholipid is embedded on the surface. The particle size ranges from 0.2 to 500 mm and provides greater stability of the drug, has a high dispersibility in an aqueous medium, and has extended-release property. An occlusive film mechanism helps to penetrate the drug through the stratum corneum (Swain et al. 2015). The physicochemical properties such as particle size and entrapment efficiency influence the delivery of the payloads into the skin. The entrapment efficiency depends on the type of lipid, the concentration of the stabilizer, and the quantity of phospholipid used (Beg et al. 2016). In contrast, smaller lipospheres improve skin penetration (Linder and Markus 2005).

14.3.3.2 Solid Lipid Nanoparticles (SLNs)

These lipids are biodegradable and form a solid lipophilic matrix in which hydrophilic or lipophilic payloads are incorporated. These lipids range from 50 to 1000 nm in size which are spherical in shape (Müller et al. 2000). SLNs provide the protection of active payloads from oxidation, light and chemical degradation, and moisture. These nanoparticles interact with the superficial junction of corneocyte clusters and channels of the stratum corneum, which improves the accumulation of payloads, thus resulting in controlled delivery (Jain et al. 2017). The practical technique for the preparation of SLNs is high-pressure homogenization (Üner and Yener 2007). Studies related to the SLNs have revealed that a particle size of 100 nm is good to penetrate into the skin through hair follicle (Adib et al. 2016). Some drugs with topical delivery in the form of SLNs are amphotericin B, terbinafine hydrochloride,

psoralen, curcuminoids, meloxicam, ketoprofen, and betamethasone dipropionate (Butani et al. 2016; Vaghasiya et al. 2013; Fang et al. 2008a, b; Zamarioli et al. 2015; Khurana et al. 2013; Kheradmandnia et al. 2010; Kong et al. 2016).

14.3.4 Nanostructured Lipid Carriers (NLCs)

The second-generation NLCs have gained attraction in the field of pharmaceuticals and cosmetic industries. Currently, there are approximately 40 cosmetic products in the market for NLC-based formulations (Obeidat et al. 2010). Compared to SLNs, NLCs have higher drug loading, which results in higher concentration and permits penetration into the skin. The smaller the particle size of NLCs, the higher the permeability (Gaba et al. 2015; Phatak and Chaudhari 2013). NLCs were prepared to improve the penetration of the drugs, drug stability, and controlled release property and increase hydration (Puglia et al. 2014; Fan et al. 2014). Researchers have claimed that NLC-loaded gels do not show an adverse effect on topical use (Gaba et al. 2015).

Different lipodermal carriers and drugs have been listed in Tables 14.2 and 14.3.

14.4 Technological Advances in Dermaceuticals

Delivery of actives by the use of skin could be the alternative pathway to the oral route. The primary task is to deliver the drugs into the skin by crossing the stratum corneum. However, in the previous four decades, technologies have modified the ancient techniques, and novel approaches gained interest. The application of dermatokinetic modeling is required nowadays to understand the drug concentration present inside the skin and the effect of toxic molecules (Thotakura et al. 2017). The kinetic study in the skin starts from the administration of the drug and with the absorption (Shah 2001). The dermal pharmacokinetic study depends on various parameters such as C_{max} (maximum drug concentration), T_{max} (time required to reach C_{max}), and AUC (area under curve) (Nicoli et al. 2009). The dermatokinetic calculation is based on the one-compartment model (Raza et al. 2013) and calculated by the following equation:

$$C_{\text{Skin}} = \frac{K_{\text{p}}.C_{\text{max}}}{(K_{\text{p}} - K_{\text{e}})} \left(e^{-K_{\text{e}}t} - e^{-K_{\text{p}}t}\right) \tag{14.1}$$

where C_{skin} is the concentration of drug at time t, K_p is the permeation constant for dermal, C_{max} is the maximum concentration achieved in skin, and K_e is the skin elimination constant. These parameters can be calculated by WinNonlin software using Wagner-Nelson method (Raza et al. 2013). There are various in vivo and in vitro techniques for dermatokinetic assessment of topical formulations. These include tape stripping method, microdialysis method, vasoconstrictor assay, and confocal scanning microscopy (Thotakura et al. 2017).

Table 14.2 Advantages and challenges of lipodermal carriers

Lipid-based carriers	Advantages	Challenges
Liposomes	Biocompatible and biodegradable Suitable for both hydrophobic and hydrophilic drug loading	High cost of lipids Poor chemical and physical stability Poor permeation Poor physicochemical characteristics
Ethosomes	Lipids are biocompatible and biodegradable Suitable for both hydrophobic and hydrophilic drug loading Higher elasticity, high efficacy with smaller vesicle size Higher skin permeation	High cost of lipids Loss of stability on long-term storage Possibility of skin irritation
Lipospheres	Biodegradable and biocompatible in nature Relatively cost-effective than other vesicles Preparation method and scale-up is easy Stability is increased for photo-labile drugs	Poor skin permeability compared to SLNs and NLCs Lack of long-term physical stability Higher particle size than SLN and NLC Poor drug loading for hydrophilic compounds
Nanostructured lipid carriers	Higher drug loading capacity compared to SLNs Water content is low compared to SLNs Particle size is less than lipospheres	Physical stability lacks on storage Lack of regulatory guidelines
Solid lipid nanoparticles	Relatively cost-effective Biodegradable and biocompatible Protect the payloads from chemical degradation Smaller particle size than lipospheres	Incorporated in the semisolid form due to the high water content Gelation and particle agglomeration Lack of physical stability on storage for long term

14.4.1 Dermatokinetic Study

Raza et al. prepared nanocolloidal carrier of isotretinoin and performed dermatokinetic modelling by using Wistar rats. The group separated the skin layers by heat treatment and then extracted the drug from each layer. They observed that nanocolloidal systems (NLCs and SLNs) have increased the rate of delivery of isotretinoin to skin layers than the marketed product. They also reported that AUC of drug was more in dermis when compared to epidermis which indicates the efficient drug supply to sebaceous glands, i.e., target site to treat acne (Raza et al. 2013).

Sharma et al. performed dermatokinetics of aceclofenac-β-cyclodextrin vesicles using Wistar rats and microdialysis technique. They observed that there was increase

Table 14.3 List of lipodermal drugs

Drug carrier system	Drugs	Disease
Liposomes	Amphotericin B	Fungal infection
	Fluconazole	Fungal infection
	Calcineurin inhibitors	Prevent DNA photodamage
	Ciclopirox	Fungal infection
	Methotrexate	Psoriasis
	Cetirizine	Pruritus
	Lauric acid	Inflammatory acne
	Tretinoin	Acne
	Idoxuridine	Herpes simplex
	Nimesulide	Inflammation and pain
	Finasteride	Acne, androgenetic alopecia
	Vitamin D analogues: Calcipotriol, tacalcitol, calcitriol	Antiparakeratosis function
	Hydroxyzine	Skin allergy
	Tamoxifen	Certain skin disorders
Ethosomes	Fluconazole	Fungal infection
	Methotrexate	Psoriasis
	Azelaic acid	Acne
Solid lipid nanoparticles	Cyclosporin A	Allergic skin disorders (atopic dermatitis)
Nanoemulsion	NB-002	Fungal infection
Nanoparticles	Tacrolimus	Psoriasis
Niosomes	Methotrexate	Psoriasis
	Dithranol	Psoriasis

in the half-life and C_{max} of drug by using liposomes when compared to marketed gel. Even they observed sudden decrease in T_{max} also. Finally, they concluded the prepared liposomes helped in better topical delivery of aceclofenac than that of marketed gel (Sharma et al. 2016a, b).

Yadav et al. developed tamoxifen-loaded polymeric micelles and evaluated the nanosystems using dermatokinetics. For this study they employed the skin of Laca mice. They reported that there was 3.4 times enhancement in the bioavailability of the drug when compared to plain drug. The skin permeation constant was higher in skin treated with drug-loaded micelles in comparison to the plain drug. Through this study they concluded that the PLGA-PEG polymer-based nanosystems can help in increasing the delivery potential of the anti-neoplastic drugs (Yadav et al. 2016).

Sharma et al. developed topical formulation of benzyl benzoate-loaded microemulsion for treatment of the scabies. They performed dermatokinetic study using the skin of Laca mice. They found that the nanoformulation helped in enhancing the C_{max} and bioavailability in both the skin layers along with the reduction in T_{max}. From the study they concluded that microemulsion is capable in

enhancing the delivery of benzyl benzoate with increased bioavailability in comparison with the conventional marketed product (Sharma et al. 2016a, b).

Sharma et al. developed and evaluated aceclofenac cocrystal nanoliposomes for rheumatoid arthritis. Through dermatokinetic study they observed that the T_{max} and half-life of the drug were reduced and enhanced, respectively. Even they observed the rise in C_{max}, in both the layers and AUC in dermis. From these results they confirmed that aceclofenac cocrystal-loaded nanoformulation has the ability to enhance the delivery of aceclofenac across the skin layers via the marketed product (Sharma et al. 2017).

Thakur et al. prepared tamoxifen-loaded chitosan conjugated PLGA polymeric micelles for treating breast cancer. From dermatokinetic study they concluded that the developed polymeric micelles are capable to deliver high concentration of drug through both layers of the skin. The bioavailability of drug in epidermis was enhanced by 3.5-fold, whereas in dermis it was 2.2-fold. There was retention in the elimination process using the designed micellar systems. These findings paved a path in developing a better marketed product of tamoxifen (Thakur et al. 2016).

Thakur et al. employed Quality by Design (QbD) approach to develop silver sulfadiazine-loaded egg oil organogel for treating infections due to burn injuries. From dermatokinetic profile they found that the delivery of drug into the skin layers was higher in comparison with the marketed formulation. In epidermis and dermis, there was an increase in the residence time of the drug along with the topical bioavailability vis-à-vis cream dosage form (Thakur et al. 2020).

14.4.2 Tape Stripping

This is one of the simplest and most straightforward methods for determining the efficacy of topical formulation. The steps follow from the application of a drug to the skin, and then the layers of the stratum corneum are removed by using the adhesive strip as depicted in Fig. 14.1. This strip calculates the ability of the formulation to penetrate by removing the corneocytes from the stratum corneum. This technological advancement in dermaceutical field provides the dermatiokinetic parameters of topical delivery (Lademann et al. 2009).

14.4.3 Microdialysis

It is an invasive technique to determine drug concentration. In this method, ultra-thin semi-permeable hollow fiber (a probe) is placed in the dermis, which acts as an artificial blood vessel and starts the diffusion process for the small molecules as depicted in Fig. 14.2. The most challenging task is the recovery of highly protein-bound and highly lipophilic drugs (Benfeldt et al. 2007).

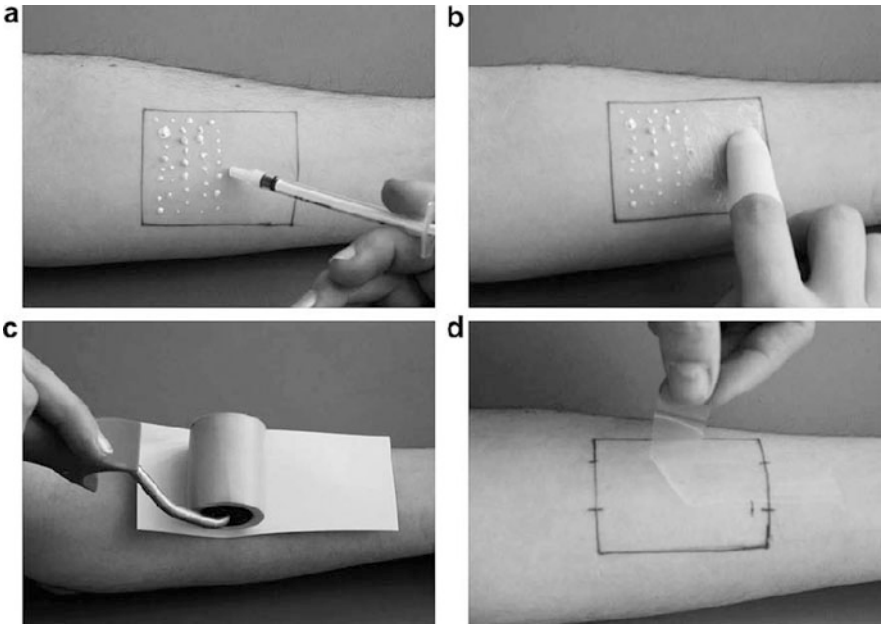

Fig. 14.1 Steps for tape stripping. (**a**) The application of the formulation on the surface of the skin, (**b**) distribution of the formulation, (**c**) the rolling of adhesive tap using a roller, (**d**) the removal of tape (reprinted with permission from Lademann et al. (2009))

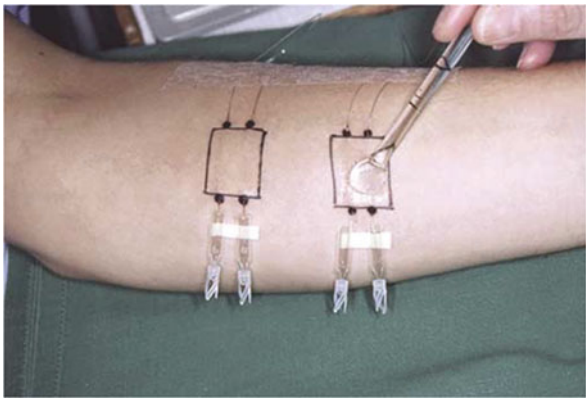

Fig. 14.2 Process for microdialysis (reprinted with permission from Benfeldt et al. (2007))

14.4.4 Vasoconstrictor Assay

This approach is used to determine the efficacy and safety of the dermaceuticals and is also known as skin blanching assay. In this, color of the skin is checked using Minolta chroma meter (Kanfer 2010). The instrument has a chamber where adhesive

tape holds the skin and the dermaceuticals are applied on it at different time intervals. The response observed will be noted according to the intensity scale of 0–4, where 0 represents no blanching and 1–4 represent the increase in the array of blanching (Clanachan et al. 1980).

14.4.5 Confocal Scanning Microscopy

The use of confocal scanning microscopy in the dermatokinetics helps in the assessment of distribution and penetration of the drug into the skin. In this, the use of dye or the fluorescent coating drug is used for easy detection of the applied product (Thotakura et al. 2017).

Technologies for enhancing the skin permeability can be classified into two active methods: (a) electrical method or (b) mechanical method.

14.4.6 Electrical Method

This method includes iontophoresis and electroporation techniques.

14.4.6.1 Iontophoresis

This technique enhances the permeation of the topically applied payloads by the application of a low electric current (Wang et al. 1993; Turner et al. 1997). This technique employs a different mechanism for different drug molecules and includes electrorepulsion (for charged solutes), electropertubation (for both charged and uncharged), and electro-osmosis (for uncharged solutes). Various parameters such as current intensity, pH, competitive ion effect, and electrode type may affect the iontophoresis process (Banga et al. 1999). E-TRANS® technology works on electrotransport principle, which is developed by the ALZA Corporation (Brown et al. 2006).

14.4.6.2 Electroporation

This technique uses a high voltage pulse for the delivery of the drug into the skin. High voltages ($\geq$100 V) are used for few milliseconds for the drug molecule for the penetration. For enhancing the permeability, lipophilicity and size with a molecular weight greater than 7 kDA play a crucial role (Denet et al. 2004).

14.4.7 Mechanical Methods

14.4.7.1 Microneedle-Based Devices

This device consists of a drug reservoir which penetrates the stratum corneum and epidermis. Macroflux® microprojection array was developed by ALZA Corporation. These needles are used topically, and the size ranges from 50 to 200 μm. These needles allow the unhindered movement of the drug (Brown et al. 2006).

14.4.7.2 Skin Abrasion

This technique is based on the removal or disruption of the upper layers of the skin. Disruption or removal of the upper layer of the skin leads to an increase in the permeability of the drug. Microdermabrasion is the technique for superficial skin resurfacing and is used in the treatment of acne, scars, and hyperpigmentation (Brown et al. 2006).

14.4.7.3 Needless Injection

It is one of the painless methods for the delivery of the drug into the skin. Several injectors for liquids (Ped-O-Jet®, Biojector 2000®, Dermajet®, Intraject®, Medi-Jector®, Injex™) and powders (PMED™) have been reported for the delivery of a drug (lidocaine hydrochloride, testosterone, insulin) into the skin (Brown et al. 2006).

14.5 Market Status and Promises

Markets for dermaceuticals and cosmeceuticals seem to be emerging around the globe. Changes in the lifestyle are due to the new era of globalization, economy, and improved technologies. India's skincare market valued about $180 million (Junaid and Nasreen 2012). Though traditional and homemade products are used for several skin-related problems, latest technologies attracted humans more nowadays. Numerous lipodermal cosmeceuticals were in the market and listed in Table 14.4 (Jain et al. 2017).

14.6 Clinical Trials

The burden of skin diseases is increasing worldwide. There are numerous marketed drugs for topical use, but some hurdles create a lot of challenges for the penetration of the drug or payloads into the skin through the stratum corneum. Currently, very few lipodermal formulations are in the clinical trial phases and were listed in Table 14.5. But, in the future, there is hope for a significant requirement of lipodermaceuticals for targeting different skin diseases with good clinical outcomes.

14.7 Future Prospects

Increase in the topical diseases (dermatitis, psoriasis, scabies, fungal skin diseases, acne vulgaris, etc.) and the skin care has made the humans rethink for their external outlook. However, several lipodermal formulations were prepared for skin care. Still, due to some limitations such as low skin permeability, poor physical and chemical stability, and high cost of lipids, skin irritations have limited the use of these formulations. The drugs in the clinical phase might show a positive outcome

Table 14.4 Marketed lipodermal cosmeceuticals

Lipid-based delivery system	Trade name	Manufacturer
Liposome	Revitalift	L'Oréal
	Rovisome ACE Plus	ROVI Cosmetics international GmbH
	Ameliox	Mibelle Biochemistry
	Lancôme Soleil Soft-Touch Anti-Wrinkle	L'Oréal
	Ageless Facelift cream	I-Wen Naturals
Ethosomes	SkinGenuity	Physionics
	Decorin cream	Genome Cosmetics
	Celltight EF	Hampden Health
	Nanominox	Sinere
	Noicellex	Novel Therapeutic Technologies
Lipid nanoparticles	SURMER Crème Contour Des Yeux Nano-Remodelante	Isabelle Lancray
	Regenerationscreme Intensiv	Scholl
	IOPE Super Vital line of: Eye cream Cream Serum Extra moist emulsion Extra moist softener	Amorepacific
	NanoLipid Restore CLR NanoLipid Repair CLR NanoLipid Basic CLR NanoLipid Q10 CLR	Chemisches Laboratorium Dr. Kurt Richter (CLR)
	Swiss Cellular White Illuminating Eye Essence	La Prairie
	Cutanova Cream Nanovital Q10 Cutanova Cream Nanorepair Q10 Intensive Serum Nanorepair Q10	Dr. Rimpler

for dermal approaches and could be fruitful for humans. Application of newer technologies has also revealed promising for the delivery of the drug into the deeper tissues. These technologies could be applicable for those drugs which are in the clinical phase for future technology with safety, efficacy, and user-friendly nature.

Table 14.5 List of drugs in the clinical trial for skin care

Drug	Identifier	Title	Year of filling	Clinical trial phase
Anthralin	NCT03348462	Formulation and clinical evaluation of ethosomal and liposomal preparations of anthralin in psoriasis	2017	Phase 4
Meglumine antimoniate	NCT01050777	Efficacy of topical liposomal form of drugs in cutaneous leishmaniasis	2010	Early phase 1
Retinyl palmitate	NCT04080869	Formulation of retinyl palmitate-loaded topical ethosomes for treatment of acne vulgaris: a split-face comparative clinical study	2019	Phase 2
Amphotericin-B	NCT02656797	Topical liposomal amphotericin B gel treatment for cutaneous leishmaniasis	2018	Phase 2
HL-009	NCT01568489	A randomized, phase 2, double-blind, placebo-controlled trial to evaluate the safety and efficacy of HL-009 liposomal gel in adult patients with mild to moderate atopic dermatitis	2012	Phase 2

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The Importance of Drug Dose Adjustment in Elderly Patients with Special Considerations for Patients on Diverse Co-medications and Antidepressants

15

Manju Bhaskar, Istvan G. Telessy, and Harpal S. Buttar

Abstract

The geriatric population is escalating globally, and the need for treating infectious and non-infectious diseases in elderly patients is also correspondingly increasing worldwide. In clinical trials and under doctor's office settings, the drug dosages are generally computed on mg/kg body wt. basis in young adults and middle-aged men and women (<40 years). It is well recognized that in comparison with the younger age counterparts, the geriatric subjects are more susceptible to drug-mediated adverse reactions due to the reduced activity in cytochrome P450 coenzymes and glucuronidation/sulfation mechanisms. Since the body mass in elderly patients, especially frail elders, is markedly reduced due to sarcopenia, progressive loss in body fat, and osteoporosis, hence, drug doses based on mg/kg body wt. usually cannot be applied in this group of patients as is done in relatively young adults. Most of the physiological functions, including drug metabolizing and excretory capacity declines in the elderly, consequently cause significant alterations in the metabolic disposition as well as changes in the pharmacokinetic (PK) and pharmacodynamic (PD) parameters of administered drugs in elderly subjects as opposed to the younger individuals. Innumerable studies have shown

M. Bhaskar
Translational Neuroscience Center, National Institutes of Neurological Disorders and Stroke, Bethesda, MD, USA

I. G. Telessy
Department of Pharmaceutics, Faculty of Pharmacy, University of Pécs, Pécs, Hungary

MedBioFit Lpc, Gödöllö, Hungary

H. S. Buttar (✉)
Department of Pathology and Laboratory Medicine, University of Ottawa, School of Medicine, Ottawa, ON, Canada
e-mail: hsbuttar@bell.net

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wide differences in xenobiotic responses due to inter-individual variation, demographics, age, gender, ethnicity, and race. These differences are attributed to a wide array of factors such as pharmacogenetics variations, gastrointestinal and microbial metabolism of drugs, bioavailability, and first-step metabolism in the liver and renal excretion. Elderly individuals are one of the most vulnerable age groups to adverse drug reactions (ADRs) due to multiple comorbidities, co-medications, and declining functions of the gastrointestinal-hepatic-renal systems. Age-related debilitating conditions tend to enhance the incidence to ADRs and hospital readmissions due to cognitive impairment, inappropriate drug use, and drug-drug, drug-diet, and drug-herbal interactions. Collectively, all these situations make it highly challenging for the physicians, nurses, pharmacists, and surgeons to make drug dose adjustment decisions for the geriatric patients. Healthcare providers should always ask their patients about herbal and dietary supplements' use and discourage concomitant ingestion of botanical products and fruit juices, especially grape fruit juice, with prescription drugs. Systematic research by various scientific groups and pharmaceutical companies has helped in the computation of drug dose adjustments and decision-making easier for drug administration in elderly and frail patients. Appropriate guidelines, equations, and formulas are available for calculating drug dosages for frail elderly patients based on serum creatinine or cystatin-C clearance or some other biomarkers. It is important that elderly patients should be enrolled in clinical trials for understanding the pharmacometabolomics and assessment of safety, efficacy, and optimal dose schedules of new drugs. The focus of this review is to address the age-related physiological, pharmacological, and toxicological changes in elderly humans as well as age-related alterations in the absorption, distribution, metabolism, and excretion (ADME) of drugs administered orally or by other routes. We will also describe the characteristics of drug molecules that influence the bioavailability, PK, PD, and potential interactions of prescription drugs or over-the-counter medications taken simultaneously with fruit juices and herbal remedies. In this review, we have selected examples of potential risks associated with the psychotherapeutic class of drugs because the antidepressant, antianxiety, and insomnia-treating medications are some of the most frequently used categories of drugs by elderly men and women.

Keywords

Phamacometabolomics in elderly · Dose adjustment in elderly patients · Geriatric pharmacology · Gut dysbiosis · Creatinine clearance · Clinical implications of polypharmacy · Antidepressants

15.1 Introduction

During the past five decades, an increasing percentage of global population has attained gerontologic status (>60 years). At the same time, advances have occurred in drug development, medical technology and practice of medicine, and

advancement in surgical procedures, which have added to the longevity and good quality of life. According to the WHO estimates, by the year 2050, the worldwide elderly population (>60 years) is expected to reach 1.4 billion, which means that 1 out of 10 people will be more than 60 years of age. In some countries, this tendency is more accelerated, e.g., in 2012, the number of people in India aged 60 years and over was 98.5 million which has increased by 54.8% in the last 15 years (World Alzheimer's Report 2018).

Prevalence of multiple drug usage or polypharmacy steadily increases with age due to the enhanced risk of non-communicable diseases (NCDs) such as neurodegenerative disorders, dementia and cognition impairment, anxiety and depression, cardiovascular diseases, diabetes mellitus, obesity, osteoarthritis, and cancer, to name a few. The incidence of infections, autoimmune disorders, insomnia, and psychiatric problems also increase in the aging population (Moxey et al. 2003). Aging is associated with liver and kidney atrophy and alterations in gastrointestinal motility, accompanied by reduced gut secretions and blood flow, as well as reduction in absorptive surface area of the gastrointestinal tract. The age-related pathophysiological changes can alter the absorption, distribution, metabolism, and excretion (ADME) of drugs, consequently affecting the PK and PD parameters. As opposed to the relatively younger age groups, the geriatric subjects are more susceptible to drug-mediated adverse reactions due to the reduced activity in cytochrome P450 coenzymes and glucuronidation/sulfation mechanisms.

Potentially inappropriate medication (PIM) is common among the elderly (Lenander et al. 2018), and some drug-related adverse effects in the elderly are due to the inappropriate use or overuse of drugs. There are neurological conditions when the risk of PIM is increased, e.g., the prevalence of PIM is high among the older adults with cognitive impairment and/or dementia ranging from 15% to 47% population (Patel et al. 2017). This means non-adherence to doctors' and pharmacists' recommendations as well as due to the use of over-the-counter (OTC) drugs and self-medication with herbal remedies or dietary supplements can alter the metabolism and PK/PD of prescribed drugs. Generally, doctors prescribe pharmaceuticals according to the instructions listed in the Drug Product Monographs, which contain the most important patient information about the needed changes in dosage if the drug is indicated for elderly patients. Nevertheless, iatrogenic effects of drugs are quite common among the elderly and frail patients when the doctors, surgeons, and pharmacists are unable to correctly assess the pathophysiological conditions and consult patients prior to ordering the appropriate medications (Hedna et al. 2015).

It is well known that marked differences exist in the metabolic disposition and PK of drugs among the infants/toddlers, young adults (<40 years), inter-individual variation, gender, ethnicity, and elderly men and women. In comparison with the younger counterparts, the activity of hepatic drug-metabolizing enzymes, especially cytochrome P450 coenzymes and phase II biotransformation, especially glucuronidation, is far lower in the pediatric and elderly populations. Hence, drug dose adjustment is an essential requirement in these special groups. Most orally administered drugs appear to be adequately absorbed in the elderly subjects.

However, absorption problem seems to occur in the elderly subjects due to the co-existing disease conditions like atrophic gastritis, bile reflux disorder, gut motility problems, constipation, dysbiosis, and insufficient circulation in the gastrointestinal tract. Aging-associated drug absorption changes can lead to day-to-day variations in the bioavailability, first pass effect, and therapeutic blood concentrations among the geriatric patients (Italiano and Perucca 2013). The total body fluid (TBF) and cell mass (CM) decrease are parallel in the aging person. Although the extracellular fluid (ECF) constitutes a large proportion of TBF in elderly, the loss of TBF significantly impacts on the intracellular fluid (ICF). Thus, the disproportional shifts between ICF and ECF are mainly responsible for age-related alterations in unchanged drug and/or its metabolite distribution in the body and PK values (Mangoni and Jackson 2003). Malnutrition, thyroid deficiency, kidney disease and hemodialysis, and metabolic disorders are frequent among elderly patients, which adversely affect not only the water balance and electrolyte homeostasis but also the quality of life (Shehab-Eldin et al. 2020).

Generally, the elderly population has increased susceptibility to the iatrogenic or toxic effects of drug, because of their diminished physiologic reserve capacity of drug-metabolizing and excretory organs. The baseline functions of the gastrointestinal tract, microbiota, liver, and kidneys decline with ageing. In addition, there is a higher incidence of concomitant disorders that require multiple drug therapy by elderly patients, resulting in increased possibility of ADRs due to polypharmacy. Self-medication with micronutrients, herbal remedies, and dietary supplements is also common among the elderly patients, and the combinations can elicit clinically important adverse drug-herb-diet interactions. To date, there is a lack of consistent criteria and evaluation of potential underlying pathophysiological conditions that are responsible for causing the large differences in the metabolic disposition and PK/PD parameters between young adult and elderly subjects (Streeter and Faria 2017). Hence, assessment of pharmacometabolomics is important for drug dose adjustment in the elderly population because every individual process associated with absorption, distribution, metabolism, and disposition (ADME) of drugs administered orally or by other routes gets altered in old age (Hilmer 2008). It is also important that elderly patients should be enrolled in clinical trials for learning more about the pharmacometabolomics and assessment of long-term safety, efficacy, and dosing schedules of new drugs. The International Association of Gerontology and Geriatrics in conjunction with WHO has suggested that the new drugs intended to be used in the older population should undergo well-controlled, randomized, double-blind clinical trials in nursing home residents. Such clinical trials are desperately needed to determine the benefits and harms of medications administered in older persons (Tolson et al. 2011). Prescribing guidelines have been developed, and several investigators have recommended reducing inappropriate polypharmacy to minimize the iatrogenic risks of drugs in frail elderly and cancer patients (Scott et al. 2015; Sharma et al. 2016; Tjia and Lapane 2017). This review provides evidence regarding the potential prevalence of polypharmacy and suggests ways to reduce drug-induced harm due to inappropriate medication use in the geriatric patients.

In a managed healthcare setting (e.g., hospital, nursing home), a medication therapy management program is an excellent way to reduce inappropriate polypharmacy that would result in cost savings. The clinicians should also consider drug-disease interactions (especially liver and kidney disorders) that may be unique to individual patients and may require dose adjustment. A proper assessment of polypharmacy interactions may provide insight into medications that are inappropriate, ineffective, and/or unnecessary for a particular patient. Such best prescribing practices would not only reduce the risk of clinical interactions and side effects of medications and herbal therapies the patient may be taking but also help in adjusting doses of interacting medications that exacerbate harmful effects.

Education of healthcare professionals is a key factor to assess the therapeutic need of frail elderly patients, and perhaps the best education is first-hand experience to talk to the patient and families with informed discussion of expected benefits and potential harms of pharmacotherapy.

The aims and objectives of this article are multi-fold: namely, (1) to review the available data on the metabolic disposition of drugs in the elderly population, and how the metabolic changes can cause adverse drug reactions and frequent hospitalizations and affect the quality of life in geriatric patients; (2) to address the issues and concerns regarding drug dose adjustment in frail elderly patients; and (3) why it is crucial to enroll elderly subjects in clinical trials to evaluate the pharmacometabolomics, long-term safety, and efficacy as well as learning about the optimal dose schedules of new drugs. All these efforts will be supported by good prescribing practices and dose adjustment examples of psychotherapeutic drugs because the antidepressant, antianxiety, and anti-insomnia medications are some of the most frequently used classes of drugs for mitigating depressive disorders in elderly men and women.

15.2 Modifications of Gastrointestinal (GI) Physiology in Elderly

The core functions of the GI tract are to ensure adequate digestion of foods and absorption of nutrients involving digestive secretions and GI motility regulated by neuronal and hormonal control. Healthy GI functions also require good dental status for chewing and swallowing of chewed food. Quite often the masticatory function, taste, and swallowing reflex get impaired in elderly subjects (Wyatt and Kawato 2019). Madhavan et al. (2016) have reported salivation reduction and swallowing problems in elderly individuals aged 65 years and over. Although salivary flow rate decreases with healthy ageing, medications with anticholinergic, psychotropic, antihistamine, and diuretic drugs reduce salivary secretion that can adversely impact on food digestion (Madhavan et al. 2016). Impairment of salivary and dental functions can contribute to food avoidance, reduce food intake, and eventually lead to poor nutritional status among elderly (Morley 2007). Reduction in esophageal functions is often observed in the elderly, which can also occur in younger age groups, about 40 years old. Deteriorated esophageal reflexes may reduce peristalsis and increase non-propulsive contractions of the GI tract (Rayner and Horowitz

2013). Healthy ageing may affect gastric emptying of solid and liquid foods. In frail elderly, compared to non-frail elderly, gastric emptying of liquids was found to be enhanced, whereas gallbladder emptying and oro-cecal transit times were similar in two groups (Serra-Prat et al. 2013; Bai et al. 2016). Decreased rectal compliance and rectal sensation associated with healthy ageing often leads to constipation. Rémond et al. (2015) found that bicarbonate and pepsin enzyme concentrations in the stomach, as well as lipase, chymotrypsin, and amylase amounts in the duodenal fluid, were lower among the healthy elderly subjects than younger adults. However, bile acid secretion was not affected by ageing. According to Demir et al. (2017), rectal incontinence due to impaired sphincter functions and dementia is one of most frequent GI tract problems in the elderly, e.g., approx. 3% in people living at home and 20% to 50% in community-dwelling and nursing homes, respectively. Further, these authors observed that polypharmacy medications are important risk factors for urinary and/or rectal incontinence. In view of these observations, it appears that defective gastrointestinal secretions and functional disorders of the GI tract can be the important risk factors causing alterations in food digestion and drug absorption in the geriatric population.

GI tract is one of the most important immune function regulator organs in the body.

The gut lymphatic system network plays a vital role in maintaining extracellular homeostasis, lipid absorption, and optimizing immunity against pathogenic bacteria, viruses, parasites, and other antigens (Breslin et al. 2019). Several studies have examined the impact of senescence on the GI tract immune functions. For example, Mabbott et al. (2015) have shown that the human GI mucosal system is compromised or morphologically changed with ageing, such as alterations in dendritic cell subsets, increased levels of tumor necrosis factor-α, reduced secretory IgA levels, decreased natural killer cells, and phagocytic cell activity. They suggested that immunosenescence contributes heavily to the increased risk of recurrent and persistent infections in the elderly. The compromised immune system of the GI tract can be further modified by immune-related comorbid conditions in frail and elderly subjects (Maijo et al. 2014). Also, immunosuppression therapy can inhibit the GI tract immune system in the elderly patients. A comprehensive review by Szalach et al. (2019) reported that the plasma levels of pro-inflammatory cytokines and the number of activated immune cells were very high in patients suffering from depression. The cytokine levels were markedly reduced in patients given antidepressant therapy, suggesting the role of immune system in depression. It is assumed that besides the nervous system, the GI tract's immunocompetence may also be modulated in patients receiving antidepressant drugs.

15.3 Age-Related Changes in Gut Microbiota Depict Health Status in the Elderly

Trillions of microorganisms or "microbial bank" of a diverse nature inhabit the human GI tract. All the microbes harboring the gut are collectively known as "gut microbiota," and their associated genomes represent the "gut microbiome." The GI bacterial load in a healthy adult is estimated to be around 3×10^{13} bacterial cells in a 70-kg adult (Sender et al. 2016). The major bacterial phyla consist of *Bacteroidetes* and *Firmicutes* and subgroups like *Fusobacteria, Cyanobacteria, Proteobacteria, Verrucomicrobia, Actinobacteria*, and few others (Clemente et al. 2012). With advancing chronological age, the gut microbiota become more diverse and variable (Kim and Jazwinski 2018). Maffei et al. (2017) found that increasing biological age is associated with gastrointestinal dysbiosis. In healthy adults, intestinal microbiota is relatively stable throughout adulthood, but with ageing drastic changes occur due to exogenous factors such as antibiotics use, dietary factors, and endogenous cellular stress. In older adults (>63–76 years), gut microbiota become unstable affecting intestinal motility and digestion. Variations in the GI microbiota adversely impact on the fermentation processes leading to immunosenescence and low-grade inflammatory responses in the gut.

The GI tract is mostly sterile in the intrauterine stage. However, *Corynebacterium* sp. is usually the early colonizers in C-section babies and *Lactobacillus* sp. in the vaginal delivery. With time the commensal bacterial community grows under the influence of solid food intake. During the initial stages of microbiota establishment, the Toll-like receptor (TLR) actions are minimal allowing growth of commensals. Eventually, the immune system develops by demarking the pathogens and commensals. Bacteroidetes dominate after 2 years of birth. The relative stability is attained at adulthood by the domination of *Bacteroidetes* and *Firmicutes*. Significant alterations occur with the use of antibiotics, obesity, GI disorders, and diet. With advancing age, the microbiota stability declines, and commensal community of *Bacteroidetes* and *Firmicutes* species decreases, and pathogenic species like *Clostridium* increase. Therefore, gut microbial dysbiosis along with malnutrition, alcohol abuse, decline in body metabolism, frequent hospitalization, and nasopharyngeal and lung infections lead to polypharmacy and ultimately causing iatrogenic-related diseases in the elderly individuals (Vemuri et al. 2018).

Biagi et al. (2010) have investigated functional differences in the gut microbiota metagenome across different age groups—young adults, older adults, and centenarians—using illumine shotgun sequencing on fecal samples. It was observed that the proteolytic activity was increased accompanied by a clear loss in the genes associated with the carbohydrate metabolism with ageing. Short-chain fatty acid (SCFA) production also declined due to the age-related reduction of genetic pathways caused by microbiome rearrangement. The authors concluded that manipulation of the intestinal microbiota and microbiome may be beneficial for maintaining health and treating age-related disorders (Biagi et al. 2010). Circadian rhythm, metabolism, and gut microbiota are intricately linked with each other. Most of the glucose tolerance occurs during sleep, influencing nocturnal brain and tissue

glucose utilization. Therefore, chronic sleep disturbances and sleep apnea in the older individuals may be associated with alterations in the body metabolism. With declined physiological functions and sleep patterns, the appetite may be altered in older adults, resulting in increased susceptibility to GI tract and other metabolic disorders. Age-related perturbations in gut microbial structure and microbiome caused by diet and other factors appear to affect the circadian rhythm, promoting metabolic disorders and obesity.

Ageing-associated alterations in GI physiology influence the amount and types of nutrients delivered to the small intestine and colon, thereby affecting the intestinal microbiota composition and functionality of these segments. A number of studies have compared the fecal microbiota's composition in elderly versus young adults. The fecal microbiota was found to be similar in some studies (Biagi et al. 2016), whereas other investigators reported significant differences in the quality of microbiomes (Mariat et al. 2009). Generally, the gut microbiota among elderly was found to be highly variable. Detailed characterization of fecal microbiota composition was described by Cătoi et al. (2020) and He et al. (2020). They found that in comparison with younger adults, the fecal microbiota in elderly consisted of a smaller amount of *Bifidobacteria*, whereas *Clostridia*, *Lactobacillus*, *Streptococcus*, *Enterobacteriaceae* species were greater in the elderly than that of younger adults. It is worth mentioning that the intestinal microbiota compositional changes occurring during ageing are most likely associated with health status of the elderly and confounding factors such as frailty, comorbidity, and living conditions (rural vs. urban), dietary intake, hygiene, or antigen exposure. Despite the typical microbiota profile observed in elderly, it is hard to change the intestinal microbiota with dietary supplements and functional foods like probiotics, prebiotics, or synbiotics. However, some studies in elderly have shown changes in fecal microbiota composition with probiotics, where pronounced changed occurred in the amounts of *Bifidobacteria* spp. e.g., *Bifidobacterium lactis* HN019, *Bifidobacterium longum* 46, and *Bifidobacterium longum* 2C (Salazar et al. 2017). Although intake of probiotics is generally considered to be safe, extra caution is warranted in subjects with lactose intolerance and impaired host defense mechanisms. Popular prebiotics containing galacto-oligosaccharides, inulin, or fructo-oligosaccharides can cause increase in defecation frequency, thus altering the bowel habits in constipated elderly (Tiihonen et al. 2010). It seems that gut microbiota among older adults is affected by a broad range of potentially confounding factors, such as lifestyle (e.g., excessive drinking and smoking), health status, sedentary habits, obesity, medical treatment (antibiotics), probiotics, healthy eating behavior, living conditions, and food insecurity rather than by ageing alone. The influence of antidepressants on gut microbiomes has been reported recently by a number of research groups, of course, with varying results (Lukic et al. 2019; Bastiaanssen et al. 2019; Mikocka-Walus et al. 2019).

While gut microbiomes are essential for maintaining good health and gut-brain interaction, the dysbiosis can cause chronic non-communicable diseases (NCDs), including diabetes mellitus, obesity, metabolic syndrome, cardiovascular diseases, and neurodegenerative disorders. For example, cyanobacteria can secrete neurotoxin

like β-*N*-methylamino-alanine, which contributes to β-amyloid accumulation in Alzheimer's disease (Bhattacharjee and Lukiw 2013). Over-production of tryptophan metabolites by the gut microbiota may increase oxidative stress resulting in cell death, tau phosphorylation, and tangle formation in Alzheimer's disease (Westfall et al. 2017). Therefore, probiotics and dietary amino acid balance, especially tryptophan, remains an important strategy to promote healthy gut microbiome environment and to prevent neurological disorders and other NCDs.

15.4 Age-Related Alterations in Drug Absorption, Distribution, Metabolism, and Excretion (ADME) and Consequences

Age-associated pathophysiological changes in the body organs not only alter ADME, but also pharmacokinetic (PK) and pharmacodynamics (PD) parameters. In simple terms, PK is defined as *what the body does to the drug* in order to make the drug metabolites water-soluble for excretion into the bile, feces, and urine, while PD is defined as *what the drug and/or its metabolites do to the body* to produce pharmacological actions. The major components of PK consist of volume of distribution (V_d), maximal concentration (C_{max}) reached after absorption, time to reach maximal concentration (T_{max}), half-life ($T_{1/2}$), area under the curve (AUC), and bioavailability. Most of the PK components get altered with age in every man and woman. Some other factors involved in causing differences in PK and PD are inter-individual variability (slow and fast metabolizers), inter-ethnic and inter-racial variations, and exposure to herbal and dietary supplements.

15.5 Absorption and Bioavailability

Aging is associated with alterations in gastrointestinal motility, gut hormones, and digestive secretions, blood flow, and absorptive surface of the GI tract. Generally, in elderly individuals, absorption problems are more likely to be related to any underlying disease conditions, e.g., diminished splanchnic blood flow (due to cardiac insufficiency) or atrophic gastritis (Murad and Kitzman 2012; Birnbaum et al. 2003). Gastrointestinal pH can markedly affect oral drug absorption and bioavailability because of the significant influence on drug dissolution, solubility, release, stability, and intestinal permeability. Atrophic gastritis affects the absorption from the stomach and bioavailability of micronutrients like dietary vitamin B12, iron, and calcium may be perturbed (Rodriguez-Castro et al. 2018). Ideally the digestion of dietary vitamin B12 takes place in the presence of gastric acid in the stomach and the binding of vitamin B12 to the intrinsic factor happens further in the small intestine. This malabsorption could be corrected by the administration of diluted HCl and with the protein-bound vitamin B12. Another alternative to enhance the vitamin B12 absorption in individuals with atrophic gastritis is by lowering bacteria in the proximal small intestine by antibiotics. However, oral vitamin B12 supplements or foods fortified with vitamin B12 can be prescribed or recommended in elderly

individuals with atrophic gastritis (Russell 2001), and in this case, the absorption process itself is hardly affected. Decreased iron absorption has been reported in achlorhydric subjects, as stomach acid serves to keep iron in ferric form until it reaches the absorptive sites in duodenal mucosa. Ascorbate can chelate ferric ions and increase absorption at neutral or slightly alkaline pH range. However, heme-iron (found in meat) does not get affected by lack of acid and is thus normally absorbed in subjects with atrophic gastritis. In elderly persons, reduced absorption of calcium is linked with decreased vitamin D absorption and lesser synthesis in skin, as well as decreased vitamin D receptor expression in the intestinal epithelial cell, and impaired conversion of 25-hydroxy vitamin D to the active hormonal form 1,25-dihydroxy vitamin D (Holick et al. 1989).

Arguably, elderly subjects do not maldigest or malabsorb macronutrients because they usually have a large reserve capacity of both the pancreas and small intestine. Therefore, taking into consideration the total pancreatic reserve capacity and total length of the small intestine, the small digestive decreases of macronutrients become clinically irrelevant (Russell 2001). In elderly individuals with achlorhydria secondary to atrophic gastritis, the orally administered drugs like ketoconazole, ampicillin, and H_2 antagonists, which require acidic environment for absorption, may show low absorption or diminished efficiency of proton pump inhibitors (Hurwitz et al. 1997). Compared to younger subjects, elderly individuals often show $>25\%$ increase in bioavailability of orally administered drugs due to reduced first pass hepatic metabolism (Wilkinson 1997), thereby suggesting the need for dose adjustment in the elderly patients. On the other hand, higher doses of prodrugs (e.g., codeine, propranolol, enalapril, perindopril, simvastatin) may be required in geriatric patients to obtain the desired AUC for the active drug as opposed to the younger individuals (Hilmer 2008). Elevated levels of gastric pH in the elderly with atrophic gastritis may also affect the bioavailability of formulations that rely on low pH to dissolve an external coat. The formulations of constant drug delivery rate that are independent of pH or gastrointestinal motility may be less affected by ageing (Hilmer 2008).

15.6 Distribution

Orally administered xenobiotics reach the circulation either free or bound to blood proteins, mainly albumin or alpha-1-acidic glycoproteins (i.e., bound drug fraction). In geriatric patients, albumin content of plasma is generally low due to the diminished liver function that increases unbound fraction of drugs for distribution. This phenomenon influences distribution properties of highly albumin-bound drugs like phenytoin, coumarins, and pethidine. The transfer of a drug from circulation or central compartment to the peripheral tissue compartments is called volume of distribution. Volume of distribution (V_d) is defined as the apparent volume into which the drug distributes to achieve the desired therapeutic plasma concentration. It is a proportionality constant related to the amount of administered drug in the body and the concentration of drug in the reference fluid (e.g., whole blood or plasma). V_d depends on the plasma protein binding, lipid to water partition coefficient,

tissue-binding properties, and transporters. Depending on the plasma protein binding, drugs can have small V_d (e.g., heparin and warfarin) or large V_d (e.g., risedronate). A typical incremental decrease in albumin and increase in α1-acid glycoprotein levels in elderly are unlikely to have large effects on drug distribution. But transmembrane protein may alter with aging. Extracellular space of the central nervous system (CNS), which is guarded by the blood-brain barrier (BBB) from non-selective crossing of xenobiotics, is a special compartment from the pharmacological point of view. The BBB impairment can affect drug disruption process in the CNS, which is generally associated with neurodegenerative disorders (memory loss and cognition) among the elderly. Besides the morphological alterations of the cellular elements (endothelial cells, astrocytes, pericytes, microglia, neuronal elements), the BBB changes also occur at the molecular level (tight junction proteins, adheres junction proteins, membrane transporters, basal lamina, extracellular matrix), and these pathologies are the major contributors to the onset and progression of neurological disorders. One of the most important transporters at the BBB is the multidrug transport protein P-glycoprotein (P-gp), encoded by MDR1/ABCB1 and belonging to the family of ATP binding cassette (ABC) transporters. P-gp is highly expressed at the BBB vessel walls of the brain capillaries, where it functions as an efflux pump to restrict the entry/distribution of many different drug moieties from blood to the brain. van Assema et al. (2012) have reported an age-associated decline in the expression and function of ABCB1 transporters at the BBB through PET studies. This evidence was further extended by Bauer et al. (2017), confirming that an age-associated reduction occurring in ABCB1 expression and function at the BBB leads to higher increase in the brain distribution of ABCB1-selective substrates like (R)-[^{11}C]-verapamil when ABCB1 is partially inhibited, viz., after the consumption of polyphenolic plant-derived fresh foods. But this could result in an elevated risk of ABCB1-mediated drug-drug interactions (DDIs) at the BBB in elderly persons, which may have important adverse consequences for pharmacotherapy in patients of advanced age. Antidepressant compounds need to penetrate the BBB to reach the site of action within the brain; therefore, inhibition of P-gp transport proteins can have marked impact on the efficacy of antidepressant drugs (O'Brian et al. 2011). Currently, CYP-450 genotyping pilot studies are being conducted to map clinical feasibility of transport-protein genotyping. Thus, the ABCB1-guided antidepressant treatment strategy is not a dream anymore (Zeier et al. 2018).

15.7 Metabolism

Metabolism involves the biodegradation of drug molecules, mainly by hepatic cytochrome 450 coenzymes, to be transformed into more polar and water-soluble compounds ready for clearance into the bile, feces, and kidney. Clearance (CL) is defined as an elimination rate constant (k_e) relating to the rate of elimination of a drug from the body and/or the plasma clearance (C_{pl}). It determines the maintenance dose required to keep optimal therapeutic drug concentration in the plasma.

Clearance by each organ (liver, kidneys) depends on the blood flow rate to the organ as well as the extraction ratio. Total body clearance is composed of the sum of individual organ clearances. Another significant parameter to be considered in drug metabolism is half-life ($t_{1/2}$). It is defined as the time taken to reach one-half of the original amounts of drug in the body or plasma level. Drug's half-life depends on V_d and CL. Since the age-related change in V_d is relatively small, mostly the CL reduction is seen with ageing, resulting in prolonged half-life of a drug in geriatric patients. Usually, it takes longer period for the drug to reach steady-state as well as longer interval for elimination in frail and elderly. Thus, dosing schedules and drug amounts must be carefully monitored to adjust dosages in elderly patients to avoid ADRs (Hilmer 2008). Liver is the major xenobiotic metabolizing organ in humans and many other species. For hepatic clearance (CL_h), the portal and arterial hepatic blood flow play a key role in determining the systemic exposure to drugs and metabolites. Aging leads to a number of significant changes in the liver, including reduction in size, blood flow, drug-metabolizing enzyme capacity and development of pseudo-capillaries. Several other factors like comorbidity, concomitant medication, frailty, and epigenetics can also influence hepatic clearance of drugs. Generally, hepatic blood flow is reduced by approx. 40% in old age with a corresponding reduction in clearance of highly extracted substrates, such as morphine, propranolol, verapamil, and amitriptyline (McLean and Le Couteur 2004). Hence, age-related changes in the liver not only cause decrease in the hepatic clearance of unbound drug, but also influence the pharmacological response to medicines in older people. Ageing may affect both phase I (hepatic metabolism by CYP450 coenzymes) and phase II metabolism (glucuronidation, sulfation, GSH-conjugation, etc.). The age-related clearance would be especially affected for drugs that undergo mandatory oxidation (dealkylation, hydroxylation, deamination) by the microsomal cytochrome P450-dependent mono-oxygenase systems. Since liver volume and blood flow decline with age, partly due to the impaired regeneration capacity, the diminished clearance would most likely occur for drugs that exhibit first-pass kinetic profiles (Schmucker 2001).

15.8 Excretion

Kidneys are the major organs for excretion of unchanged drug and/or metabolites via glomerular filtration, tubular secretion, and tubular reabsorption. A gradual decline in the renal function with advancing age reduces the excretory capacity of the kidneys. Hence, in order to assess the excretion of xenobiotics and their metabolites, evaluation of blood flow levels and renal function become crucial parameters to determine the glomerular filtration rate (GFR). Irrespective of intestinal absorptive defects, creatinine clearance and decline in urinary D-xylose excretion account for the reduction in renal clearance (Russell 2001). One of the major contributing factor to reduced metabolic disposition of drugs in old age may be decreased tissue perfusion, particularly to less perfused organ/tissue such as skeletal muscle (Payne and Bearden 2006), and fat tissue (Nnodim 1988). One of the most important

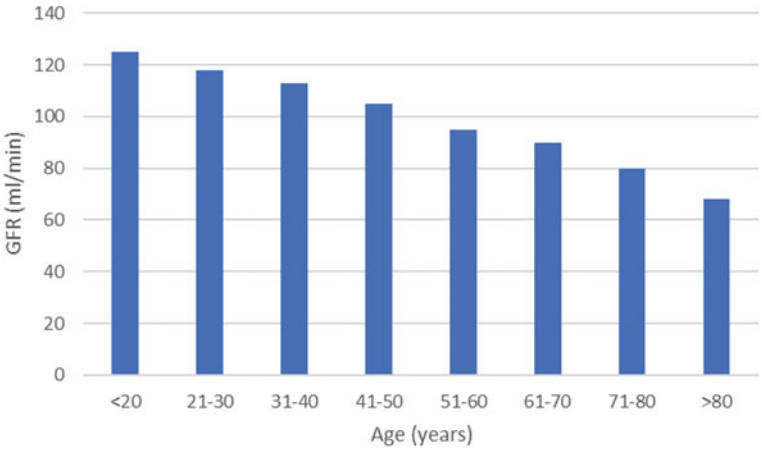

Fig. 15.1 Age-dependent GFR functions (based on cumulated data of several studies: Coresh et al. 2003; Berg 2006; Pottel et al. 2017)

changes that occur in old age is decline in GFR (Fig. 15.1). To account for the reduced GFR, the Cockroft and Gault Equation (see Eq. (15.3)) is widely used for dose adjustment of drugs mainly excreted in the urine of elderly patients. This equation is applied for dose adjustment of gentamicin, digoxin, and lithium, which are primarily eliminated via the renal route and have narrow therapeutic margins (McLean and Le Couteur 2004). The monitoring of GFR is very important prior to the initiation of lithium therapy, particularly when given in the presence of comorbidity and polypharmacy. Ideally, lean body weight and creatinine clearance predict steady-state lithium concentrations. The ratio of dose-serum lithium concentration may be significantly correlated with age during chronic dosing (Vestergaard and Schou 1984). Hence, assessment of GFR and close monitoring of therapeutic drug levels are recommended for elderly subjects to avoid any ADRs due to declined renal functions.

In summary, the most important PK and PD changes among the elderly are related to ADME alterations: namely, reduced absorption of drugs from the GI tract, decreased blood flow in liver and kidney, lesser hepatic metabolism, and renal excretion. Age-dependent kidney hypofunction expressed by GFR steadily declines by ageing (Fig. 15.1). While the impact of decreased GFR is highly significant among elderly men and women, it is also substance and patient dependent. Thus, there is no universal rule to calculate how to adjust drug dosing by age. Monitoring the optimal therapeutic drug levels by the healthcare workers at least in the dose adjustment period and in case of changes in health parameters or living conditions would be an excellent strategy to avoid ADRs in the frail and elderly patients. Extra precaution should be exercised for administering drugs in single kidney patients as well as patients undergoing dialysis. Such approach would amount to a personalized medicine. It is also important that elderly patients should

be enrolled in clinical trials to learn more about the pharmacometabolomics and assessment of safety, efficacy, and optimal dose schedules of new drugs.

15.9 Miscellaneous Factors Involved in ADME, PK, and PD Alterations in Elderly

Elderly population is particularly susceptible to ADRs because of comorbidity, polypharmacy, and age-associated changes in ADME and PK/PD properties of drugs. A thoughtful strategy should be used to avoid drug-drug and drug-herbal/food interactions and compliance problems. Physicians should design a simple drug regimen with one or two treatments per day and decide whether a multidrug therapy is necessary or the exposure can be avoided. The first issue regarding orally administered drugs is gut metabolism prior to absorption that impacts on the bioavailability of drugs. Most often, the role of gut metabolism by the CYP 450 coenzymes and transporters like P-gp is ignored by the physicians. Heart failure and achlorhydria have been mentioned earlier as two reasons requiring drug dose adjustments in treating elderly patients. In addition to oral drug administration, it is worth mentioning about the topically applied medications. The epidermal and subdermal layers of skin get atrophied in elderly because of reduced tissue blood perfusion; hence, the rate and extent of transdermal drug absorption are expected to be reduced in the skin of old age patients (Kaestli et al. 2008). Some barrier for absorption is, however, observed in the subcutaneous and muscular tissue. It is therefore recommended that subcutaneous and intramuscular injections should be avoided in frail patients because of the erratic absorption and high risk of sterile infiltrates (Trautinger 2001).

Decrease of muscle mass and relative increase in fat tissue may also alter *Vd* of drugs. Therefore, this concern should be taken into consideration in light of the lipophilicity of drug molecules. Presently, body impedance analysis (BIA) is an economic and reliable method to assess the ratio of fat mass vs. fat free mass, and thus failure in dosage can be avoided (Sampson 2013). Use of body mass index (BMI) or body composition parameters for daily drug dose calculations may be illusive due to the accidental presence of ascites or other fluid retentions. However, PK and PD alterations of lipophilic agents have been demonstrated in obese patients. For example, in obese patients (BMI > 30 kg/m^2), dose adjustments are required for several classes of drugs, such as general anesthetics, opioids, analgesics, anticoagulants, antidiabetics, oral contraceptives, neuromuscular blockers, β-blockers, antibacterials, anticancer drugs, psychotropics, and anticonvulsants (Telessy and Buttar 2017). Usually, the whole body water content falls by 10–15% in 80-year-old person. Therefore, the V_d of hydrophilic drugs is expected to decrease in elderly; hence, administration of equivalent doses given to younger individuals will result in higher plasma concentrations among elderly. This has been observed in the case of aspirin, d-tubocurarine, edrophonium, famotidine, and lithium therapy. Use of diuretics reduce the extracellular spaces and further accentuate risk for drug intoxication (Turnheim 1998).

The working capacity of liver in geriatrics should also be considered for estimation of safe and effective drug doses. It is well known that liver dysfunction, as briefly mentioned before, not only reduces the first-phase metabolic clearance of certain drugs, but also the biodegradation and/or biliary excretion of unchanged drugs and their metabolites. Also, low production of plasma proteins by the liver, especially albumin, can have strong influence on drug transport and distribution in the body (Verbeeck 2008). No consistent relationship has been observed between age and microsomal cytochrome P_{450} coenzymes that are mainly responsible for hepatic phase I metabolism. However, under certain in vivo conditions, metabolic clearance of some drugs like amiodarone, amitriptyline, triazolam, fentanyl, nifedipine, warfarin, and verapamil are decreased by 20–40%, whereas the clearance remains unchanged for alfentanil, diazepam, paracetamol, diclofenac, and citalopram, irrespective of which CYP450 coenzyme system is involved. These changes could be attributed to high or low drug extraction by the liver. As the blood flow through the liver declines in elderly, drugs extensively cleared by the liver display an age-related decrease in metabolic clearance. Generally, interindividual variations in metabolic drug degradation by CYP450 coenzymes exceed the decline caused by aging. The nutritional status of a patient also has a marked influence on the rate of drug metabolism. In frail elderly, drug metabolism is diminished to a greater extent than in elderly with normal body weight (Walter-Sack and Klotz 1996).

15.10 Formulas for Drug Dose Adjustments in Frail Elderly With Special Reference to Age-Dependent GFR Functions

As alluded to earlier, one of the most significant changes that occur in old age is decline in GFR. The age-dependent GFR functions are illustrated in Fig. 15.1. Reduction in kidney blood flow is accompanied by decreased drug elimination and impaired transmembrane transporter functions in the kidneys as well as PK & PD alterations among elderly. Disregarding the renal drug elimination will result in increased drug serum levels, because age-related decline in renal functions are closely related to high incidences of ADRs (Morley 2007). For drugs following linear pharmacokinetics, a reduction in renal clearance can be compensated by correcting the maintenance dose by a dose adjustment factor (Q) and by correlating endogenous creatinine clearance (CL_{CR}) as shown in Eq. (15.1).

$$D'_m = D_m k'_e / k_e = D_m Q, \tag{15.1}$$

where k_e is the elimination rate constant. The prime ($'$) designates value in old age.

Creatinine clearance (CL_{CR})-based drug elimination is calculated as follows:

$$Q = Q_0 + (1 - Q_0)\, CL'_{CR}/CL_{CR} \tag{15.2}$$

where Q_0 is the non-renal elimination fraction and Q is dose adjustment factor.

CL'_{CR} as a function of age and serum creatinine concentration can be calculated from the Cockroft and Gault Eq. (15.3):

$$CL'_{CR} = (140 - \text{age}) \times \text{body weight (kg)}/72\ C_{CR} \quad (15.3)$$

where age represents the patient's age in years and CL_{CR} is creatinine concentration in serum (mg/dL).

This equation gives creatinine clearance for men, and for women the clearance can be obtained by multiplying the value by 0.85 to account for the lower skeletal muscle mass in women. Nevertheless, the search for a better equation to evaluate kidney function and GFR is still going on for computing drug dosages in diverse populations (Diao et al. 2021).

Elderly patients with comorbid conditions generally receive multiple drugs on a daily basis. In majority of these patients, the kidney diseases complicate the admonition of prescription drugs. Hence, family physicians, surgeons, and pharmacists are always in a dilemma how best to measure renal function and decide drug dose schedules. Impaired kidney function results in reduced drug clearance, drug accumulation in the body, and risk of ADRs, which could sometimes be life-threatening. Therefore, over-estimation or under-estimation of kidney function can lead to inappropriate drug dosing or unnecessary discontinuation of potentially essential drugs. Serum creatinine-based GFR or creatinine clearance prediction equations [Modification of Diet in Renal Disease (MDRD) and Cockroft-Gault (CG)] are important tools for identifying geriatric patients with chronic kidney disease (CKD) and for selecting proper drug doses in these patients. Serum cystatin-C is an alternative biomarker for impaired renal function in elderly patients that ranges between slightly and clearly better than the diagnostic accuracy of creatinine. It is considered to be a suitable biomarker in elderly, since it is less sensitive to metabolic and extra renal factors than creatinine. Hojs et al. (2010) did analysis of 234 patients aged 65-years or older and found that serum creatinine-based formulas had slightly lower diagnostic accuracy than cystatin C-based formulas using ^{51}Cr EDTA clearance as a reference standard. The Hoek et al. (2003) formula had the highest accuracy, whereas the Grubb et al. (2005) formulas showed reasonable accuracy compared to ^{51}Cr EDTA clearance (Hoek et al. 2003; Larsson et al. 2004).

15.11 Dose Adjustment Suggestions for Antidepressant Drugs Commonly Prescribed to Elderly

Depression is the frequently encountered psychiatric disorder among the elderly with 10–20% cases of major depressive disorder and 10% having depressive symptoms (von Moltke et al. 1993; Barua et al. 2011). In the USA, more than 58 million Americans are inflicted with mental disorders. Elderly depressed individuals usually have altered physiological functions from normal ageing and concomitant medical conditions leading to changes in the metabolic disposition of psychotropic drugs. Therefore, dose adjustments are required to avoid ADRs. Meta-analysis of

21 antidepressants showed that in order to derive better clinical benefits, the dose adjustment of antidepressants should be based on both age and dose combined covariates than age or dose separately (Holper 2020). Presently, there are four major categories of antidepressants, viz., selective serotonin reuptake-inhibitors (SSRIs), monoamine oxidase-inhibitors (MAOI), the presynaptic noradrenalin-receptor antagonists, and others. Due to limitations of space, we are not able to discuss every member of the antidepressants, but some typical examples of the main representatives of the groups involved are described in detail.

Tricyclic antidepressants (TCAs) such as amitriptyline, imipramine, desipramine, nortriptyline, and tetracyclic maprotiline constitute old and traditional agents used in the pharmacological treatment of depression in elderly patients. These molecules are characterized by incomplete absorption, varying systemic availability secondary to hepatic first-pass extraction, lipid solubility, and high degree of tissue and protein binding (Ereshefsky et al. 1988). The use of these drugs is of highest concern in elderly as a result of increased drug sensitivity or because of increased plasma concentrations secondary to altered drug disposition. CNS toxicity, orthostatic hypotension leading to dizziness or falls, and direct electrophysiological cardiac effects (QT prolongation) that may trigger cardiac arrhythmia are the major concerns with these agents (Taylor 2015). CNS-induced toxicity by TCAs is generally under-reported, because it may be difficult to diagnose in the early stages when it may be mistaken for increasing depression or emerging psychosis. In severe form, it may appear as overt delirium or seizures. Several clinical studies have concluded that both TCA plasma concentrations and age are risk factors for TCA-induced delirium; however, all antidepressants can contribute to delirium (Alagiakrishnan and Wiens 2004).

At present, selective serotonin reuptake inhibitors (SSRIs) are the most prescribed class of antidepressants, and newer molecules often have more selective benefits (MacQueen et al. 2016). The main members of SSRIs are fluoxetine, paroxetine, sertraline, citalopram, and escitalopram, which are frequently used in clinical practice. Due to the weak receptor affinity, they have better tolerability than TCAs. However, fluoxetine due to its longer half-life and subsequent higher side effects and paroxetine due to the high anticholinergic effects are not recommended for elderly patients. SSRIs are usually well absorbed from the GI tract and represent high protein binding in the blood stream. Venlafaxine, duloxetine, and reboxetine produce dual effect s (serotonin and norepinephrine), and are selectively norepinephrine reuptake inhibitors (SNRIs) with similar efficacy to TCAs, but have weaker autonomic side effects. Venlafaxine may increase blood pressure. Fluoxetine and paroxetine are also strong inhibitors of CYP450 coenzymes; therefore, they have high potential to cause pharmacokinetic drug-drug interactions. Less potent inhibitors of this class are citalopram and escitalopram. The abovementioned representatives of SNRIs exert weak interaction activity with other drugs.

Because of the high rates of side effects and drug-drug interactions (DDIs), the monoamine oxidase inhibitors (MAO) are not considered the first- or even the second-line of treatment for depression in the elderly. Thus, we do not intend to go into many details about this group of drugs. Owing to their synergistic effect with

Table 15.1 Basic reference data of frequently used antidepressant drugs obtained from young healthy volunteers (data source: Drug Product Monographs)

Ingredient	Usual maintenance dose (mg/day)	Terminal HL (hours)	Elimination (organ, %)	Main metabolic pathway (CYP isoenzyme)	Its inhibitory effect on CYP enzyme	Need for age-dependent dose adjustment
amitriptyline	100-250	25	kidney	2D6, 2C19, 3A4	**2C19**, 1A2,(2D6)	dose reduction recomm.
imipramine	150-200	9-28	kidney 80%, liver 20%	2D6,	**2C19**, 1A2, (2D6)	careful dose escalation
desipramine	100-250	7-60	kidney 80%, liver 20%	2D6, 1A2, 3A4, 2C	(2D6, 2C19)	
nortriptyline	100-150	25	kidney	2D6	(2D6, 2C19)	dose reduction recomm.
doxepin	150-200	6-24	kidney	**2D6**	2D6, 2C19	
fluoxetine	5-40	96-144	kidney, 60%	**2D6**	**2D6**, **2C19**, 2C9,(3A4)	0
paroxetine	20-30	24	liver 46%	2D6	**2D6**, (1A2, 2C9,2C19)	0
sertraline	50-150	26	kidney 40%, liver 40%	3A4, 2C19	2C19, (1A2, 2D6,3A4)	0
citalipram	20-40	36	hepatic 85%, kidney 15%	2C19, 3A4, 2D6	(1A2)	dose reduction recomm.
escitalopram	10-20	30	kidney	**2C19**, 3A4, 2D6	2D6	dose reduction recomm.
venlafaxine	150-225	5	kidney, 87%	(2D6)	(2D6)	0
duloxetine	40-120	8-17	kidney	1A2, 2D6	2D6	0
mirtazepine	15-45	20-40	kidney 75%, liver15%	2D6, 1A2, 3A4	(2D6)	0
reboxetine	8-12	13	kidney 78%	3A4	0	0
agomelatine	25-50	1-2	kidney, 80%	**1A2**, (2C9, 2C19)	(1A2)	0
tianeptine	25 - 37,5	3	kidney	non-CYP	0	dose reduction recomm.

1A2 strong effect, *2D6* medium effect, *(1A2)* weak effect, *0* no effect/not required

other agents, the MAO inhibitors exert undesirable pharmacodynamic interactions with all other antidepressants, especially with TCAs and SSRIs, including the high risk for serotonin syndrome with mirtazapine, reboxetine, venlafaxine, and tianeptine. Mirtazapine and mianserine are relatively safe alpha-2-adrenoreceptor antagonists in the elderly. Both are well tolerated by patients with cardiovascular disease, their use results in weak weight gain and sedation, but they neither have influence on the CYP 450 coenzyme system nor is their metabolism affected by majority of other drugs.

Finally, the mixed group of antidepressant drugs include agomelatine and tianeptine. Both are relatively newly approved substances with less clinical experience compared to the members of aforementioned groups. Agomelatine is a melatonergic analogue acting as MT1/MT2 agonist and 5-HT2C antagonist. It is 90% metabolized by CYP4501A2 coenzyme and should be used carefully together with CYP4501A2 strong inhibitors (e.g., celecoxib) because serum levels of agomelatine may rise (He et al. 2018). Tianeptine is a mu-opioid receptor agonist (MOR) that elicits its effects via modulation of glutaminergic pathway (Samuels et al. 2017). This compound has demonstrated efficacy in patients resistant to SSRI therapy and avoids some negative side effects (e.g., sexual dysfunction) seen with SSRIs. Its therapeutic safety in the elderly has been affirmed by some investigators. The main PK and PD parameters of the frequently used antidepressants are summarized in Table 15.1. Clinical and pharmacological data, including PK details of few novel antidepressants were recently reported by Faquih et al. (2019). Due to the scarcity of population-based data, these new compounds were not included in the tables.

Age-associated metabolic changes in the PK and PD characteristics of psychotropic medications cause increased prevalence of iatrogenic effects or unwanted ADRs. Much pronounced biochemical and pathophysiological changes have been observed in the CNS of older persons regarding neuro-hormones and neurotransmitters:

including acetylcholine, dopamine, serotonin, melatonin, and kynurenine. The deficiency of these neurotransmitters is associated with depression, dementia and cognition decline, insomnia, and disturbance in circadian rhythms affecting the sleep and wake-fullness cycles (Moore and O'Keeffe 1999; Nayak et al. 2019). Serotonin is responsible for the modulation of some developmental events, such as neuron migration, cell differentiation, cell division, or synaptogenesis. Serotonin is also involved in the control of appetite, sleep, memory and learning, temperature regulation, mood, behavior (including sexual function), cognition or mental disorders among others (Nayak et al. 2019). Additionally, there are reports indicating changes in the blood-brain barrier (BBB) among aged people, especially with neurological disorders (Alzheimer's disease, Parkinson's disease) which influence the traversing of drugs from plasma through BBB and reaching CNS. The BBB changes are bound to cause unwanted responses to psychotropic drugs (Mehta et al. 2015). As mentioned earlier, inappropriate drug use and high incidence of ADRs are the major reasons for high hospital admissions or discontinuation of treatment among elderly. Geriatrics with dementia or cognitive impairment are at a much greater risk to drug-related ADRs. The main PK and clinical features along with typical ADRs associated with antidepressant drugs in elderly versus young adults are summarized in Table 15.2.

15.12 Clinically Significant Interactions Between Antidepressant Drugs: Mechanisms of Interactions and Prescribing Strategies

Drug-drug interactions (DDIs) are caused via ADME alterations, consequently producing changes in the bioavailability and PK profiles of co-administered drugs. Some DDIs may produce therapeutic benefits; however, most often DDIs cause unwanted side effects. Some DDIs can cause clinically adverse reactions resulting from PK changes. The PK interactions include all alterations during the ADME phase of one drug induced by another co-administered drug. Mostly, DDIs occur due to drug-induced inhibition or induction of CYP450 isoenzymes. The interactions may be additive or potentiative as well as agonistic or antagonistic in manner and may occur at the cellular or molecular level, or at the receptor site, and mechanistically can be classified as PK & PD interactions. There is some general information available about the CYP450 effects of antidepressants: there are known potent inhibitors of different CYP450 3A4 and CYP450 2D6 isoenzymes. For example, fluoxetine and paroxetine are potent inhibitors of CYP450 2C19 and can also act as inhibitors of fluvoxamine and TCA (amitriptyline and imipramine). As a rule, TCAs are victims of metabolism via hydroxylation; therefore, every CYP450 2D6 inhibitor can increase the serum levels of tricyclic antidepressant drugs. Combined administration of TCAs and SSRIs often results in strong PK interactions, which require careful titration of TCAs with SSRIs, and avoiding the combination may offer the best solution, too (English et al. 2012). DDIs are an important reason for hospital

Table 15.2 Clinical features of antidepressant drugs: safety and efficacy assessment in young adults versus elderly persons

Antidepressant	Clinical study	Clinical observations
Amitriptyline	Schulz et al. (1983)	The disposition of a single parenteral or single oral dose of amitriptyline was followed in seven young (mean age 22 years, range 21–23) and five elderly (mean age 71 years, range 62–81) healthy men. The mean systemic clearance did not change with age (10.8 ± 2.1 mL/min/kg in elderly and 12.5 ± 2.3 mL/min/kg in young subjects). Mean $t_{1/2}$ was longer in the older (21.7 ± 2.9 h) than in the younger group (16.2 ± 6.1 h) as a result of an increase in the volume of distribution (17.1 ± 2.4 and 14.1 ± 2.0 L/kg). The bioavailability and the fraction of the drug bound to plasma proteins did not change with age
	Ghose and Spragg (1989)	Elimination half-life of amitriptyline was 31 h in elderly subjects. PK parameters of amitriptyline were comparable to other published studies involving elderly people. Compared to placebo and lofepramine, amitriptyline produced drowsiness and dry mouth, reduced salivary volume and increased movement reaction time. These effects correlated with the plasma amitriptyline levels
	Henry et al. (1981)	The PK of amitriptyline (AMI) was evaluated in six healthy elderly volunteers (72–83 years of age) after a single dose of 125 mg of AMI-HCl. AMI was absorbed rather slowly (mean peak time 10.4 ± 1.6 h) but very efficiently (F: 0.59–0.75). The rate of formation of nortriptyline (NT) and the appearance clearance values (0.18–0.45 L/h/kg) of AMI were significantly lower than those previously described for younger subjects. Reversible alterations in ECG were observed in five cases concomitantly with AMI peak plasma concentrations. The results indicate the desirability of reduced and/or divided daily doses of AMI in the elderly
Imipramine	Gram et al. (1977)	C^{SS} of imipramine in 76 patients (20–65 years) who were given a range of dosages (150–225 mg/day) for 2–5 weeks were recorded. Women aged 30–39 years had lower concentrations than women 20–29, 40–49, and 50–59, or men 50–59 and 60–65 (all at the $p < 0.05$ level, or better). Men aged 30–39 years had lower concentrations than men 60–65 years
	Bjerre et al. (1981)	Six patients (64–78 years) received imipramine 50–200 mg/day. Plasma concentrations were determined by quantitative TLC and compared with six patients (62–79 years) receiving nortriptyline 40–100 mg/day. An increase in imipramine dosage generally resulted in a corresponding increase in imipramine concentrations; and a disproportionate rise in the metabolite desipramine was observed, making imipramine a

(continued)

Table 15.2 (continued)

Antidepressant	Clinical study	Clinical observations
		"difficult drug to use in the elderly," when compared with nortriptyline
	Glassman et al. (1979)	A group of 44 inpatients (34–76 years, mean 58.5 years) received imipramine to achieve a therapeutic imipramine plus desipramine concentration of at least 200 μg/L. An orthostatic change of 26 mmHg was seen during treatment, compared with a pretreatment drop of 10.9 mmHg
	Benetello et al. (1990)	26 patients received a single 25 mg dose of imipramine by intramuscular injection. 14 patients were between the ages of 66 and 77 years. A significant age difference in total body CL was observed only in males. The six younger men had a mean CL of 0.851 L/h/kg compared with 0.396 L/h/kg for the seven older men. The extent of formation of desipramine was also significantly reduced in the elderly regardless of gender
Desipramine	Antal et al. (1982)	Calculated CL after a single desipramine 75 mg oral dose in 12 non-institutionalized elderly patients (55 to 86 years, mean age 72 years). Based on calculated oral CL values, maintenance doses were chosen to produce C^{SS} at the low end of the presumed therapeutic range (30–50 μg/L). Mean oral CL was 1.78 L/h/kg (range 0.60–8.36), and mean $t_{1/2}$ was 21 h (range 9.4–36.5 h). Predicted CSs correlated with observed CSs ($r = 0.967$, $p < 0.0005$)
	Abernethy et al. (1985)	35 healthy volunteers aged 21–85 years received 50 mg orally. The $t_{1/2}$ was somewhat prolonged in elderly men compared with younger men (31 vs 21 h, $p < 0.05$), with oral CL slightly and not significantly reduced. For women, $t_{1/2}$ and CL did not change significantly with age. There were no changes in protein binding. Thus, little change in desipramine dose is required to obtain similar desipramine plasma concentrations during long-term administration for both young and elderly patients
	Kutcher et al. (1986)	ECG or EKG changes in 10 elderly patients 60–85 years of age (no younger control group) who were undergoing treatment with desipramine was analyzed. Significant changes in EKG parameters were prolongation in PR interval and QRS complexes, QTc was decreased, and a further observation that a significant correlation existed between PR and QTc changes and 2-hydroxy-desipramine concentrations. These investigators suggest the necessity of regular ECG monitoring for elderly patients
Nortriptyline	Rubin et al. (1985) Kragh-Sorensen and Larsen (1980)	The relationship between dose and C^{SS} in 33 patients (24–86 years) as well as the relation between age and CSs in 116 hospitalized patients (18–86 years) was evaluated. Groups were comprised of participants in different studies and concentrations were determined

(continued)

Table 15.2 (continued)

Antidepressant	Clinical study	Clinical observations
		by different analytical methods. The results indicated that nortriptyline C^{SS} were proportional to dosage. Furthermore, dose normalized C^{SS} appeared to increase with age, suggestive of altered disposition of nortriptyline in older patients
	Dawling et al. (1980)	Patients received a single dose of nortriptyline before starting long-term therapy; plasma nortriptyline concentrations were determined by GC with nitrogen-phosphorus detection CL of the initial dose was used to predict the regimen needed to bring C^{SS} into the 50–150 μg/L range. Predicted and actual mean C^{SS} were significantly correlated ($r = 0.71$, $p < 0.002$), suggesting this as a possible method for avoiding toxicity in the elderly
Doxepin	Ereshefsky et al. (1988)	A group of 61 patients (mean 57 years) undergoing plasma concentration were monitored from facilities throughout the state of Texas. Over 80% of the plasma concentrations were generated from inpatients. Based on C^{SS} of doxepin and dimethyl-doxepin, the investigators reported a weak but significant reduction in oral CL vs age, with $r = 0.16$ ($p < 0.002$). A comparison of mean oral CL values in patients less than 55 years vs those >55 years suggested that lower doses would be needed to achieve a targeted C^{SS} in elderly patients. In their database, doxepin was frequently prescribed for the elderly. Of the 211 patients treated with either imipramine ($n = 151$) or doxepin ($n = 61$), the mean age for doxepin recipients was 57 years compared with 28 years for imipramine
Fluoxetine	Altamura et al. (1994)	Fluoxetine has a nonlinear PK profile. Therefore, the drug should be used with caution in patients with reduced metabolic capability (i.e., hepatic dysfunction). In contrast, the PK values of other antidepressants are not affected by age. This finding together with the better tolerability profile of fluoxetine (compared with tricyclic antidepressants) makes this drug particularly suitable for use in elderly patients with depression. Furthermore, the PK parameters of fluoxetine are neither affected by obesity nor renal impairment
	Ferguson and Hill (2006)	Plasma concentration of fluoxetine and norfluoxetine in geriatric patients was higher than previously reported in the literature. Elderly women had a significantly higher serum level of norfluoxetine than men. The terminal half-life of norfluoxetine was longer in patients over the age 75; elderly women had a significantly slower rate of norfluoxetine elimination than similarly aged men
	Harvey and Preskorn (2001)	Fluoxetine AUC0-24, C_0, and C_{max} did not differ in young and elderly subjects. The norfluoxetine C_0 was 22% lower in elderly subjects ($p < 0.05$), with

(continued)

Table 15.2 (continued)

Antidepressant	Clinical study	Clinical observations
		comparable decreases in AUC0-24 and C_{max}. In the elderly volunteers, the $t_{1/2}$ for fluoxetine was 25% longer (5.0 vs. 4.0 days) and for norfluoxetine was 33% longer (20 vs. 15 days), although variability and sample size precluded statistical significance. Fluoxetine dosing inhibited CYP2C19 activity in both age groups, increasing the (S)- to (R)-mephenytoin ratio three- to four-fold ($p < 0.01$). The half-lives of fluoxetine and norfluoxetine at 40 mg/day were longer than commonly reported in the literature and may be longer in elderly subjects. Fluoxetine substantially inhibited the metabolism of the CYP2C19 substrate (S)-mephenytoin
	Gibbons et al. (2012)	Response and remission rates at 6 weeks were analyzed for 2635 adults. The antidepressant fluoxetine and venlafaxine are efficacious for major depressive disorder in all age groups, although more so in young and adults compared with geriatric patients
	Goldstein et al. (1997)	The high BMI group, but not the low/normal BMI group, had a statistically greater proportion of fluoxetine-treated patients who lost at least 5% of their baseline weight
Paroxetine	Bourin et al. (2001)	Paroxetine is well absorbed orally and undergoes extensive first pass metabolism that is partially saturable. Its metabolites are pharmacologically inactive. Steady-state levels are achieved after 4–14 days and an elimination half-life of 21 h is consistent with once-daily dosing. There is a wide inter-individual variation in the PK of paroxetine in adults as well as in the young and the elderly with higher plasma concentrations and slower elimination noted in the latter. Serious adverse events are, however, extremely rare even in overdose.
	Feng et al. (2006)	A two-compartment nonlinear PK model with additive and proportional error provided the best base model for description of the data of 171 subjects with mean age of 77 years. Weight and CYP2D6 polymorphisms were found to have a significant effect on maximal velocity (V_m), whereas sex had an effect on volume of distribution of the central compartment. The Vm estimates in each of the CYP2D6 phenotypic groups were 125 μg h^{-1} in poor metabolizer ($n = 1$), 182 μg h^{-1} in intermediate metabolizers ($n = 28$), 454 μg h^{-1} in extensive metabolizers ($n = 36$) and 3670 μg h^{-1} in ultra-rapid metabolizers ($n = 5$)
	Kaye et al. (1989)	In elderly subjects, there is wide inter-individual variation in steady-state PK parameters, with statistically significantly higher plasma concentrations and slower elimination than in younger subjects,

(continued)

Table 15.2 (continued)

Antidepressant	Clinical study	Clinical observations
		although there is a large degree of overlap in the ranges of corresponding parameters. In severe renal impairment, higher plasma levels of paroxetine are achieved than in healthy individuals after single dose. In moderate hepatic impairment, the PK after single doses are similar to those of normal subjects. Paroxetine is not a general inducer or inhibitor of hepatic oxidation processes and has little or no effect on the PK of other drugs examined
Sertraline	Oslin et al. (2000)	There were no differences in the tolerability of sertraline vs. nortriptyline. However, in this group of frail older adults, sertraline was not as effective as nortriptyline for the treatment of depression
	Ronfeld et al. (1997)	The terminal elimination half-life ($t_{1/2}$ beta) of sertraline was similar in young females, elderly males and elderly females (mean $t_{1/2}$ beta ranged from 32.1 to 36.7 h in these groups), but shorter (22.4 h) in the young males. The mean maximum plasma sertraline concentration (C_{max}) and the mean steady-state area under the plasma concentration-time curve from time zero to 24 h post-dose (AUC0-24) were also similar between the young females, elderly males and elderly females, but were approx. 25% lower in the young males. The time to C_{max} was unaffected by age or gender and ranged from 6.4 to 6.9 h
	Saiz-Rodriguez et al. (2018)	PK and PD parameters were similar in men and women. Polymorphisms in CYP2C19 and CYP2B6 genes influenced sertraline PK, with a greater effect on CYP2C19. Individuals carrying defective alleles for CYP2C19 and CYP2B6 showed higher area under the curve (AUC) and $T_{1/2}$. Moreover, CYP2C19*17 was related to a decreased AUC and $T_{1/2}$. No significant effect was found for polymorphisms in CYP2C9, CYP2D6, and ABCB1 on sertraline PK.
	Bondareff et al. (2000)	Nortriptyline treatment was associated with a significant increase in pulse rate, whereas sertraline was associated with nonsignificant decrease
Citalopram	Wu et al. (2020)	Most of the predicted PK values of citalopram after single oral dose administration were within the 70–130% range of the corresponding PK values obtained from observed data from eight studies. After multiple oral administrations, the percentage of C_{max} and AUC ranged between −21% and +25% and −31% and +21%, respectively
	Bezchlibnyk-Butler et al. (2000)	Citalopram is reasonably safe for elderly populations vulnerable to PK effects
	Cipriani et al. (2014)	Some older people may be more vulnerable to side effects associated with citalopram antidepressant, and decreased dosage is often recommended for them

(continued)

Table 15.2 (continued)

Antidepressant	Clinical study	Clinical observations
Escitalopram	Rao (2007)	Adolescents, elderly individuals, and patients with hepatic impairment do not have clinically relevant differences in PK compared with healthy young adults, implying that adjustment of escitalopram dosage is not necessary in these patient groups. Escitalopram is metabolized by CYP isoenzymes like CYP2C19, CYP2D6, and CYP3A4. However, ritonavir, a potent inhibitor of CYP3A4, does not affect the PK values of escitalopram
	Chung et al. (2017)	The (S)-enantiomer of citalopram has a potential QT prolonging effect. In a clinical study, 12 healthy elderly individuals received a single oral dose of escitalopram (20 mg), and their pharmacokinetics and QT effect data were compared with data from 33 younger adults obtained in a previous study. Serial blood samples for PK analysis were collected and ECG was performed up to 48 h post-dose. The elderly and younger adults showed similar PK profiles. The mean baseline-adjusted QT (dQT) time profiles were similar and the mean values of maximum dQT were not significantly different between the elderly and the younger adults
Venlafaxine	Hansen et al. (2017)	The median dose-corrected serum level for venlafaxine was 1.49 nmol/L/mg, while the dose-corrected serum level of men and women were 1.21 nmol/L/mg and 1.60 nmol/L/mg, respectively, after a median daily dose of 225 mg. The dose-corrected sum of venlafaxine and o-desmethyl-venlafaxine (ODV) was 8.91 nmol/L/mg versus 5.52 nmol/L/mg for patients above 64 years and below the age of 65 years, respectively. Dose-corrected plasma concentrations of venlafaxine and ODV are increased to a clinically significant degree in patients above the age of 64, and initiation of venlafaxine therapy in the elderly should be made cautiously and supported by drug measurements
	Allard et al. (2004)	Benefits of venlafaxine treatment in elderly patients with major depression were similar to those observed in younger adults as were reported adverse events or side effects
	Hefner et al. (2019)	In elderly inpatients aged $\geq$65 years, amitriptyline and venlafaxine induce significant QT prolongation depending on drug concentrations in blood
Duloxetine	Knadler et al. (2011)	Duloxetine achieves a maximum plasma concentration (C_{max}) of approx. 47 ng/mL (40 mg twice-daily dosing) to 110 ng/mL (80 mg twice-daily dosing) around 6 h after dosing. The elimination half-life of duloxetine is approximately 10–12 h and the volume of distribution is about 1640 ng/L. Patient demographic characteristics found to influence the PK of duloxetine include gender, smoking status, age, ethnicity, CYP450 2D6 genotype, hepatic function, and renal function. Of these, only

(continued)

Table 15.2 (continued)

Antidepressant	Clinical study	Clinical observations
		impaired hepatic function or severely impaired renal function warrant specific warnings or dose recommendations. Specifically, following oral administration in the presence of fluvoxamine, the area under the plasma concentration-time curve and C_{max} of duloxetine significantly increased by 460% (90%: CI 359, 584) and 141% (90%: CI 93, 200), respectively. In addition, smoking is associated with a 30% decrease in duloxetine concentration.
	Skinner et al. (2003)	Elderly participants >65 years of age had a safety profile of duloxetine comparable to their younger counterparts. Specific dose recommendations for duloxetine in the elderly are not warranted
Mirtazapine	Timmer et al. (2000)	Mirtazapine binds to plasma proteins (85%) in a nonspecific and reversible way. The absolute bioavailability is approximately 50%, mainly because of gut wall and hepatic first-pass metabolism. Mirtazapine shows linear pharmacokinetics over a dose range of 15–80 mg. The presence of food has a minor effect on the rate, but does not affect the extent, of absorption. The PK profiles of mirtazapine are dependent on gender and age: Females and the elderly show higher plasma concentrations than males and young adults. The elimination half-life of mirtazapine ranges from 20 to 40 h, which is in agreement with the time to reach steady state (4 to 6 days). Total body clearance as determined from intravenous administration to young males amounts to 31 L/h. Liver and moderate renal impairment cause an approx. 30% decrease in oral mirtazapine clearance; severe renal impairment causes a 50% decrease in clearance. Biotransformation is mainly mediated by the CYP2D6 and CYP3A4 isoenzymes. Inhibitors of these isoenzymes, such as paroxetine and fluoxetine, cause modestly increased mirtazapine plasma concentrations (17% and 32%, respectively) without leading to clinically relevant consequences. Enzyme induction by carbamazepine causes a considerable decrease (60%) in mirtazapine plasma concentrations
	Hilas and Avena-Woods (2014)	Nearly 15–20% of older adults experience unintentional weight loss and require intervention to maintain quality of life. In recent years, mirtazapine has gained attention not only for its antidepressant effects but also for its potential benefits in underweight patients. This agent has been found to increase appetite and weight in adults compared with placebo and other antidepressants
	Begg et al. (1989)	All of 12 elderly patients enrolled (aged 60–86 years) showed half-lives greater than or equal to 2.5 days with a mean of 6 ± 2.8 (SD) days. In six of the patients, the

(continued)

Table 15.2 (continued)

Antidepressant	Clinical study	Clinical observations
		profile of elimination was suggestive of saturable elimination. There was a marked variability in the elimination of mirtazapine in elderly patients
Reboxetine	Poggesi et al. (2000)	Reboxetine displayed linear PK, with dose-proportional changes, in elderly depressed patients. Mean total urinary recovery ranged from 4.06% to 6.17%. The mean area under the plasma concentration-time curve (AUCtau) and the maximum plasma drug concentration (C_{max}) showed considerable variation between patients given a dose of 4 mg/day, AUCtau was 1466–6866 ng h/mL, and C_{max} ranged from 169 to 663 ng/mL. It means C_{max} and AUCtau values are higher (and more variable) than in young adults. These observations support the use of a lower starting dose (4 mg/day) of reboxetine in the elderly
	Bergmann et al. (2000)	C_{max} in the healthy elderly was 271 ± 86 ng/mL, compared with 111 ± 28 ng/mL in the young subjects after a single 4 mg dose, although in both groups C_{max} was observed after 2 h. the AUC infinity was nearly four times that in the younger subjects (8345 ± 3107 ng h/mL vs. 2106 ± 881 ng h/mL) and the $t_{1/2}$ was twice as long (24 ± 6 h vs. 12 ± 3 h). Renal clearance was also reduced
	Hajós et al. (2004)	Unlike conventional tricyclic antidepressants (TCAs), reboxetine had only minimal sedative and cardiovascular liabilities, probably due to increased pharmacological specificity of reboxetine as compared with TCAs. Unlike serotonin reuptake inhibitors, this selective and specific norepinephrine reuptake inhibitor demonstrated a distinct side-effect profile with diminishing sexual dysfunction and GI tract side effects. The starting dose of reboxetine should be reduced by 50% in the elderly patients with renal or hepatic impairment or in patients receiving potent CYP3A4 inhibitors
Agomelatine	Fornaro et al. (2010)	Melatonin and its receptor agonists (e.g., agomelatine) help to correct age-related changes in circadian rhythm response to environmental stimuli in rodents and could prove to be useful in treating/preventing or delaying disturbances of circadian rhythmicity and/or sleep disorders in older people. In humans, agomelatine is well absorbed following oral administration, but absolute bioavailability is about 5–10% due to its high first-pass effect, which may be considered in special populations such as the elderly or hepatic disordered patients. Volume of distribution of approx. 35 L, and is 85–95% bound to plasma proteins. Extensively metabolized by the CYP450 isoforms 1A1, 1A2, and 2C9. The mean terminal elimination half-life is 2.3 h

(continued)

Table 15.2 (continued)

Antidepressant	Clinical study	Clinical observations
	Pei et al. (2014)	The intra-subject coefficient of variability calculated for C_{max} and AUC 0-T was 78.34% and 43.52%, respectively, in Chinese healthy subjects
Agomelatine	He et al. (2018)	A single dose of 30 mg/kg celecoxib significantly increased the area under the concentration-time curve (AUC) and maximum concentration of agomelatine. In addition, celecoxib inhibited the metabolism of agomelatine in the in vitro studies, which was determined to be by a competitive mechanism on CYP2C9 isozyme. These results indicated that celecoxib has an inhibitory effect on the metabolism of agomelatine both in vivo and in vitro
	Freiesleben and Furczyk (2015)	Agomelatine was found to be associated with higher rates of liver injury than both placebo and the four active comparator antidepressants used in the clinical trials for agomelatine, with rates as high as 4.6% for agomelatine compared to 2.1% for placebo, 1.4% for escitalopram, 0.6% for paroxetine, 0.4% for fluoxetine, and 0% for sertraline. As agomelatine has a potential risk of liver injury, clinicians must carefully monitor liver function throughout treatment. However, agomelatine's unique mechanism of action and favorable safety profile render it a valuable treatment option
Tianeptine	Zheng and Kim (2014)	The PK parameters were assessed in the 40 subjects after taking a single dose of 12.5 mg tianeptine sodium. In the randomized, 2-sequence, 2-treatment crossover study, tianeptine C_{max} for the test formulation was 283.13 ± 57.58 ng/mL (mean ± SD), and for the reference formulation was 272.50 ± 59.00 ng/mL. The AUC of tianeptine was 803.24 ± 180.94 ng h/mL for the test formulation and 792.27 ± 180.93 ng h/mL for the reference formulation. The geometric mean ratio (%) of the test to reference formulation was 104.04 (90% CI: 99.66–108.61) for C_{max} and 101.30 (98.01–104.71) for AUC. No clinically significant adverse events were observed during the study
Tianeptine	Saiz-Ruiz et al. (1998)	In a group of 63 elderly patients (mean age, 68.8 years; range, 65–80 years) with depressive symptoms (major depression, 55.6%; dysthymia, 44.4%) were included in a 3-month open multicenter study with tianeptine (25 mg/day). Forty-three patients (68.2%) completed the study. There were no drop-outs due to side effects. Total Montgomery and Asberg depression rating scale scores were significantly decreased ($p < 0.01$) on day 14, with a response rate of 76.7%. Improvements were also observed in anxiety and cognitive performance. Side-effects were seen only in 11.7% of patients, with no changes in laboratory or ancillary safety parameters. Tianeptine is thus effective and well tolerated in this category of patients

admissions amongst elderly population, and nearly 4.8% of admissions in elderly are caused by DDIs (Hines and Murphy 2011).

It has been reported that around 8.7–10% hospital admissions in older persons are frequently related to ADRs caused by NSAIDS, beta-blockers, and polymedication. In most cases, ADRs are preventable if the doctors are aware of prescribing the interacting drugs and inappropriate medications to older patients (Oscanoa et al. 2017). Increased risk of bleeding, prolongation of QT interval, and hyperkalemia are examples of the DDI consequences that expose elderly people with dementia and cognitive impairment to unnecessary risks if drug use is not properly monitored (Hosia-Randell et al. 2008). Many hospitalizations caused by DDIs could be avoided by careful selection of antidepressant medications (Bogetti-Salazar et al. 2016). Some clinically relevant interactions may result from the simultaneous intake of antidepressant drugs with herbal remedies (St. John's wort, Ginseng, Ginkgo biloba, Ashwagandha) and fruit juices (grape fruit, orange, and pomegranate). Healthcare providers should ask questions to their patients about the use of herbal remedies, dietary supplements, and fruit juices, and discourage concomitant ingestion of botanical products with psychotropic drugs.

Keeping in mind the scope of this review, we will focus on the DDIs targeting more precisely on the psychotropic drugs, which are related to antidepressants. DDIs are frequently observed in the geriatric population due to comorbidity and a wide range of polypharmacy. The selection of these kinds of interactions is rather subjective, but we will describe the DDIs which are more serious and most frequently observed within the elderly populations. It is worthy to note that many compounds may produce similar pharmacological effects due to their close chemical structures, CYP-metabolic pathways, or some other attributes mentioned in Table 15.3.

Balanced and safe prescribing is difficult to achieve in frail older adults affected by multiple comorbid conditions, especially with renal and hepatic disorders. The improvements in medical technologies and better nutrition and sanitation have expanded the life span of people worldwide. At the same time, the proportion of elderly people inflicted with multiple chronic diseases and requiring multiple drug therapies has also increased. Unfortunately, older patients with comorbid conditions are often excluded from clinical trials, and as a consequence the evidence coming from diverse studies may not be generalized to this population. In addition, the application of clinical practice guidelines, which are based on the evidence coming from randomized trials and meta-analyses, is problematic because such studies usually focus on specific disease and do not take into account the presence of comorbid conditions. The problem is further exacerbated by the fact that multiple conditions are often treated by different specialists, many of whom do not communicate with one another. As a result, the guidelines-driven therapeutic approach in elderly patients with multiple chronic abnormalities may produce undesirable consequences resulting from multiple drug regimens with increased risk of drug-drug or drug-disease interactions. In view of these circumstances, the International Association of Gerontology and Geriatrics in conjunction with WHO has suggested that drugs which are intended to be used in older population should undergo double-blind and randomized controlled trials in nursing homes and long-term care

Table 15.3 Clinically relevant interactions among antidepressant drugs and other medications: mechanisms of interactions and potential adverse consequences in the elderly

Reference	Drug combination	Potential consequences
PK/PD and mechanistic interactions		
Saraghi et al. (2018)	SSRI (fluoxetine, paroxetine)	Diminished therapeutic effect of drugs being substrates of CYP2D6, avoid combination with tramadol and codeine
Saraghi et al. (2018)	SSRI (sertraline, paroxetine) + warfarin	Increased risk of bleeding due to inhibition of CYP 2C9
Ellis et al. (2015)	Duloxetine + isocarboxazid, linezolid, procarbazine, rasagiline, selegiline, tranylcypromine	CNS toxicity or serotonin syndrome
Ellis et al. (2015)	Duloxetine + thioridazine	Risk of arrhythmia due to increased thioridazine levels
Ellis et al. (2015)	Duloxetine + metoclopramide	Increased risk of extrapyramidal reactions, or neuroleptic malignant syndrome
Ellis et al. (2015)	Duloxetine + clozapine	Increased risk of QT prolongation due to increased plasma-clozapine levels
Ellis et al. (2015)	Duloxetine + escitalopram, antiplatelet drugs	Increased risk of bleeding
Ellis et al. (2015)	Duloxetine + citalopram, fluoxetine, fluvoxamine, paroxetine, sertraline, tramadol, triptanes, venlafaxine	Increased risk of serotonin syndrome
Gareri et al. (2014)	Fluvoxamine + TCAs (amitriptyline, imipramine, clomipramine), mirtazapine	Increased serum-concentration due to inhibition of CYP1A2 isoenzymes
Gareri et al. (2014)	Carbamazepine, phenytoin, rifampin + TCAs (amitriptyline, imipramine, clomipramine), mirtazapine	Decreased serum-concentration due to induction of CYP1A2 isoenzymes
Gareri et al. (2014)	Fluvoxamine, fluconazole, ketoconazole, itraconazole, erythromycin + venlafaxine, clomipramine, citalopram, mirtazapine	Increased serum-concentration of antidepressants due to inhibition of CYP3A4 isoenzymes
Gareri et al. (2014)	Barbiturates, carbamazepine, phenytoin, rifampicin, topiramate + venlafaxine, clomipramine, citalopram, mirtazapine	Decreased serum-concentration of antidepressants due to induction of CYP3A4 isoenzymes
Gareri et al. (2014)	Fluoxetine, paroxetine, perphenazine propafenone, quinidine, thioridazine + TCAs (amitriptyline, clomipramine, imipramine, desipramine,)	Increased serum-concentration of TCAs due to inhibition of CYP2D6 isoenzymes

(continued)

Table 15.3 (continued)

Reference	Drug combination	Potential consequences
PK/PD and mechanistic interactions		
Zieglmeister and Hein (2003)	Ritonavir, ketoconazole, itraconazole + mirtazapine	Increased side effects of mirtazapine due to CYP3A4 inhibition
Zieglmeister and Hein (2003)	Ciprofloxacin, fluvoxamine + agomelatine, duloxetine	Increased side effects of agomelatine and duloxetine due to CYP1A2 inhibition
Zieglmeister and Hein (2003)	Carbamazepine + citalopram/ diazepam/felodipine/olanzapine	Reduction in clinical efficacy of citalopram, diazepam, felodipine, olanzapine
Zieglmeister and Hein (2003)	SSRI (citalopram, fluoxetine, paroxetine) and amitriptyline + warfarin	Increased risk of GI bleeding at initiating antidepressant therapy with the mentioned combination
Zieglmeister and Hein (2003)	Citalopram + simvastatin/ diltiazem	Increased efficacy of simvastatin, diltiazem; risk of myopathy
Bahar et al. (2018)	Paroxetine, fluoxetine, citalopram + metoprolol	Increased metoprolol AUC and early discontinuation of metoprolol therapy in the elderly
Gjestad et al. (2015)	Esomeprazole, omeprazole, lansoprazole + SSRIs (escitalopram, citalopram, sertraline)	Increased plasma levels of SSRIs due to inhibition of CYP2C19; risk of QT prolongation
Saraghi et al. (2018)	NSAID + SSRI (sertraline, paroxetine) and SNRI (venlafaxine, duloxetine)	15-times increase of risk of GI bleeding
Saraghi et al. (2018)	SSRI, SNRI, TCA + pro-serotoninergic medication (phenylpiperidine opioids like meperidine, fentanyl), ondansetron, metoclopramide, erythromycin, metronidazole, triptanes, clozapine, olanzapine, quetiapine, risperidone	Increased risk for precipitating serotonin syndrome
O'Brian et al. (2013)	Escitalopram + cyclosporine, verapamil and other P-gp receptor inhibitors	Increase of escitalopram delivery to the brain
O'Brian et al. (2013)	Ciprofloxacin + escitalopram/ citalopram/donepezil/haloperidol	Increased risk of QT-prolongation
Zieglmeister and Hein (2003)	Mirtazapine, duloxetine + antiepileptics, benzodiazepines, opiates	Increased risk of sedative side effects of antidepressant drugs due to additive CNS effect
Zieglmeister and Hein (2003)	MAOi + duloxetine, mirtazapine, reboxetine, venlafaxine, tianeptine	Increased risk of serotonin syndrome, hypertension, agitation, diarrhea, delirium
Zieglmeister and Hein (2003)	TCAs + amiodarone, quinidine, disopyramide, lidocaine	Dysrhythmia, dizziness, headache, vomiting
Sparkman and Li (2012)	Ciprofloxacin + haloperidol, olanzapine	Ciprofloxacin enhanced antipsychotic efficacy

(continued)

Table 15.3 (continued)

Reference	Drug combination	Potential consequences
PK/PD and mechanistic interactions		
van der Lee et al. (2007)	Antiretrovirals (ritonavir, fosamprenavir) + paroxetine	Antiretroviral compounds reduce total paroxetine exposure by 55% due to the inhibition of CYP2D6 isoenzyme
Gärtner et al. (2010)	SSRI + acetylsalicylic acid, clopidogrel	SSRIs increase the risk of post-operative bleeding due to the action on platelets' cell membrane serotonin transporters

residences. Such trials are desperately needed to determine the long-term safety and efficacy of medications to be used in frail and older persons (Tolson et al. 2011).

15.13 Discussion

There are three groups of populations who are most sensitive to ADRs: the pregnant mothers, the children, and the elderly. In this review, we have concentrated on drug dose adjustment in the geriatric population group in which susceptibility to drugs does not depend only on the age but also on comorbidities and concomitant medications as well as the socioeconomic conditions. As highlighted in Fig. 15.1, physiological age-dependent changes occur in GFR and other body organs such as liver and GI tract. Elderly subjects, in comparison with their younger counterparts, are more susceptible to drug-mediated changes due to reduction in CYP450 isozymes activity. Drug-induced side-effects and ADRs are mostly related to chronic illnesses and polypharmacy.

We have described several examples in this review to illustrate changes in ADME as well as in the PK and PD parameters in frail and older persons. Detailed description has been given in the field of antidepressant drugs and the precautions to be taken while prescribing antipsychotic therapy and monitoring of adverse events in elderly and frail patients. It should be mentioned that individual's pharmacogenetics make-up sometimes overrides the framework based on general population of elderly patients. For instance, the prevalence of CYP 2D6 polymorphism is different in ethnic populations: "fast and slow metabolizer" genotype in Europe is around 5–10%, whereas in South-East Asians it may reach as high as 20%. Consequently, this pharmacogenetics factor can potentially influence the metabolism of CYP2D6 substrates. Further, the polymorphism of ABCB1 transporters indicating allele variations (e.g., in C1236T: 34–42% in Caucasians; and 60–72% in Asians) make pharmacokinetics variations in the metabolic disposition of certain groups of xenobiotics or endogenous compounds (Lam and Scott 2019). Pharmacogenetics can strongly influence the predisposition fate of several antidepressant drugs and alter the PK and PD values these drugs. In order to minimize any adverse events, the dosing schedule of antidepressants should be individualized,

particularly during the initial phase of therapy, when the treatment is intended for long-term intensive psychiatric care, together with laboratory facilities to monitor optimal drug levels in plasma (Jin et al. 2010; Stout et al. 2010; Drozda et al. 2014; Kratz and Diefenbacher 2019).

The therapeutic index [TI = toxic dose/effective dose] is an important indicator for the safety of xenobiotics. There are groups of drugs with narrow TI (e.g., antiepileptics, warfarin, digoxin), and even in case of antidepressants, TI has a pivotal significance in the geriatric population, especially if co-medications are prescribed. In two drug safety communications (2011–2012), the US-FDA announced that co-administration of omeprazole (CYP2C19 inhibitor) with citalopram causes abnormal heart rhythms and QT interval prolongation in geriatric patients. As a result, maximal dose of omeprazole was restricted to 20 mg/day for patients above 60 years. Omeprazole is a proton pump inhibitor that decreases HCl production in the stomach and is used for treating heartburn and duodenal ulcers. Citalopram and escitalopram are frequently prescribed in the elderly subjects, and their interaction with omeprazole may lead to serious cardiac arrhythmias, Torsade de Pointe and sudden cardiac arrest (Lozano et al. 2013). Patients on chronic treatment with antidepressants should be regularly monitored for interacting drugs mentioned in Tables 15.2 and 15.3 as well as over-the-counter medicines and newly marketed drugs where dose adjustments may be necessary for antidepressants.

In Tables 15.2 and 15.3, we have shown examples of the safety and efficacy of antidepressant drugs as well as the clinically relevant interactions of antidepressants with other medications prescribed to elderly patients. The PK and PD parameters and metabolic profiles of drugs depend up on patient's drug metabolizing capacity, renal and hepatic functions, pharmaceutical formulation of the drug, comorbidities, and co-medications. Best practice to reduce the risk of drug-drug and drug-herbal interactions requires thorough assessment of medications the patient may be taking, and then adjust doses of medications or reduce the number of unwanted medications accordingly. Healthcare providers should ask their elderly patients about herbal and dietary supplement use and discourage concomitant ingestion of botanical products, including fruit juices (grape fruit, orange, pomegranate, tomato), with pharmaceutical medications. The clinicians and pharmacists should also consider drug-disease-interactions (especially liver and kidney disorders), and drug metabolizing capacity of individual patients that may be unique to Caucasians, Asians, Hispanics, Blacks, etc. and may require drug dose adjustments based on these multiple factors.

In summary, we have demonstrated via examples that special attention should be paid to ADME of orally administered antidepressant drugs, and physiological functions of liver and kidney should be taken into consideration while prescribing these drugs to elderly and frail patients. Overwhelming evidence suggests that drug dose adjustments are necessary in patients >65 years. More personalized medication is needed compared to the actual mechanistic prescription praxis! Control of therapy output and recognition of early signs of accidental side effects or toxic symptoms are very important in patients with comorbidity and polymedication. Therapeutic drug monitoring, especially in the case of narrow therapeutic index drugs, is recommended in any uncertain situation.

15.14 Concluding Remarks

The global elderly population is increasing at a tremendous pace and many elderly patients require multiple drugs for treating comorbid conditions. Several factors like declining liver and kidney functions, comorbidity, polypharmacy, and drug interactions lead to ADRs in the elderly patients. Drug dose adjustments are absolutely necessary, in order to avoid any iatrogenic or drug-induced harmful effects in the elderly and frail patients. Custom designing of personal therapeutic regimens as well as precision medications is the need of the hour to suit individual patients. Hence, it is the professional responsibility of healthcare providers such as physicians, surgeons, nurses, pharmacist, and para-medical fraternity to change the current scenario and provide properly suitable medications which would improve the quality of life of frail and elderly men and women.

Adverse drug reactions, sometimes with life-threatening situations, occur among elderly patients due to inappropriate drug doses, polypharmacy, comorbid conditions, dementia, and inability to read drug labelling. Drug doses based on mg/kg body wt. cannot be applied in frail and elderly patients due to reduction in body mass and compromised hepatic drug metabolism and renal excretory capacity, which consequently cause significant alterations in the metabolic disposition of drugs as opposed to the younger individuals. Overwhelming number of studies have shown that elderly men and women are the most vulnerable group to iatrogenic effects due to comorbid conditions, co-medications, and declining functions of the gastrointestinal-hepatic-renal systems. Age-related debilitating conditions and self-medication with micronutrients (vitamins, minerals), herbal remedies, and dietary supplements tend to enhance clinically important ADRs due to drug-herbal-diet interactions. All these situations make it highly challenging for the physicians, nurses, pharmacists, and surgeons to make drug dose adjustment decisions for the geriatric patients.

Systematic research by various scientific groups and pharmaceutical companies has helped in the computation of drug dose adjustments and decision making easier for drug administration in elderly and frail patients. Appropriate equations and formulas are available for calculating drug dosages for elderly patients based on serum creatinine or cystatin-C clearance as well as some other biomarkers. It is important that elderly patients should be enrolled in clinical trials for learning more about the pharmacometabolomics and therapeutically optimal dose levels without any ADRs. In this review, we have focused on the dose adjustments of psychotherapeutic drugs because the antidepressant, antianxiety, and insomnia treating medications are most frequently used by elderly men and women. The prescribers need education, training, and motivation about reporting ADRs and pharmacovigilance to ensure patient safety.

Conflict of Interest The authors declare no conflict of interest.

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Role of Microfluidics and Nanofluidics in Managing CAD

16

K. Tankeshwar and Sunita Srivastava

Abstract

A comprehensive understanding of interaction between molecules and pathways is essential to understand complex biological systems and their associated diseases paving way to develop methods of their treatment. Qualitative methods alone are not sufficient enough unless and until they are supplemented by quantification of various involved parameters and consequently cultivate various techniques to treat the diseases. Coronary artery diseases (CAD) are found to be quite prevalent affecting about 523 million people globally every year. Quantitative methods can help identify CAD in initial stages and assist in managing it. With recent technological advances, the control and monitoring of complex cellular processes at the molecular level has been made possible. This article describes the role of microfluidics and nanofluidics in managing CAD. Heart-on-a chip device has also been described which essentially integrated nanofluidics into microfluidics. A microscopic theoretical model has also been presented which gives insight of CAD and may help in effectively guiding the researchers to develop various methods to quantify its various aspects. Various useful software have also been listed in a subsection.

Keywords

Microfluidics · Nanofluidics · CAD · Compartmental model · Confinement

K. Tankeshwar · S. Srivastava (✉)
Guru Jambheshwar University of Science and Technology, Hisar, India

Panjab University, Chandigarh, India

Central University of Haryana, Mahendergarh, Haryana, India
e-mail: sunita@pu.ac.in

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16.1 Introduction

To make biology more predictable, advanced engineering technologies can be advantageously used not only to understand the conventional qualitative biology but also to estimate (Lopatkin and Collins 2020) functionality of various components of complex living organisms. Quantitative biology helps us to comprehend the intricate network of functioning of basic components of living organisms like genes, proteins, and various involved pathways. It helps us to appreciate biology modules and networks and hence facilitate in controlling the environments minutely (Azuaje et al. 2009; Turku PET centre n.d.). One can carry out relative measurements of various parameters at very small scales with higher resolution by employing these advanced engineering techniques encompassing microfluidics and nanofluidics wherein one has flexibility to manipulate the fluids at a scale which is less than tens of micrometers or nanometers respectively. It first started in the 1950s when these were fabricated on Si substrate and subsequently gained momentum with the advent of soft lithography in the 1990s, which considerably reduced the cost as well as the level of difficulty in its production. Integrating microfluidics and nanofluidics with external instruments goes a long way in investigating organism and components ranging from subcellular to multicellular.

There could be two different approaches in microfluidics, viz., channel based and droplet based. Real-time observation could be easily carried out in channel-based microfluidics which can easily provide a long-term living environment enabling it to capture even subtle changes of cell behaviors. In droplet-based microfluidics, each well-defined water droplet with surrounding oil phase could be regarded as isolated reactors for cell living inside or for biochemical reactions. These water droplets could also provide 3D microenvironment.

Coronary heart disease is a very common disease, which kills about 19 million people every year. Nearly 18.2 million adults are suffering from coronary artery disease (CAD) which include people aged 20 and above. About 2 out of 10 deaths from CAD happen in adults who are below 65 years of age. In 2016, the estimated prevalence of coronary diseases in India was estimated to be 54.5 million which resulted in one in four deaths in India. Above 80% of deaths resulted from ischemic heart disease and stroke. These diseases tend to affect patients in their most productive years of their lives which has disastrous social and economic consequences. Individuals with possibility of coronary-related diseases may have related symptoms which may include overweight, obesity, as well as raised blood pressure, glucose, and lipids. Identifying all those persons who are at highest risk of this disease and also ensuring that they are appropriately given medical treatment can certainly prevent premature deaths. In the present chapter, important role of microfluidics and nanofluidics has been discussed which help in quantifying various parameters of this heart disease and help the medical world in managing CAD. Subsequent sections of this chapter deal with the introduction to CAD, understanding basics of microfluidics and nanofluidics, a dynamic microscopic theoretical model, methods of quantifying various involved biological parameters, and available clinical and research software packages. The chapter concludes with possible future scope of this field.

16.2 Coronary Artery Diseases

Coronary arteries of a physically fit person are normally smooth and elastic. The endothelium lining protects the walls enabling effective working of the arteries and thus permitting a free flow of blood through them. Coronary artery disease may begin at a very young age with the appearance of streaks of fat in the walls of the blood vessel which, in turn, may result in buildup of fat with passage of time resulting in minor damage to the blood vessel walls. Other constituents of blood streams, viz., proteins, calcium, inflammatory cells, and other cellular waste products, stick to the vessel wall and combine with the fat to form a plaque as shown in Fig. 16.1. These plaques could differ in size and softness and could be covered with a hard fibrous cap which may eventually crack or tear with time. Though excessive plaque ultimately restricts the blood flow in arteries, it is often hard to diagnose early. Platelets migrate to these areas and build up blood clots around the plaque. In addition, the endothelium too may get irritated and consequently stop functioning and thus coercing artery to squeeze inconsistently resulting in further narrowing down of the artery. At times these clots may break apart resuming the blood flow in the region, and at other times the blood clot may completely block the supply of blood to the heart muscle, initiating a severe disorder known as acute coronary syndrome. Thus, coronary artery disease leads to atherosclerosis—narrowing of coronary arteries by plaque which is depicted in Fig. 16.2. Arteries get clogged by formation of these plaques and hence get damaged by the

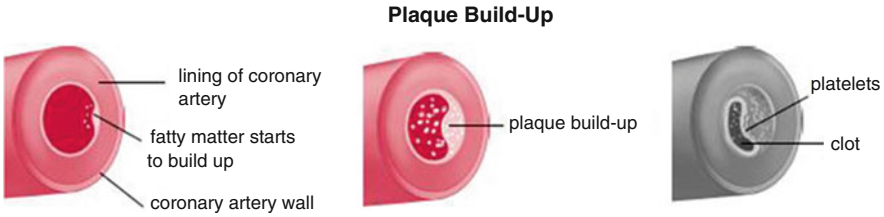

Fig. 16.1 Plaque buildup by combination of fat with constituents of blood stream

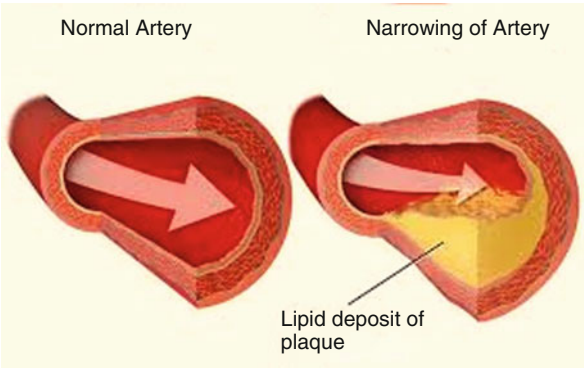

Fig. 16.2 Lipid deposit of plaque in the artery

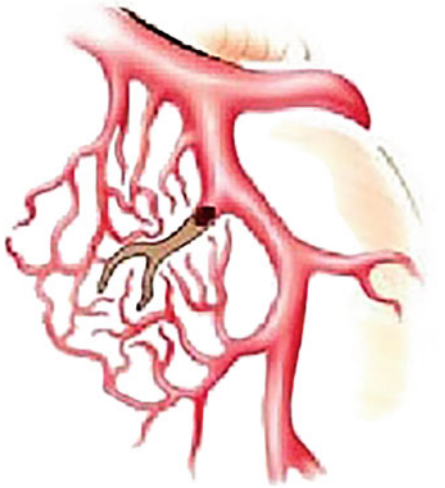

Fig. 16.3 Collateral circulation developing around an area of reduced flow

restrictive blood flow to the muscles of the heart. The lack of blood deprives heart muscles from enough oxygen and nutrients to carry out its work properly leading to chest pain or angina.

When blockage becomes bigger in the coronary artery, there could be re-routing of new blood vessels around the blockage which is known as collateral circulation. Figure 16.3 shows collateral circulation developing around an area of reduced blood flow. However, they may not be capable enough to carry sufficient blood to the heart in a state of stress. When the heart muscles lack adequate supply of oxygen and nutrients for its efficient working through its nano-arteries, the cramping of these muscles may take place, and such a condition is termed as ischemia. Consequently, the person may suffer pain in the chest in addition to other symptoms. Such a condition may be experienced often when one is active, excited, stressed, or exposed to cold. This condition may last for a brief time (~10 min) and may improve only after taking rest. Such a condition may be termed as a stable angina. However, the symptoms may persist even after taking a rest. For people with diabetes, the symptoms may not surface, and such a cause could be termed as silent ischemia (Gatimu et al. 2006; Nabel 2003; Louridas et al. 2010; Kemp and Conte 2012). In Fig. 16.4, one can observe a situation where enough plaque builds up in artery and makes it harder for blood to get through the artery leading to the state of angina in a person. The lower portion of the figure displays a condition of heart attack where a blood clot completely blocks the artery resulting from cracking of deposited plaque.

16.3 Role of Microfluidics and Nanofluidics

Surface properties of a system at micro- and nanoscales play an indomitable role as its surface to volume ratio is quite large and the pressure drop is also quite significant. Hence, it becomes quite imperative to transport biomaterial electrokinetically

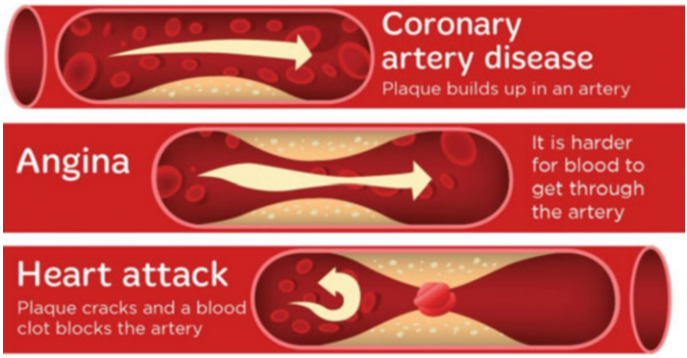

Fig. 16.4 Coronary artery disease: angina and heart attack

(Rems et al. 2016). In addition, a deeper understanding of fluid flow, mass of heat transfer, electrokinetics, electrochemistry, and molecular biology becomes mandatory in the designing of micro- and nano-devices.

Microfluidics deals with the systems which process minimal quantities of fluids through channels with microscale dimensions—ranging from 10 μm to 100 μm. Their properties result in very useful applications in varied fields which include biology, chemistry, information technology, optics, and many more. It not only saves money but also enormous time expended in research.

Study of behavior, manipulation, and control of fluids confined to nanostructures (1–100 nm) is termed as nanofluidics (Eijkel and van den Berg 2005). Fluids confined to nano-structures exhibit distinctive physical parameters for a truly valid reason that the magnitude of their dimensions is of the same order as that of its characteristic's physical scaling length (e.g., Debye length, hydrodynamic radius, etc.). As the dimensions of nanostructure correspond to molecular scaling lengths, the physical constraint results in new properties not observed in bulk.

Enormous increment is observed in viscosity in proximity of the pore wall affecting its thermodynamic properties as well as chemical reactivity at the fluid-solid interface. For example, in nano-capillary array membrane (NCAM) (Joshi et al. 2021), surface charges start playing a dominant role at the electrified interface.

Figure 16.5 displays one such NCAM which consists of parallel nanocapillaries each with pore radius, $a/2$, ~Debye length, κ^{-1}. The Debye length is a characteristic distance over which ions and electrons can be separated in a plasma and is a ratio of electron thermal velocity divided by the plasma frequency. Significant applications of nanofluidics lie in its potential to integrate into the microfluidic system resulting in lab-on-a-chip devices such as PCR which could be employed as analytical systems. When integrated with microfluidic devices, NCAMS could be advantageously used as a digital switch to transfer fluids between one microfluidic channel to another, proficiently segregate and relocate the analytes on the basis of size and mass, mix reactants, and separate out characteristically dissimilar fluids. A natural analogue of fluid-handling capabilities of these nanofluidic structures could be found in

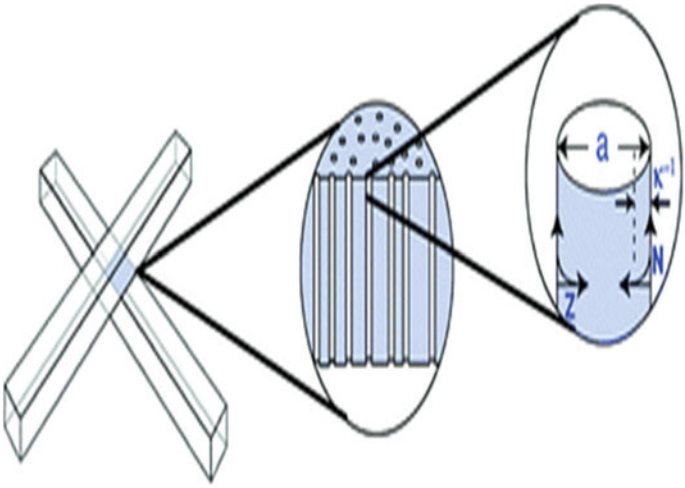

Fig. 16.5 The NCAM (parallel nanocapillaries), each with pore radius, $a/2$, ~Debye length, κ^{-1} [Adapted from Ugolini et al. 2017]

regulating the flow of charge carriers by electronic components. This particular analogy has helped in realizing functions like rectification and transistor action of field effect transistor (FET) and bipolar junction transistor (BJT) with ionic currents.

16.3.1 Advantages of Microfluidic and Nanofluidic Systems

In research labs, only infinitesimal amount of samples and reagents are required which could result in the cost reduction of reagents specifically the expensive one. As a result, a high degree of sensitivity as well as resolution is achieved while working with molecules. This further reduces extensive usage of complex equipment and technologies. Time involved in the analysis also gets reduced considerably. The nature of the flow of fluid in these micro-/nano-channels are laminar and smooth which facilitates greater control of flow of fluid and various other experimental parameters at this micro-/nano-scale.

16.3.2 Applications

Central usage of microfluidic systems could be recognized (Zhang and Austin 2012) in numerous processes such as flow cytometry, capillary electrophoresis, immunoassays, isoelectric focusing, sample injection in mass spectrometry, DNA analysis, PCR amplification, cell patterning, and separation and management of cells. These findings could be advantageously used (Beebe et al. 2002) in research of antibiotic drug-resistant bacteria, observation of the chemical reaction kinetics,

and nanoparticle transport in blood. It could also be used as a diagnostic tool in cancer and pathogen detection.

Its analytical (Bai et al. 2018) usage lies in biopharmaceutical production where one can monitor and optimize protein drug production as well as in the analysis related to human cells. Microfluidic devices could also be employed in assessing diffusion coefficient, visibility, pH, and chemical binding coefficients.

Nanofluidics has the potential to integrate into microfluidic systems resulting in structures which are broadly called as lab-on-a-chip. One such example could be when NCAMs are integrated with the microfluidic devices and hence could be used as a digital switch which can easily transfer fluid from one microfluidic channel to another. In the process, it could selectively isolate and transfer analytes proficiently on the basis of size and mass mix reactants. It can also help separate fluids with disparate characteristics. One can draw a natural correlation between the capabilities of nanofluidic structures in handling fluids and thus controlling the flow of electrons and holes by electronic components. Such an analogy has been fruitfully used to understand active electronic functions which could be rectification and field effect and bipolar transistor action. In addition, nanofluidics find applications in nano-optics wherein microlens array could be produced which are tunable. With the advent of lab-on-a-chip devices, nanofluidics find effective role not only in medicine and biotechnology but also in clinical diagnostics for PCR and related techniques.

16.4 Quantification of PET Myocardial Blood Flow

For patients with alleged coronary artery disease (CAD), it becomes imperative to manage and diagnose them by noninvasive method along with their risk assessment. To get an estimate of myocardial ischemia and the involved risk, a quantitate assessment of myocardial perfusion is carried out with the help of numerous techniques (Waller et al. 2014; Nesterov et al. 2016) such as positron emission tomography (PET), cardiac magnetic resonance (CMR), single-photon emission computed tomography (SPECT), and cardiac computed tomography perfusion (CTP). Such techniques prove to be very helpful in assessing the extent of CAD, especially in patients with multivessel diseases by measuring myocardial blood flow and coronary flow reserve. In addition, these imaging techniques have proficiency to demarcate the level and severity of diffuse atherosclerosis and microvascular dysfunction. This also eliminates any biasing of intermediate observer.

In a healthy coronary vessel, any change in the myocardial oxygen triggers a local endothelial facilitated release of nitric oxide and subsequent arteriolar dilation along with reduced resistance in the microvasculature and thus ensuing increased myocardial perfusion. However, in case of patients with atherosclerosis, microvascular dysfunction results in a restricted coronary vasodilator response to an increased demand of oxygen resulting in myocardial ischemia. Clinically, help is taken either through exercise or pharmacological vasodilators to achieve the coronary hyperemia.

16.4.1 Compartmental Model

A compartmental model is a form of mathematical model wherein simulation is carried out to analyze interaction between individuals in different "compartments." Here, it is assumed that the people (or animal) in each compartment are same as all the other people (or animals) in that compartment. These compartments could (Bassingthwaighte 2012) either flow between each other or they could interact with each other, and these rates of flow as well as interaction rates between compartments may be taken as "parameters" of the model ascertained from observational studies of the species which could, in turn, estimate the average lifetime of the species. One example is the finite element analysis model used in engineering and biomedical engineering where an object is divided into small representative bits to carry out an analysis of the changing forces on each element as the object moves. The whole idea is to get a model which could simulate the reality and accordingly vary those parameters to examine "alternate realities." Finally, it could be used to devise improved disease intervention strategies and other situations like optimizing pest control, optimizing fisheries, etc.

Compartmental models prove to be very effective in carrying out simulation of the spread of disease in a population. These models give us deep understanding of the mechanisms and subtleties of the spread of disease (specifically when comparison is carried out with epidemic data). This, in turn, would help us develop intervention strategies which could be more effective in managing the diseases. One could also easily employ these disease models to successfully forecast the course of an epidemic or a pandemic. The support parameters of compartmental disease models are the "Susceptible" (S), "Infected" (I), and "Recovered" (R) variables developed by Kermack and McKendrick (1927). The **SIR model** is a basic form of compartmental model which works very efficiently for several diseases including mumps, rubella, measles, influenza, etc. Many researchers have successfully made use of SIR model to analyze the propagation of COVID-19 pandemic and predict its future course.

The models could be described either by employing deterministic ordinary differential equations or with the help of more realistic but complicated stochastic (random) framework. Models can easily predict disease spreads, epidemic duration, and the total number infected, and also it can evaluate epidemiological parameters such as the reproductive number. Such models could also be used to demonstrate the effect of outcome of different public health interventions in an epidemic. For example, it can help us decide for most efficient technique of distributing a limited number of vaccines to a given population.

The physiological system under consideration could be divided into number of interacting compartments in order to study its dynamic processes. Such a compartment could be considered as a chemical species in a physical place with a uniformly distributed tracer. In a given compartmental model:

- Within the tissue of interest, an injected isotope would be present in a well-defined number of interconnected physical or chemical states.

- One could make use of linear, first-order ordinary differential equations (ODE) to describe these compartmental models.

In a given tissue compartment, variation in tracer concentration could be expressed as a linear function of the concentrations in remaining compartments:

$$\frac{dC_i(t)}{dt} = f_i(C_0(t), C_1(t), C_2(t), \ldots) \tag{16.1}$$

Hence, by taking the convolution of the tracer input function and the response function, kinetic measurements could be carried out. By employing the measured kinetics of the system, one can obtain the response function by de-convolving the input function.

The compartment model could be classified into two types:

1. *Catenary model*: Here one considers series connection of one-dimensional chain of compartments.
2. *Mammillary model* consists of a central compartment surrounded by other parallel-connected compartments. In nuclear medicine, one has to work with mixed mammillary/catenary models.

16.4.1.1 Applications

Simulation studies of given tissue data can be carried out with the help of this compartmental model and hence could be employed to

1. Examine simplified analysis methods and software.
2. Calculate parameters of compartmental model by making use of available PET data.

The parameters of a model which define the dynamic progressions could be calculated from available dynamic PET data and metabolite corrected arterial blood curve starting from the time of injection and covering all observed significant modifications in tracer kinetics. With good sufficient information, one could calculate all the parameters of dynamic processes which would include reaction rate, perfusion, transport, blood volume involved in tissue vasculature, specific binding, etc. However, most of the time only one key parameter is required to correlate with the desired property under normal conditions.

16.4.1.2 Two-Compartment Model

The two-compartment model, as shown in Fig. 16.6, is the simplest compartmental model where the input function (measured) is given to the first compartment which could be plasma or blood curve. The second compartment could be used for the isotope label in tissue. It is also known as one-tissue compartmental model (1TCM). Connection between the two compartments could be defined by two rate constants,

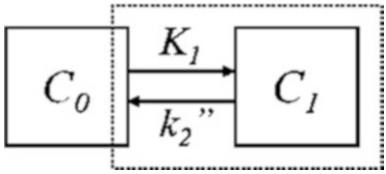

Fig. 16.6 Two-compartment model

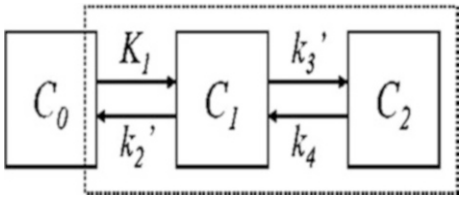

Fig. 16.7 Three-compartment model

K_1 and k_2''. The change with time in the tissue compartment, C_1, could be described by differential equation

$$\frac{dC_1(t)}{dt} = K_1 C_0(t) - k_2'' C_1(t) \tag{16.2}$$

With this two-compartment model, one could quantify perfusion (blood flow, f) with spontaneously diffusible tracers such as H_2O with ^{15}O, butanol [with both possibilities ^{11}C and ^{15}O], and fluoromethane [with ^{18}F].

16.4.1.3 Three-Compartment Model (Two-Tissue Compartment Model)

The three-compartment model, also known as two tissue compartments (2TCM), could be described by two differential equations:

$$\frac{dC_1(t)}{dt} = K_1 C_0(t) - (k_2' + k_3')C_1(t) + k_4 C_2(t) \tag{16.3}$$

$$\frac{dC_2(t)}{dt} = k_3' C_1(t) - k_4 C_2(t) \tag{16.4}$$

Figure 16.7 shows an arrangement of three-compartment model defined by the rate constants K_1, k_2', k_3', and k_4. These equations can be employed to analyze the concentration curves in both of the tissue compartments. This three-compartment model could be used to measure the transport of glucose and phosphorylation rate in the brain. For irreversible tracer uptake, one can set $k_4 = 0$. This model could also be used for analyzing brain receptor radioligands. The two parallel tissue compartments are kinetically indistinguishable, and hence one could further simplify the model and its constrained parameters.

The series configuration of tissue compartments could be applied in the metabolism studies where perfusion uptake gets limited more than the transport across capillary endothelium.

The rate constant, K_1, represents the delivery rate which defines the unidirectional transport of the tracer from plasma or blood compartment to the first tissue compartment. Using the Fick principle and Renkin-Crone model, K_1 depends on perfusion, f (mL × mL^{-1} × min^{-1}), and the product of capillary permeability P (cm/min) and capillary surface area S (cm^2/cm^3), $P \times S$:

$$K_1 = f\left(1 - e^{-PSf}\right) \tag{16.5}$$

Transport must happen during the time that the blood stays in the capillaries (1–3 s). Even if tracer resides in both blood plasma and blood cells, for many radioligands only the ligand that is in blood plasma is available for transport; then K_1 depends on plasma flow instead of blood flow. The rate constant k_2 represents unidirectional transport back from tissue to the blood and is defined in terms of K_1 and distribution volume, V_1, of the tracer in the first tissue compartment:

$$k_2'' = K_1 V_1 \tag{16.6}$$

Both K_1 and k_2 depend on perfusion. In the case of central nervous system, if K_1 gets strongly limited by the value of $P \times S$ of the capillary endothelium owing to the blood-brain barrier, one can assume the first tissue compartment (C_1) to denote the interstitial and intracellular spaces, whereas the second tissue compartment (C_2) can indicate a metabolic or a receptor-bound compartment within the tissue. Rate constant defines the fractional tracer leaving the compartment per unit time measured in units of s^{-1}. For a zero concentration, the rate constant can have values over 1.0 because if flow of material is zero, then the quantity of material transferred per unit time is given by the product of rate constant as well as the amount of tracer in the initiating compartment. K_1 takes into account the perfusion-dependent component expressed in units of milliliter plasma (or blood) per minute per milliliter tissue (mL min^{-1} mL^{-1}), whereas k_2', k_3',... represents fraction of mass transferred per unit time with unit min^{-1}.

Compartmental modeling with the two-tissue model has been implemented in **syngo MBF** which is an FDA-approved commercial software package.

16.4.1.4 Perfusion Limited Uptake

For blood flow limited uptake, if $P \times S \gg f$, it reduces $e^{-PS/f}$ to zero and hence $K_1 = f$. This further leads to, $k_2 = f/V_1$, and $C_V(t) = C_1(t)/V_1$. With one-tissue compartmental model, i.e., when all tissue compartments are lumped into one, this would equal the model for radiowater where rapid diffusion of water takes place in the tissues. In the case of liver with large openings of capillary endothelium, the organ is largely porous to all radiotracers. Therefore, blood flow is the first restrictive feature for uptake, and the first tissue compartment (C_1) represents extracellular volume which comprises of both vascular and interstitial space. The second tissue compartment (C_2) characterizes the intracellular space in hepatocytes. Thus, $K_1 = f$, and rate constants k_3 and k_4 denote the transport rates among extracellular and intracellular compartments. Comparable models could be applied to tissues where

perfusion is very low as in the case of resting skeletal muscle with tighter than in the liver.

16.4.2 Plasma Compartment

The concentration of tracer in the plasma is an identified input function which drives the system, and the metabolite-amended arterial plasma curve forms the input to the compartment model. If the intravascular activity is considered in the calculation, one should use the whole blood concentration, having metabolites. If metabolite-corrected plasma curve is employed to correct for vascular blood volume fraction, blood contribution at later intervals could be undervalued resulting in the false inclusion of an apparent additional tissue compartment.

16.4.3 Retention Model

Our experience of the world is not of a series of unconnected moments. Indeed, one could not experience the world if we are not aware of a sense of temporality (which is traditionally the linear progression of past, present, and future). Perception we have is an impression to our minds which solely is determined by upon both retention and protention.

Retention is a progression by which a phase of a perceptual act is recalled in our consciousness. It is a demonstration of that which does not exist before us any longer and is different from immediate experience. For example, if we watch an object being thrown, we would retain where the object was in our minds to comprehend its momentum as we observe it in the immediate present. Retention (Geng and Regnier 1984) is certainly different from representation or memory and is simply a presentation of a temporally long-drawn-out present which spreads beyond the few short milliseconds that are recorded in a moment of sense perception. *Protention* is our expectation of the ensuing moment—the moment that has yet to be perceived. Again, relating to the example of the thrown object, our focus shifts along the expected path the object would take.

Husserl defined three temporal aspects of perception, viz., retention, the immediate present, and protention along with the flow through which each moment of protention gradually turns into the retention of the next. The main benefit of the retention model for clinical measurement of MBF is the easy PET protocol and minimized computing demands for reconstructing and processing the image as compared with compartmental modeling.

The retention model crafts flexibility for simplicity and efficiency and hence addressing the integral technological limitations for former generations of clinical PET scanners, which had made it very difficult to employ dynamic PET and full compartmental modeling for routine purposes. Another significant potential advantage of this model is to decrease variability of the MBF estimates at the expense of increased bias due to the use of approximations and fixed correction elements. In

daily clinical applications, utility is very frequently defined by physiological and methodological variability rather than systematic error (bias).

16.4.3.1 Limitations of the Retention Model

Principal limitations of the retention model are:

- One assumption made in this model is that there is no wash-out of tracer. Though this approximation holds good for only normally perfused, feasible myocytes, nonetheless tracer eventually gets washed out in the case of severe ischemia or non-transmural scar.
- Assumption that the entire integrated arterial input function can be captured during the initial fixed 2-min blood pool image may not hold good in case of some physiological delay or low heart function.
- Evaluation of the partial volume correction factors used in the model should be done with the help of phantom scans for each PET scanner and radionuclide.

16.4.4 Comparison Between Retention Model and Compartment Model

A comparison of these two models can be made as follows:

- The short-term repeatability expressed as 95% repeatability coefficient, RPC, is found to be 15–20% for retention model and about 20% for compartmental modeling.
- Plot in Fig. 16.8 shows Bland-Altman global left ventricular flow data depicting repeatability limits of 95% (RPC ± 20%, dashed lines) (R-flow simplified retention model, C-flow compartment model). 27% of rest scans and 32% of stress scans were found to lie outside the repeatability limits (Moody et al. 2020).
- Thus, it signifies a prominent lack of agreement in about 33% of patients considered.

16.5 Software Model and Tools

Based on simplified retention model, the following commercial and academic software packages are available:

1. *HeartSee*: This software has been developed by the University of Texas, Houston. This software used for cardiac positron emission tomography (PET) determines both local and global complete rest and stress myocardial perfusion. It maps in patients with suspected or known coronary artery disease (CAD) and hence is useful in quantifying clinical interpretation of PET perfusion images and hence predicting their severity.

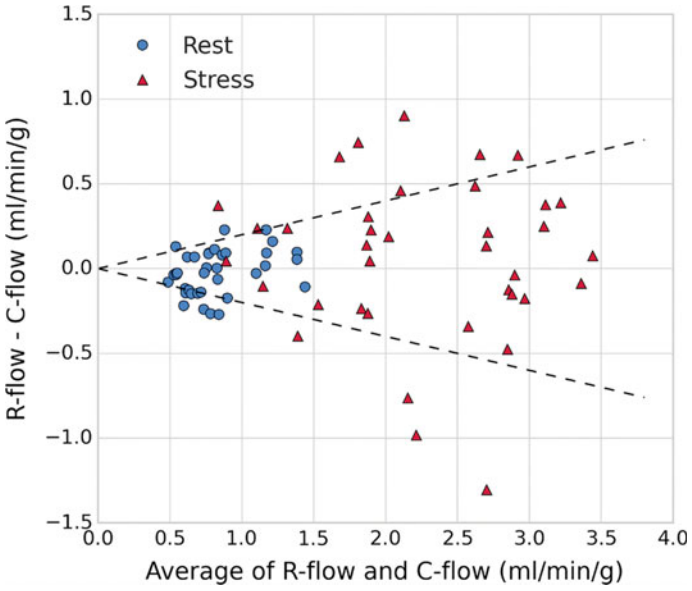

Fig. 16.8 Bland-Altman global left ventricular flow data (Adapted from. Moody et al. 2020)

2. *FlowQuant*: This has been developed and established at the University of Ottawa Heart Institute by Robert A. deKemp, Cardiac PET Centre, which makes use of nuclear medical images to quantify myocardial blood flow (MBF).
3. *ImagenQ* (CVIT).
4. *MunichHeart* (Technical University of Munich).
5. *4DM* (INVIA)
6. *PMOD*: Details have been discussed in the next subsection about this popular software used for quantification of various involved parameters.

16.5.1 PMOD Software

The PMOD software proposes tools for the model-based analysis of PET and SPECT data. One can carry out modeling of MR signals and also construct geometric models for simulations. PET kinetic analysis can also be done with an excellent program PKIN which works with tremendous speed.

16.5.2 Tools

PXMOD tool: A complementary tool to the PKIN is PXMOD with which one can carry out similar modeling techniques resulting in images known as parametric maps displaying the model parameter value in each image pixel. Thus, one can visualize quantitative tissue properties and compare them with results obtained

from other sources, such as autoradiography. Similarly, quantitative maps could also be statistically analyzed. Thus, this tool could carry out quantitative parametric mapping for more than 25 models.

PCARDP tool: PCARDP tool offers a comprehensive environment for static, dynamic, and gated analysis of cardiac PET images. One can easily understand different processing stages in a streamlined workflow. The involved crucial steps are provisioned with automatic procedures which include short-axis reorientation of the images and myocardial segmentation However, there is freedom for the user to interactively modify the outcomes. Dynamic data could be easily modeled in PKIN and leveraging the PET quantification solution for the cardiac environment. One obtains results in the form of comprehensive reports which could be exported numerically or in standard report formats. Numerous powerful analysis tools are available for investigating cardiac research environments.

PCARDM tool: It is a combination of PMOD's modeling expertise with ETH's leading-edge MR methodology for qualitative and quantitative CMR image analysis by employing the state-of-the art perfusion quantification approaches to the available data. This tool in conjunction with accelerated MR acquisition sequences can possibly image the full extent of the heart in a single breath hold.

PGEM tool: This tool is helpful in carrying out analyses with different types of geometry. **Fiber tracking and track visualization** (PMOD) can be done by using DWI MR images. By measuring the velocity vector of fluids in vessels as a function of time, one can possibly analyze and have a streamline visualization of **4D flow MR** images. It can also create **geometric models** to carry out different simulations. It also facilitates comprehensive support for **computational fluid dynamics (CFD) research** in the form of vessel models and interface with the OpenFOAM simulation system. It can also assist in quantitative image processing.

Other software packages include Carimas, Corridor4DM, HOQUTO, MunichHeart, QPET, syngo MBF, UW-QPP, etc.

The subsequent section deals with a microscopic theoretical modeling which explains the flow of fluid in different nano-geometries.

16.6 Theoretical Model: Flow of Fluids in Nano-Geometries

Motion of a nanoconfined fluid gets strongly affected in layers near the wall which modifies its diffusive and viscous properties. The structural and dynamical properties of liquids have been found to experience changes when liquids are confined even by smooth walls. Moreover, at the nanolevel, the Reynolds number reduces considerably resulting only in laminar flow. The continuum behavior of the liquid is unsuccessful in explaining fluid transport. Thus, theoretical modeling and its analysis become a challenging task as one has to incorporate the complexity of the fluid in the model. In this section we shall discuss an attempt to include all these aspects in a microscopic model developed by Tankeshwar and his research group.

To analyze the effect of nano-confinement on the self-diffusion coefficient, a dynamical model was proposed (Tankeshwar and Srivastava 2007) wherein the effect of confinement on molecular motion was analysed. The model was built up on the model of a microscopic (local) self-diffusion coefficient varying as a function of distance taken from the walls of the channel.

16.6.1 Model for Many-Body Liquid Problem

The configuration space of a many-body fluid system could be thought of as being divided into a number of cells where each cell may be described by a fixed configuration corresponding to the local minima on the potential energy hypersurface of the system. Inside the cell, motion of the liquid configuration is harmonic around a local minima characterized by a well-defined frequency or band of frequencies. The system could jump from one cell to other with a specific jump frequency τ^{-1}. Use has been made of basic definitions and expressions of diffusion and velocity autocorrelation functions which can be expressed as follows:

$$D = \frac{k_B T}{m} \int_0^\infty V(t)dt \text{ and } V(t) = \frac{1}{3}\sum_{i,\alpha} \langle v_{i\alpha}(t) v_{i\alpha}(0) \rangle \tag{16.7}$$

where $v_{i\alpha}(t)$ is the αth component of velocity for a ith particle at time t and such that $\langle v_{i\alpha}(0) \rangle^2 = 3$ kBT/m. Calculation of the velocity autocorrelation function (VACF) for any real system is probable only through a simplified picture within the framework of many-body theory.

16.6.2 Various Cases of Microscopic Model

1. *Effect of Confinement*: Solution is taken in the form of harmonic oscillator in nonconfined state with motion along z-axis characterized by amplitude A and frequency ω expressed as $z(t) = A \sin(\omega t)$. When width of the channel is of the order of nano-/microscale, particles would inevitably experience a compression-like situation which would eventually decrease its amplitude by d (as shown in Fig. 16.9) and also change its frequency to Ω which becomes a function of z

$$z(t_1) = A - d = A \sin(\omega t_1) \tag{16.8}$$

where $t_1 = \frac{1}{\omega} \sin^{-1}(1 - d/A)$, leading to a new frequency of motion

$$\Omega = \frac{\pi}{2t_1} = \frac{\pi\omega}{2 \sin^{-1}(1 - d/A)} \tag{16.9}$$

whose corresponding curve is depicted in Fig. 16.10. Expressing ratio d/A as a function of z, i.e.

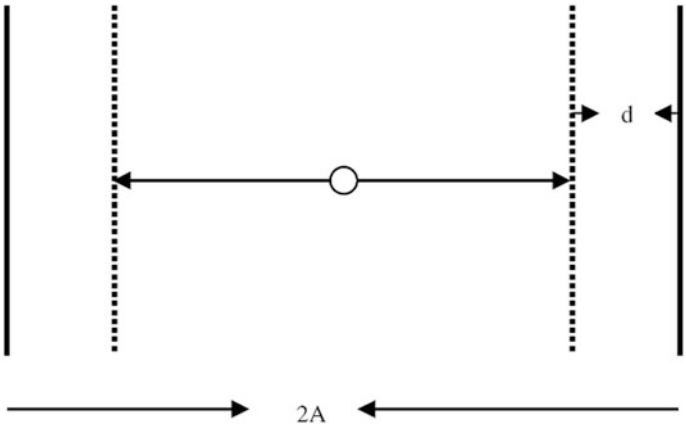

Fig. 16.9 Particle in a compressed situation

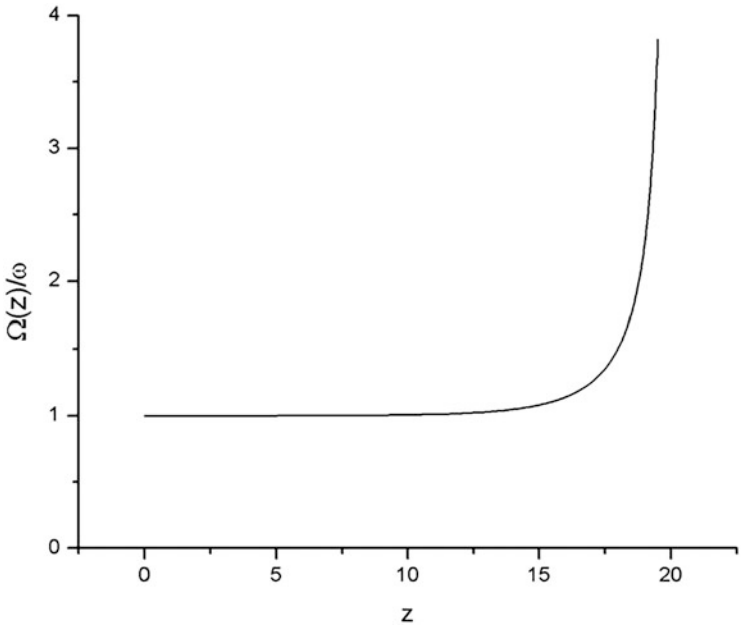

Fig. 16.10 Ratio of local frequency to the bulk frequency as function of z

$$c(z) = \exp(-(l - z)). \tag{16.10}$$

We get self-diffusion coefficient as

$$D(z) = \frac{\pi}{2}\frac{k_B T}{m}\tau \mathrm{sech}(\pi\Omega(z)\tau/2) \tag{16.11}$$

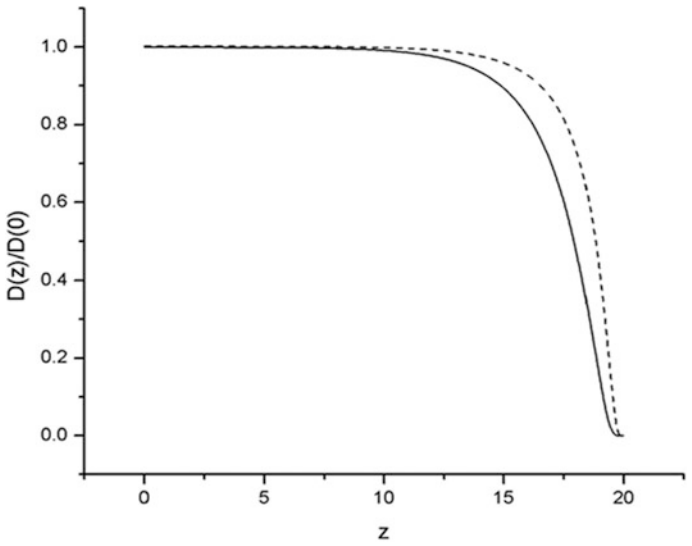

Fig. 16.11 Variation of ratio of $D(z)$ to the bulk value as function of z for two different densities. Solid line corresponds to more dense system

The self-diffusion coefficient was found to decrease in the proximity of the confining walls of nano-channel and hence affected the fluidity of the fluid. Thus, we observe that when the particle is within 10 atomic distances from the wall, the frequency gradually increases and reaches a maximum value in a region adjacent to the wall which indicates trapping of the particle resulting in reduced self-diffusion coefficient. This effect on the macroscopic self-diffusion coefficient gets more pronounced for the denser fluid as seen in Fig. 16.11. In fact, the denser fluid contributed in the formation of an extra artificial wall which further restricts the flow of fluid. This realization and analysis find a great relevance to the study of flow of blood like fluid in arteries. In general, this model could be easily applied to any complex liquids since the structural complexity of the liquids defines the frequencies ω and τ^{-1}. A similar and significant analysis has also been carried out for blood flowing in capillaries/arteries/arterioles of varying concentrations. The analysis demonstrated that the denser the blood, the lower the diffusion coefficient near the wall, thus resulting in narrower effective width of an artery or meta arteriole which, in turn, would further restrict the flow of blood. Thus, we observe that fluid in proximity to the wall works as if it is in super cooled state or frozen to the solid state. These findings have an important application in biological studies. The present results imply that thicker blood (with large cholesterol) reduces the effective width of an artery.

2. *Double Confinement in Rectangular Nanotube*: Particle in a specific cell experiences a compression owing to its confinement and hence causes an effective decrease in its amplitude (Aggarwal et al. 2007; Devi et al. 2011) by $d1$ and $d2$ along perpendicular y and z directions, respectively, as shown in Fig. 16.12. Here, Fig. 16.13 represents a 3D plot of diffusion of particle. Behavior of

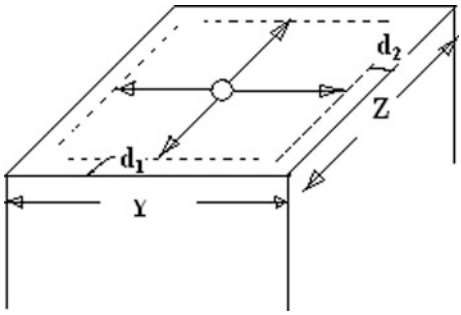

Fig. 16.12 Particle in a compressed cell

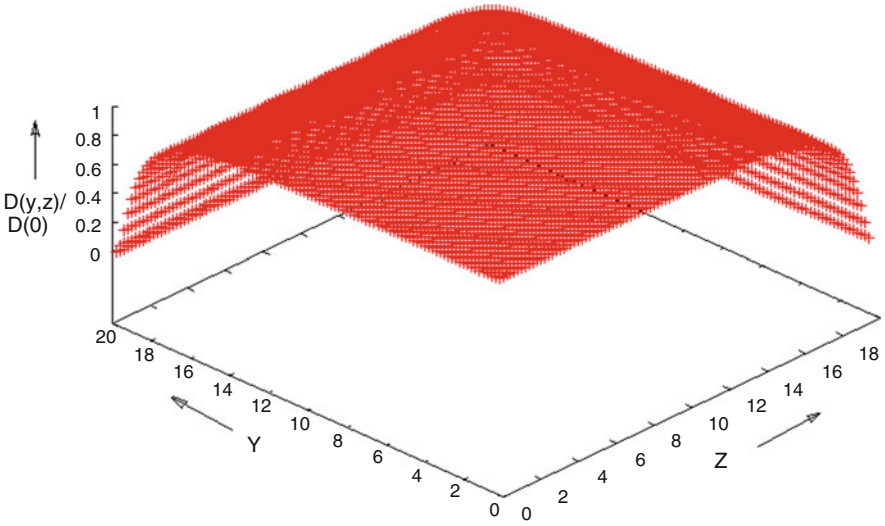

Fig. 16.13 3D Plot of diffusion

normalized velocity autocorrelation function (VACF) of fluid confined to rectangular nanotube as a function of z has been depicted in Fig. 16.14, whereas ratio of $D(y, z)$ to the bulk value as a function of z (walls of tube are at $z = \pm 20$) for $y = 20$ has been displayed in Fig. 16.15. The model envisages that the self-diffusion near the walls (of the order of few atomic layers) of the nanotube falls off significantly. The effect of such confinement on dynamic motion could lead to solidification of liquid close to the walls, and such a transition is effectively dynamical in nature. It is also discovered that effect of confinement on dynamics also determined by the radii of the particles. Subsequently, width of the tube cannot be treated as absolute and must always be quantified in terms of radius of particles contained in it. It is also observed that effect of confinement is more pronounced on denser fluids as compared to that on dilute fluids.

3. *Wall-Fluid Interaction*: The self-diffusion of fluid is calculated by incorporating the fluid-wall interaction at the atomic level (Devi et al. 2015). The fluid-fluid and fluid-wall interactions have been assumed to be LJ 12-6 potential and are given

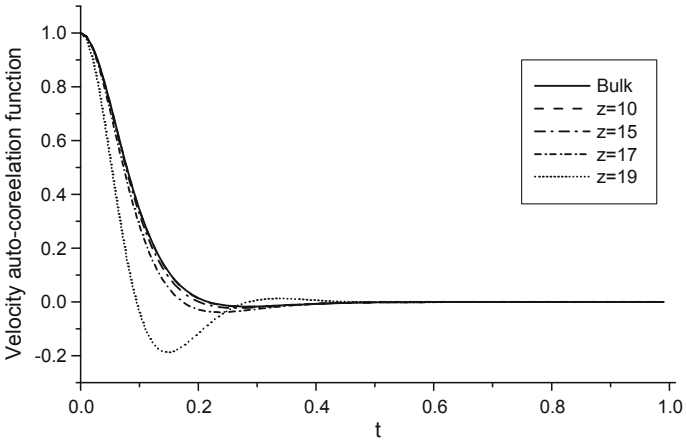

Fig. 16.14 Variation of normalized velocity autocorrelation function of fluid confined in rectangular nanotube

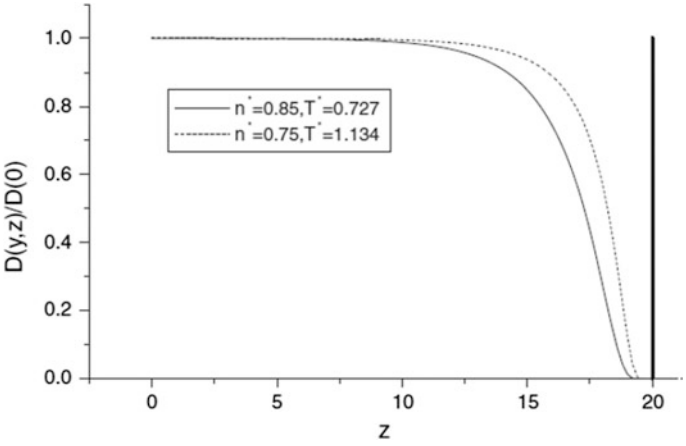

Fig. 16.15 Variation of ratio of $D(y, z)$ to the bulk value with z for fluids of different densities

by $U = U_{ff} + U_{fw}$, where U_{ff} is fluid-fluid interaction potential and U_{fw} is fluid-wall interaction potential expressed as

$$U_{ff} = 4\varepsilon_{ff}\left[\left(\frac{\sigma_{ff}}{r_{ij}}\right)^{12} - \left(\frac{\sigma_{ff}}{r_{ij}}\right)^{6}\right] \quad U_{fw} = 4\varepsilon_{fw}\left[\left(\frac{\sigma_{fw}}{r_{ij}}\right)^{12} - \left(\frac{\sigma_{fw}}{r_{ij}}\right)^{6}\right]. \qquad (16.12)$$

Here, ε_{ff} and σ_{ff} are the parameters of LJ potential for fluid-fluid interactions. Fluid-wall potential's parameters are represented by ε_{fw} and σ_{fw}. The ratio $\varepsilon_{ff}/\varepsilon_{fw}$ is an important parameter representing wettability of fluid. For example, for water, larger values of $\varepsilon_{ff}/\varepsilon_{fw}$ represent a hydrophilic wall, whereas its smaller value relates to a hydrophobic wall. Figure 16.16 shows a 3D plot of such a fluid-

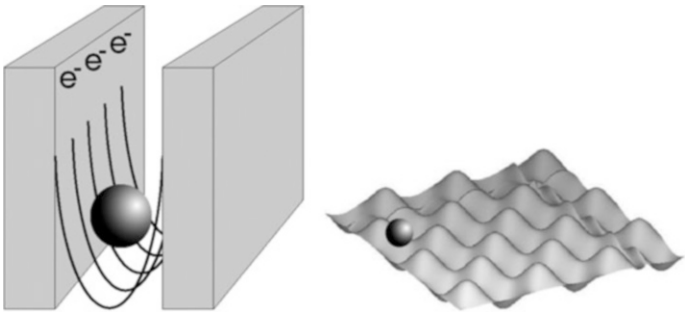

Fig. 16.16 3D plot depicting fluid-wall interaction

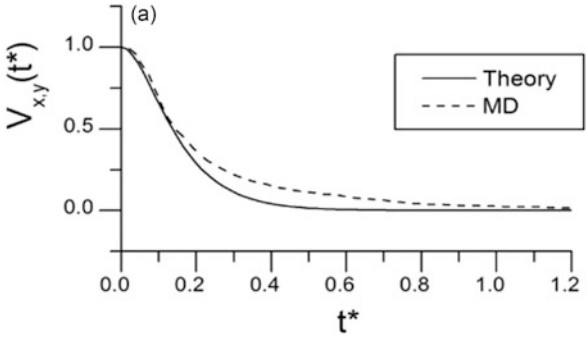

Fig. 16.17 Normalized velocity autocorrelation function $V_{xy}(t^*)$ versus reduced time (Devi et al. 2010)

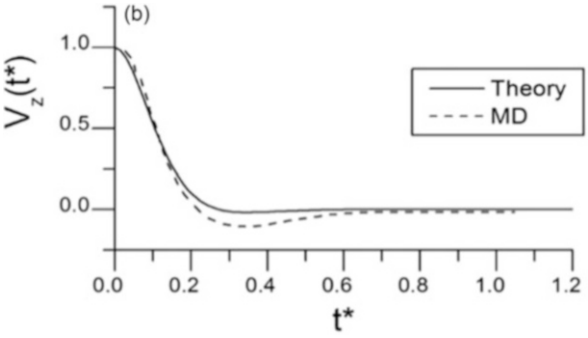

Fig. 16.18 Normalized velocity autocorrelation function $V_z(t^*)$ versus reduced time (Devi et al. 2010)

wall interaction. Normalized velocity autocorrelation function in parallel as well as perpendicular direction has been computed using Eq. (16.7) and illustrated in Fig. 16.17 and Fig. 16.18, respectively. A comparison of numerical results with available MD data has also been carried out which shows very good agreement.

4. *Confinement (Effect of Roughness)*: Plaque deposited in arteries over passage of time could be regarded as a source of roughness which is likely to slow down the blood flow. The roughness could be incorporated in the microscopic model which can be represented as

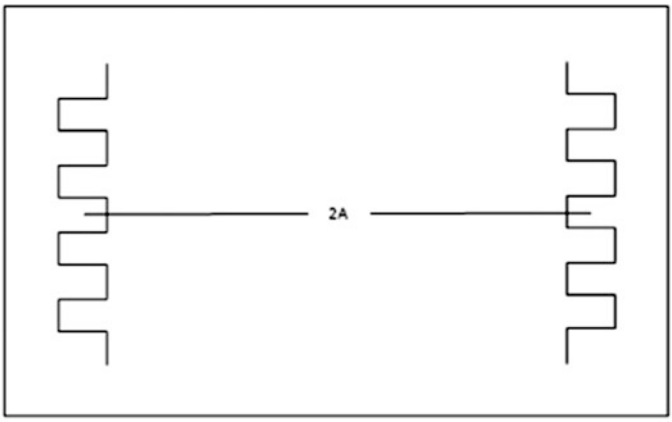

Fig. 16.19 Rectangular Roughness. Step like surface with $\delta e = 1$

$$C_{xy}(z) = \exp\left(-l(x,y) - z\right) \tag{16.13}$$

$$\text{Where } l(x,y) = l + \delta\cos(x)\cos(y) \tag{16.14}$$

where δ is the delta function with the property that it is nonzero at a given x and y and zero at all other values of x and y which defines step like surface giving a structure of rectangular roughness to the wall as shown in Fig. 16.19. The product $\cos(x)\ \cos(y)$ in Eq. (16.14) represents sinusoidal roughness illustrated by Fig. 16.20. With this representation of roughness, the diffusion coefficient thus calculated from Eq. (16.7) has a behavior represented by Fig. 16.21 drawn as a function of z.

5. *Effect of Elastic Confinement*: The arteries have elastic walls which can be further incorporated in this microscopic model by considering compression represented by "*d*" and recovery represented by "*b*" due to elastic wall as shown in Fig. 16.22. Thus the modified frequency takes the form

$$\Omega = \frac{\pi}{2t_1} = \frac{\pi\omega}{2\sin^{-1}(1 - d/A + b/A)} \tag{16.15}$$

Corresponding velocity autocorrelation function and diffusion coefficient have been calculated using Eq. (16.7), and corresponding results obtained are displayed in Fig. 16.23 and Figs. 16.24 and 16.25, respectively. Figure 16.24 shows how the diffusion goes to a non-zero minimum before rising again for an elastic wall where the diffusion completely dies down if the artery walls are hard in nature. Figure 16.25 depicts diffusion behavior of liquid pertaining to walls of different elastic strength. The walls with more elastic strength reached a comparatively higher minimum as compared to the other wall with lower elastic strength. Thus, more elasticity of wall would lead to reduced freezing of the confined liquid. In analogy, one can take the example of flow of blood in arteries which are referred to as microtubes/nanotubes,

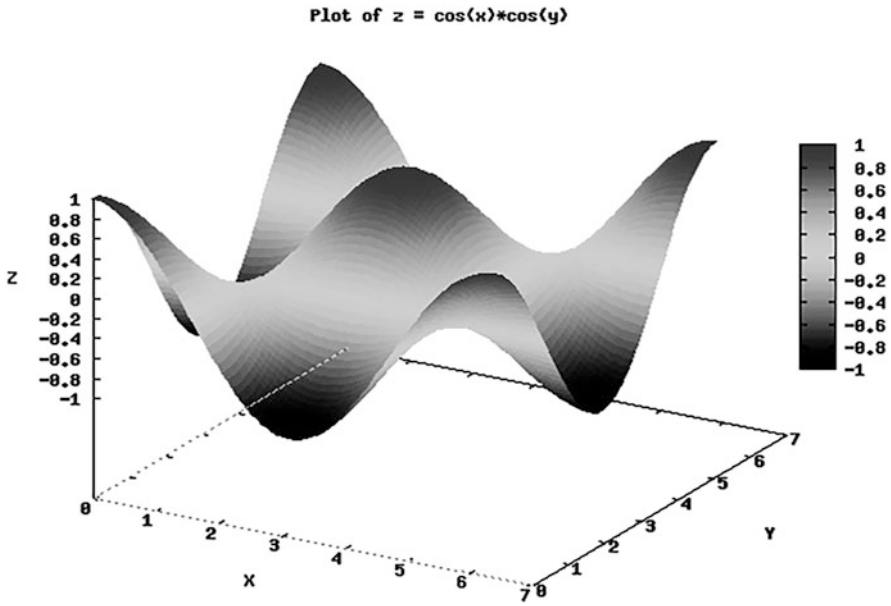

Fig. 16.20 Sinusoidal roughness. Plot of cos(x)cos(y) representing rough surface

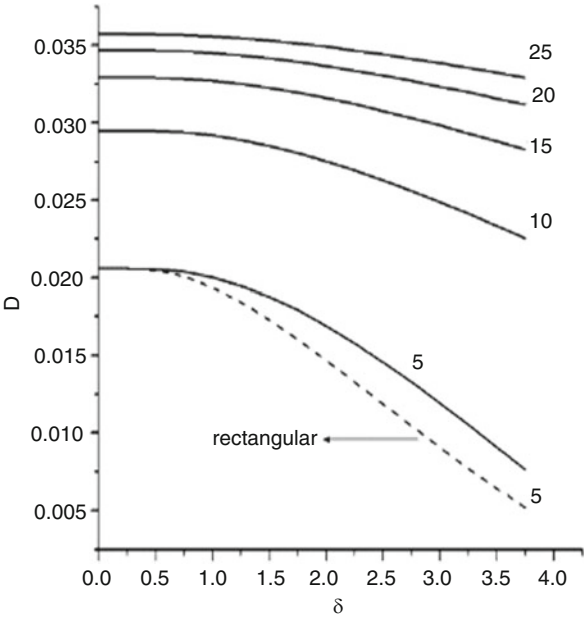

Fig. 16.21 Diffusion coefficient as function of z by including roughness of the wall

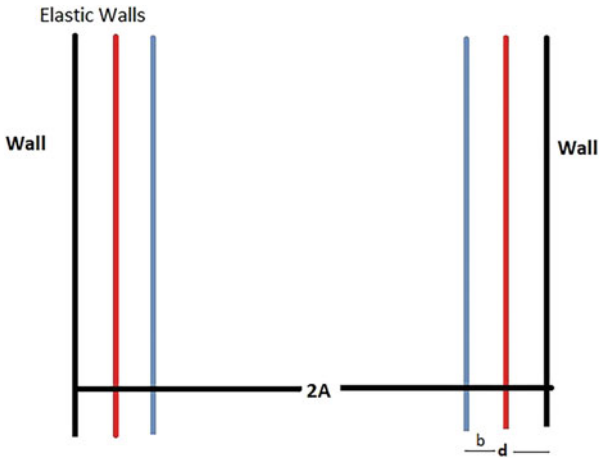

Fig. 16.22 Elastic walls

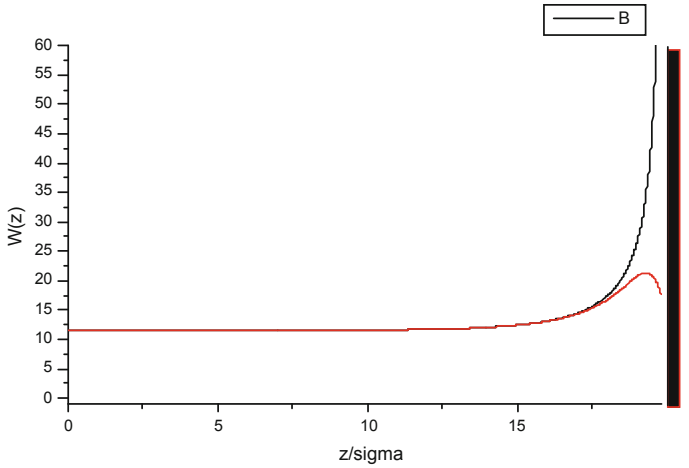

Fig. 16.23 Velocity autocorrelation function

and the phenomena of freezing could be recognized as clotting process of blood. If arteries are kept elastic, clotting occurring due to hardness can be avoided.

16.6.3 Results and Conclusions

- As one approaches the confining wall, the self-diffusion starts reducing, and within two to three atomic layers. The fluid starts exhibiting solid-like behavior.
- The denser the fluid, the more is the tendency to freeze.

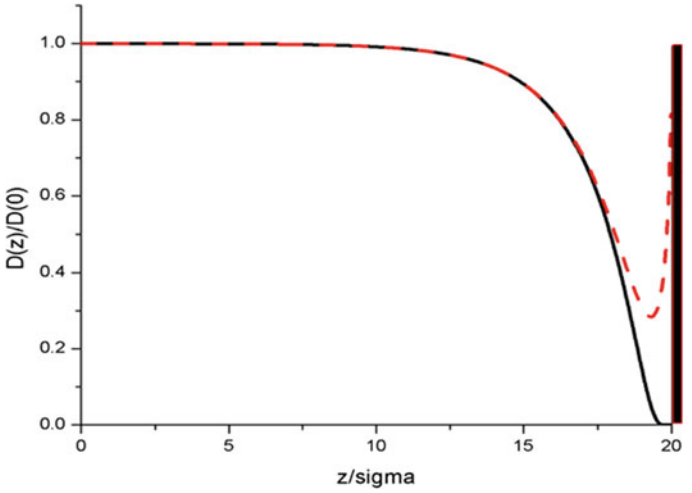

Fig. 16.24 Elastic Wall Vs Hard Wall

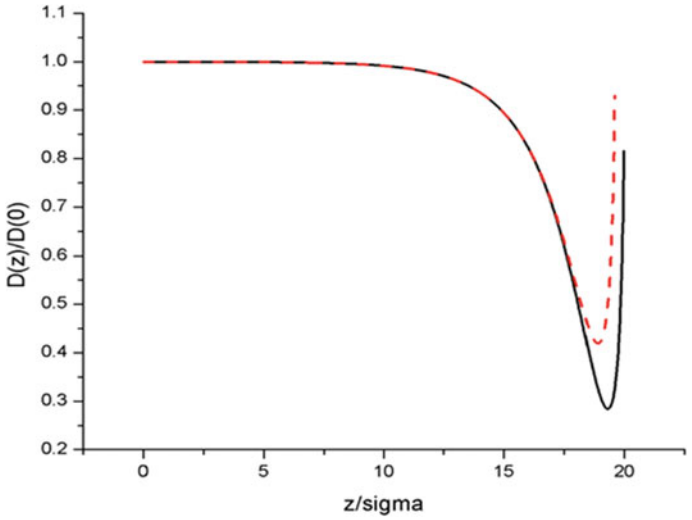

Fig. 16.25 Different Elastic Strength

- Roughness of the wall increases the tendency of the liquid to freeze more quickly.
- The elastic property of the wall helps to reduce the freezing of the confined liquid.
- One can avoid high viscous behavior due to confinement by mixing lower mass isotopic fluid. It helps in reducing friction.

The above explained model could be applied to any complex liquid like blood as complexities of the fluid enter in our model only through the sum rules. This could be further understood as arteries are of similar size (30 times of size of blood cells) when it is compared with the average size of blood particles. It could be extrapolated that thinner blood could provide a smooth flow. However, as the blood gets thicker (denser), it has great probability of clotting (freezing) near the wall. Hence, on freezing, it could provide an artificial wall, which decreases the width of a channel and could trigger more restrictions on its flow. Beyond a critical value of thickness of this artificial wall, the arteries could automatically get blocked.

16.7 Synthesis of Microfluidic Systems

Microfluidic systems can be built by employing technique called photolithography originally used in the semiconductor industry to create small features on circuits. The process involves transferring geometrical shapes present from a mask onto the

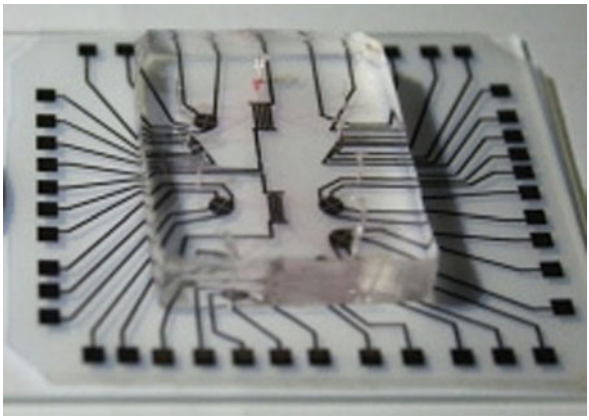

Fig. 16.26 Lab-on-chip (Esfandyarpour et al. 2017)

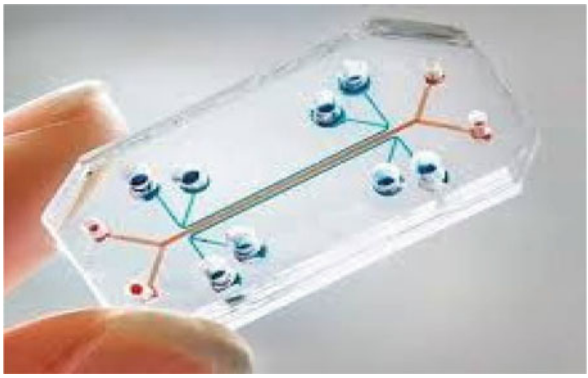

Fig. 16.27 Microfluidic chip (Image: darwin-microfluidics.com)

surface of an appropriate substrate with the support of special polymers which are sensitive only to the well-defined wavelengths of light which would finally create desired geometric patterns on a substrate. These microfluidic systems are employed (Ma et al. 2017) to create lab-on-a-chip devices which are illustrated in Figs. 16.26 and 16.27. The next section deals with a specific application known as heart-on-a-chip which would help and go a long way to find solutions for CAD.

16.7.1 Heart-on-a-Chip

The standard static cell culture approach lacks in fully capturing the intricate in vivo environment. In addition, the current drug discovery process is currently a very difficult and costly process. Moreover, majority of the drug candidates fail to enter even clinical trials. In such a scenario, microfluidics play a vital role in biological research domains which include diagnostic sector, disease modeling, and therapeutic approaches. These include cardiac research area also. Microfluidic technology along with stem cell technology has been playing a revolutionary role in the cardiac tissue engineering which has resulted in fabrication of cardiac lab-on-a-chip which is also known as heart-on-a-chip device (Kitsara et al. 2019). It has been made possible to recreate cardiac tissues using cell culturing techniques in a highly spatiotemporally controlled microenvironment (Chan et al. 2015) from patient-specific models to mimic the natural habitat of the heart cells. This has resulted in heart-on-a-chip devices consisting of numerous imprinted microchambers as well as microchannels on a polymer which is bonded on another material (normally a glass). Transparency and biocompatibility (which is defined as the extent of its permeability to oxygen and carbon dioxide) (Crone 1963) are two required properties of the polymeric material to be used for this application. Such characteristic properties are easily met by the material polydimethylsiloxane (PDMS).

Cells are housed in microchannels and microchambers to which reagents like growth factors, cytokines, and nutrients are delivered. ECM-derived hydrogels (biological reagents) and enzyme/protein stick the cells to the surface and monitor the dynamics of cell culture. Microchambers are usually lined with primary heart cells and controlled with the help of microchannels, microvalves, and actuators. Monitoring is done with the help of digital and biological sensors in addition to the imaging devices (Zhang et al. 2020; Lammertsma 2002).

Flow in the main and side microchannels usually control transportation of nutrition (Renkin 1959) as well as waste discharge. Real-time monitoring of the cells in these microfluidic devices can be carried out by engaging pressure and flow sensors. Electrophysiology and mechanobiology of the experiment could be controlled by tailoring the chip (Liu et al. 2020) by adding electrical and mechanical components. This could also help in mimicking the in vivo conditions. These are known as electrical and mechanical actuators. This can help in actually placing different electrodes in the chip for stimulating the cell as well as using it as a readout system. This device is very helpful in probing the cellular behavior against a number of stimuli.

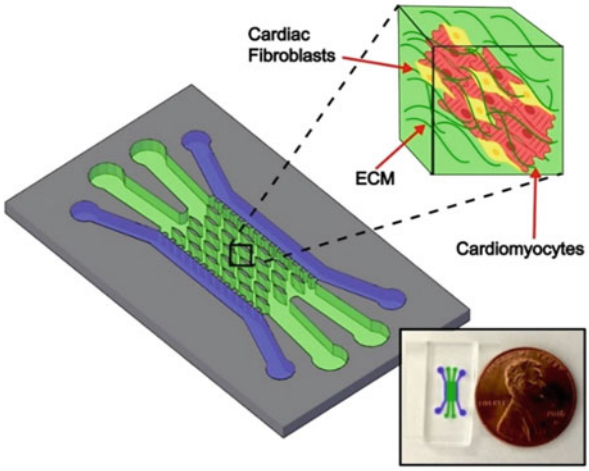

Fig. 16.28 Microfluidic chip for 3D cardiac tissue modeling: schematic of the microfluidic chip with inset of US penny for scaling (Veldhuizen et al. 2020)

A novel way to analyze the functioning of the heart is by making use of miniature microfluidic chips. Mehdi Nikkhah and his group from Arizona State University designed and validated a new heart-on-a-chip platform (Kitsaraa et al. 2019). These are small rectangular silicone-based pieces of the size of a soda can tab and have specially designed channels where cells are deposited. These cells organize and develop into tissues mimicking organs at a much smaller scale (Zhang and Radisic 2017; Sidorov et al. 2017; Marsano et al. 2016). These tissues respond in the same manner as a human heart would to pharmaceuticals and diseases (Selimović et al. 2013). This facilitates researchers to closely detect the responses without the potential for harmD cardiac tissue modeling is shown in Fig. 16.28. The next section deals with possible future applications of microfluidics and nanofluidics.

16.8 Future Possible Expansion of Microfluidics and Nanofluidics

Few possible applications in the field of Microfluidics and Nanofluidics in near future have been listed below:

1. Accelerated progress in sophisticated and innovative technologies can be very helpful in designing and manufacturing of microfluidic and nanofluidic systems which could also promote their commercialization.
2. The first commercial 3D fluidics printer has been launched which could be advantageously used for fluidically sealed devices (Shahzadi et al. 2021; Esfandyarpour et al. 2017) such as valves, chips, connectors, fluid manifolds, and other medical devices.
3. Recently, a manufacturer, Fluigent, has developed the MFCS™ series of microfluidic systems built on its patented FASTAB™ technology which

incorporates pressure driven flow control resulting in a pulseless flow and a greater responsiveness to manage fluid volume manipulation issues.
4. Embedding microfluidic devices may be possible with the help of Biocompatible polymers such as PDMS for carrying out biomedical analysis in the forthcoming years. Microfluidics has immense potential to facilitate single-cell or single-molecule analysis enabling researchers to organize fundamental investigations in cell and molecular biology.
5. Novel microfluidic tools cultivated in research labs could be profitably used in proteomics, genomics, and metal.

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Targeted Gene Delivery Through Magnetofection: The New Face of Medicine

17

Jagmohan Singh, Ipsita Mohanty, R. C. Sobti, and Satish Rattan

Abstract

Lesser effective gene delivery by viral and nonviral vector techniques is one of the most daunting challenges faced by researchers. Overcoming these fundamental challenges, magnetofection has emerged as a cutting-edge technology. The scientific premise is that DNA, RNA, biomolecules, or simply the drugs are either linked to, or encapsulated within, magnetic nanoparticle. The whole therapeutic agent—magnetic nanoparticle (magnetofectin) complex—is then administered in vitro or in vivo under the influence of a high gradient magnetic field. The superior high-grade magnetic field generated forces the magnetofectin complexes to sediment near in vitro cell lineage or localize around the targeted region of the body, thus increasing the uptake of the therapeutic agent and preventing leaching of the complex to surrounding areas. This technology has shown a lot of potential and is gathering attention of the scientists for the diagnostics and theragnostics of the diseases. The current chapter discusses in brief the principles of the magnetofection, designing of the magnetofectin complex, its usage both in vitro

J. Singh
Department of Pharmacology and Experimental Therapeutics, Thomas Jefferson University, Philadelphia, PA, USA
e-mail: Jagmohan.Singh@jefferson.edu

I. Mohanty (✉)
Departments of Pediatrics, Children's Hospital of Philadelphia Research Institute, Philadelphia, PA, USA
e-mail: mohantyipsita8@gmail.com

R. C. Sobti
Department of Biotech, Punjab University, Chandigarh, India

S. Rattan
Department of Medicine, Division of Gastroenterology and Hepatology, Thomas Jefferson University, Philadelphia, PA, USA

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and in vivo settings, as well as its prospects in the field of modern science and medicine.

Keywords

Magnetofectin · Targeted gene delivery · Magnetofection · Iron nanoparticles

17.1 Introduction

Targeted drug delivery has always been the goal of researchers to minimize side effects of the therapeutic agents. Ideally, a preferable drug delivery system is non-immunogenic, nontoxic, biocompatible, cost effective with ease of localized delivery, as well as suitable for human use (Coelho et al. 2010). Researchers all over the globe are tirelessly working to design such drug delivery system, which is both safe and can target the area of interest. One of these drug delivery approaches is magnetic nanoparticle (NP) (Nandi et al. 2017).

Recently, magnetic nanoparticles (NPs) have gained popularity and are the focus of discussion among researchers. Magnetic NPs have a biodegradable iron oxide core and an outer polymer coating. This polymer is often loaded with the drug of choice using certain types of linkers or even conjugated to the magnetic nanoparticles (Fig. 17.1). Once coated these nanoparticles can be delivered into the systemic circulation via intravenous injections or administered near target site in the body. A strong magnetic field around the target tissue is applied that facilitates magnetic NPs to accumulate around the targeted area and perform desired effect instead of leaching into the systemic circulation, thus preventing undesirable side effects in other organs of the body. This approach is being preferred by researchers,

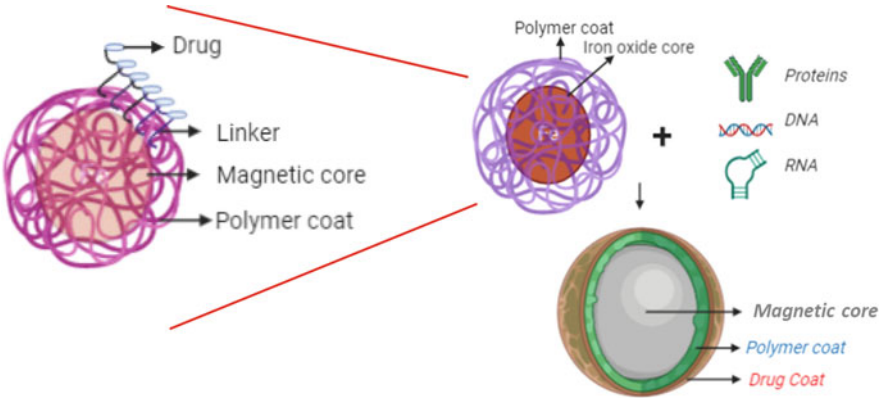

Fig. 17.1 Design and concept of magnetic nanoparticles. Typical magnetic nanoparticle has iron oxide magnetic core and polymer coating outside. Drugs/biomolecules are linked to polymer via a linker. Once administered into the body, the cell's linker gets digested with enzymes or by heat, and drug/biomolecules are released inside the cells (Chen et al. 2017; Wang et al. 2018)

clinicians, and other health professionals throughout the world, and continual efforts are being made to improvise this approach. Researchers are using this magnetic nanoparticle as an effective tool in the in vitro experiments to deliver the nucleic acids into the cells which are hard to transfect (Marcus et al. 2016). Its use has also been extrapolated for targeted delivery of other biomolecules such as antibodies, plasmids, proteins, microRNA (miRNA), silencer RNA (siRNA), noncoding RNA, and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) to the targeted regions in the body (Hryhorowicz et al. 2019; Jin et al. 2018; Rohiwal et al. 2020; Zhu et al. 2019; Kaushik et al. 2019). In this chapter we will discuss different aspects of this emerging new technology including its basic principles, different types of magnetic nanomaterials used, its application in the ex vivo and in vitro studies, as well as its future implications.

17.2 Discovery, Basic Principle, and Technique

The use of magnetic nanoparticle for drug delivery was first proposed in 1978 by Widder et al. (Widder et al. 1978) The principle behind the magnetic nanoparticles is biomolecules or drugs that are attached to polymer-encapsulated nanoparticles with a magnetic core made of nontoxic iron oxide or porous polymer structure that can accommodate iron oxide magnetic NPs which get precipitated within the pores (McBain et al. 2008). The drug is generally attached to the polymer with appropriate linker, and then the therapeutic agent NP (magnetofectin) complex is injected into the bloodstream, or near the disease target site in the body. Magnetic fields created by powerful rare earth neodymium magnets is applied over the target site to attract the magnetofectin leading to their accumulation at the target site. While this is effective for targets near the body's surface, it may be hard to apply magnetic field at sites deeper within the body. Researchers have been working relentlessly to devise a solution which can resolve this issue. The use of magnetized stunts, implantation of magnets near the target site, or magnetic probes within the body (Kubo et al. 2000) have been beneficial in coping with the limitations. Besides targeted delivery, NPs have also been employed for diagnostic purposes like magnetic bioseparation and purification of biomolecules, magnetic biosensing, magnetic imaging and treatment for hyperthermia (Wu et al. 2019).

17.3 Applications

In vitro applications: Magnetofection has emerged as a useful method in cells which are difficult to transfect. OZ Biosciences INC USA (San Diego, USA) has the flagship product line for developing and commercializing molecular delivery systems specialized in transfections of nucleic acids, viral vectors in models which are difficult to transfect such as stem cells, microglial cells, neurons, or endothelial cells. Transfection efficiency depends on many factors including cellular binding and internalization of reagent-gene complexes, delivery of nucleic acids into the

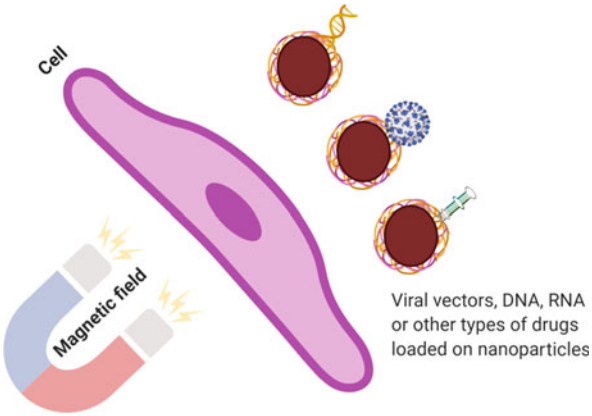

Fig. 17.2 Principle of magnetofection. Magnetic nanoparticles are coated with different types of drugs and delivered near the cells under the influence of an external magnet. Magnetic field directs the drug-coated magnetic nanoparticles toward the cell and facilitates fast, effective, and localized drug delivery in in vitro models

cytoplasm, nuclear uptake and expression of the gene, in addition to the cell's metabolic activity, and reproduction state. The primary mechanism for biomolecules releasing into the cells involves cell surface receptors that latch onto the cell-like liposomal reagents, triggering receptor-complex interactions and internalization through endocytosis. Most of the cells except a few are covered with these surface receptors and have rapid division, high rate of endocytosis, and high metabolic activity, thus making them a good model for transfection. On the other hand, immature cells, including stem cells, and uncommitted progenitor cells are devoid of these features. Similarly, primary cells often employed as in vitro models in basic research and drug discovery have lower endocytosis uptake and less reproduction activity and are unable to adhere to transfection complexes. In these models, magnetofection is an effective approach to deliver biomolecules such as DNA, RNA, and protein for in vitro and in vivo applications, enabling incorporation of the transfection complexes without physically introducing pores on cell membrane or causing damage in the cells. It uses metallic NPs coated with cationic molecules complexed with biomolecules including naked, packed, or virus-enveloped portion, which are bound by electrostatic and hydrophobic bonds. These magnetofectin complexes attach loosely to the cells but, under the influence of a magnetic field created by placing a magnet under the culture dish, are localized, concentrated onto the cell surface, and eventually internalized through endocytosis (Figs. 17.2 and 17.3). In contrast to other mechanical techniques such as gene guns, electroporation, and sonoporation, magnetofection does not compromise the cell membrane or cause cell death; instead it imparts the lowest level of stress, along with maximum efficiency of transgene expression.

Another advantage of magnetofection is consistency. Once the protocol for the gene incorporation or protein yield is optimized, conditions are reproducible for yielding identical results. Considering the numerous benefits, it is expected that application of magnetofection will broaden in biomedical science in future. They also have a remarkable potential in clinical setting or bedside use. These magnetofectin complexes can be modified in vitro as CART (chimeric antigen

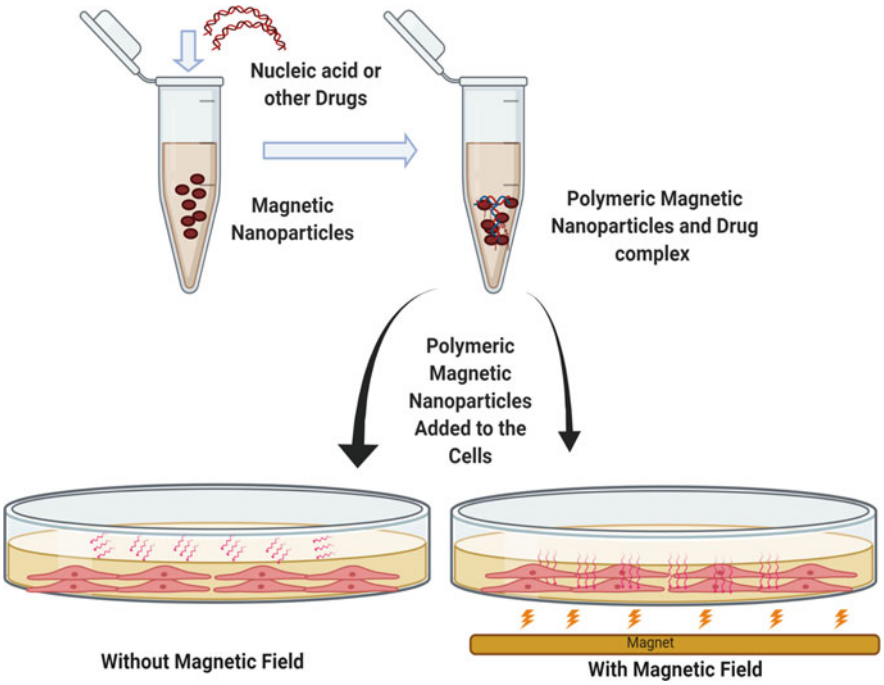

Fig. 17.3 Magnetofection increases the transfection efficiency. Magnetic nanoparticles are mixed with the nucleic acids or other drugs and added to the cell culture plates, and a strong magnetic field is applied under the tissue culture plates with a magnet. This helps in overcoming resistance by cell membrane for hard to transfect cells lines leading to rapid uptake of magnetic nanoparticles by the cells and release of coated drug inside the cells

receptor T) cell therapies and gene therapy and can be re-injected into the patients for therapeutic or prophylactic uses in complex genetic disorders.

17.3.1 Ex Vivo Applications

17.3.1.1 Alternative Approach to Viral Vector Delivery of CRISPR/Cas-9 in Animal and Plant Cells

Recently, CRISPR/Cas9 has emerged as a unique genome editing tool that enables researchers to edit parts of the genome by deleting, modifying, adding, or inserting desired DNA sequences. Although virus transduction is being used extensively in delivery of CRISPR/Cas-9 in various organisms, they have some disadvantages, such as the risk of cytotoxicity, immunogenicity, expensive large-scale production, limited insertion size, and risk of integrating viral sequences into the target genome (Glass et al. 2018). Therefore, nonviral delivery systems for CRISPR/Cas9 are a promising alternative. Magnetofection has proved to be better than electroporation,

nucleofection, and chemical delivery such as lipofection techniques as it has no impact on cell viability and has high transfection efficiency (Glass et al. 2018). Currently, nonviral delivery routes for the CRISPR/Cas9 components via the use of magnetic NPs have the potential to overcome these pitfalls of different techniques enumerated above. The day is not far when CRISPR/Cas-9 with magnetofection will revolutionize the field of medical science (Glass et al. 2018; Huth et al. 2004; Berry et al. 2003).

A proof-of-principle study in an in vitro model showed that stimulating magnetic NPs with a magnetic field facilitates particle migration across the blood-brain barrier (Kaushik et al. 2019). After passing the blood-brain barrier, a CRISPR plasmid was released by an alternate magnetic field trigger.

Magnetofection is an effective approach for primary endothelial cells. Other applications include advances in ex vivo tissue engineering, designing of tumor vaccines, targeted therapy for cancer, and cardiovascular therapy. In parallel, an independent study in a porcine airway model, authors have reported a significant and rapid improvement in the expression of reporter gene through magnetic NP, which they attributed to an increase in contact time with the mucociliary cells, thereby reducing their clearance from the target site (Xenariou et al. 2006).

17.3.1.2 Patient-Derived Xenografts and 3D-Bioprinted Prosthetics

3D-bioprinted organs have huge translational capabilities across in vivo, in vitro, and ex vivo applications (Ramadan and Zourob 2021). Magnetofection can be a futuristic tool for the successful delivery of 3D-bioprinted scaffold or prosthetics to the organ before transplantations into the experimental models.

17.3.2 In Vivo Applications

Magnetofection has been widely used for different biological agents (viral and nonviral vectors, and for the delivery of DNA, nucleic acids, and siRNA) in living animals. In living animals drug-coated nanoparticles can be injected into systemic circulation or locally near the disease-affected region. Consequently, magnetic NPs are attracted and retained in the area of interest in the body by the application of magnetic field (Fig. 17.4). Magnetofection is a convenient and more effective tool than electroporation or other chemical methods for the biomolecule delivery to target cells on different internal organs such as the lungs, kidneys, spleen, GI tract, and blood vessels. It offers numerous advantages for antisense ODNs (antisense oligodeoxynucleotides) delivery requiring higher cellular uptake of vector in minutes and gene expression targeted at the desired site of action. The first report for the use of in vivo magnetofection was demonstrated by Plank et al. using magnetic NP complexed with pDNA injected into the pig ear vein (Plank et al. 2003; Scherer et al. 2002). They showed a confined localization of the reporter gene around the ear vein with magnetic NPs (Scherer et al. 2002). This concept was further explored in several clinical trials in cats. Currently, the magnetic NPs with doxorubicin as an anticancer drug are also under clinical trial (Mukherjee et al.

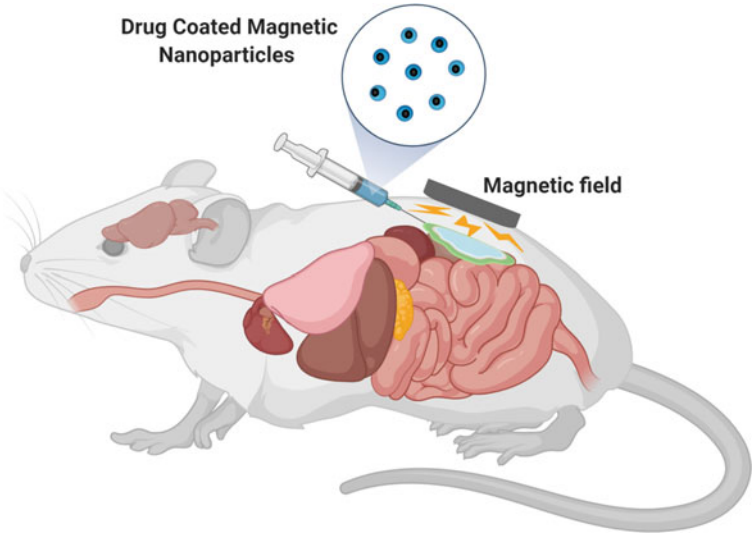

Fig. 17.4 Principle of *in vivo* drug delivery via magnetofection. Drug-loaded magnetic nanoparticles are injected at the site of tumor or disease, and magnet is placed over the site of injection. This facilitates localized delivery of drug and prevents its leaching into systemic circulation

2020). A study carried out using magnetic albumin microspheres with entrapped doxorubicin in the rat model for tumors has proved that magnetofection has potentials to deliver to a relatively narrow area of tumor preventing drug leaching into other organ systems (Laurent et al. 2011; Titze de Almeida et al. 2018).

17.3.2.1 Lungs

Iron oxide NPs could be used in inhalers for magnetic delivery into the lungs under the influence of an externally applied magnet. Price et al. observed targeted delivery of nano-in-microparticles (NIMs) containing superparamagnetic iron oxide nanoparticles (SPIONs) and doxorubicin (DOX) in the lung with an external magnet (Price et al. 2017). They showed that when mice were administered fluorescently labeled NIMs as a dry powder through endotracheal mode in the presence of an external magnet placed over one lung, DOX-loaded NIMs were therapeutically efficient, thus allowing for the targeted delivery (Price et al. 2017; Price et al. 2018).

17.3.2.2 Eye

Of late the use of magnetic NPs in ophthalmology has gained massive interest by clinical trials because of its easy accessibility and immunosuppressive barrier (within the vitreous and aqueous humors), thus facilitating the use of noninvasive maneuvers for the therapeutic uses. Applications of iodate and gene therapy remain the most promising approach against acute macular degeneration and retinitis pigmentosa. Adeno Associated Virus (AAV) is one of the most promising gene augmentation tools for the treatment of ocular diseases and magnetic NPs offer an added

advantages in targeted gene delivery to the posterior eye, thus limiting the use of multiple invasive injections. Luxturna (voretigene neparvovec-rzyl) is a FDA approved Adeno Associated Virus (AAV) gene therapy to treat eye disorders in humans (Smalley 2017). Moreover, there are a number of other clinical trials going on around the world to treat eye diseases through gene therapy using magnetofection (Bordet and Behar-Cohen 2019; Czugala et al. 2016).

17.3.2.3 Gastrointestinal Tract

Recently, we have demonstrated successful delivery of microRNA-139-5p (miR-139-5p) into the rat internal anal sphincter (IAS) (Singh et al. 2018) using in vivo magnetofection. The IAS tone plays a major role in the rectoanal continence via activation of RhoA-associated kinase (ROCK2), miR-139-5p targeting Rho kinase 2 (Singh et al. 2017). Using a multi-pronged approach of confocal microscopy showing confined delivery of miRNA around IAS through immunofluorescence images as well as ex vivo physiological and biochemical validation showing that miR-139-5p decreased the basal IASP (internal anal sphincter pressure), the basal IAS tone, and the rates of contraction and relaxation which are associated with fecal pellet output, we demonstrated that magnetofection is a novel method of in vivo gene delivery for the site-directed therapy of the rectoanal motility disorders. These studies have direct therapeutic implications in rectoanal motility disorders especially associated with IAS (Fig. 17.5) and potentially other gastrointestinal motility disorders.

Another group of researchers have reported the potential usage of magnetofection for in vivo delivery of silencer RNA (siRNA) using magnetic crystal-lipid nanostructures in cancer gene therapy (Namiki et al. 2009). Authors here used a magnetite nanocrystal coated with oleic acid and a cationic lipid shell and complexed it to EGFR-specific siRNA, which was injected to the mice. Following administration of siRNA complexed to the magnetic core-encapsulated cationic lipid shell, authors observed the distribution in the spleen followed by the liver and lung. For in vivo magnetofection, titanium nitride-coated magnets were internally implanted under the skin peripheral to tumor lesions or were externally placed onto the skin. Authors observed a significant reduction in tumor volume compared to the control group following internal and external applications of a magnetic field 28 days after the initiation of treatment.

17.3.3 Magnetic Implants

While stationary external magnets are useful in superficial drug delivery under the skin, it can be challenging to deliver drugs into the deeper layers of the skin and internal tissues. Here, use of magnetic implants deep under the skin and deep in the body shows a promising solution (Shapiro 2009). Ge et al. provided a proof of concept for the magnetic implant-directed nanodrug delivery substituting the need for an external magnetic field (Ge et al. 2017). They used a biocompatible magnetic implant scaffold made of a magnetite/poly (lactic-co-glycolic acid) nanocomposite

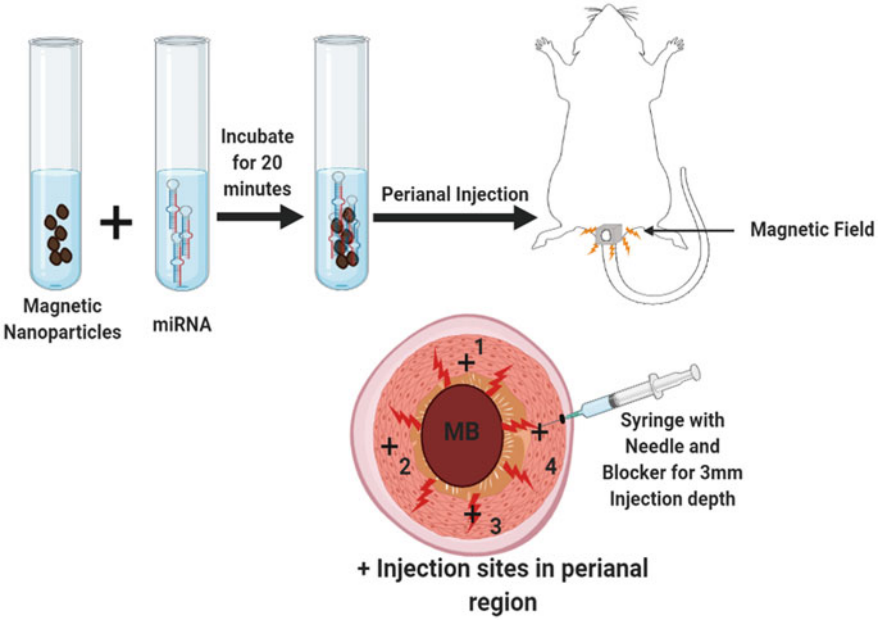

Fig. 17.5 Protocol for *in vivo* magnetofection (MF) of miRNA in the internal anal sphincter. (**a**) miRNA is mixed with magnetic nanoparticles and injected in the perianal region. Magnetic field is applied inside the anal canal to hold the magnetic nanoparticles at the injection site. (**b**) Model shows the positions of perianal injection in circular smooth muscle layer of internal anal sphincter. Length of injection needle was adjusted with a rubber blocker to ensure that needle only penetrates unto circular smooth muscle layer

which effectively attracted nanodrugs to its surface, thereby killing cancer cells. These magnetic implants show an optimistic approach in targeted chemotherapy. Moreover, magnetic implants can be embedded in the deeper body regions such as fatty tissue to manage obesity (Saatchi et al. 2017) and in the inner ear to treat deafness (Le et al. 2017).

17.3.4 In Vivo Magnetofection of Viral Vectors as Virus Stamping

In vivo magnetic NPs associated with different viruses are used to regulate expression of fluorescent markers or calcium markers in an in vitro or an in vivo model. This technique employs an electromagnet and is often termed as virus stamping; it enables targeted single-cell infection with different viruses at the same time (Schubert et al. 2018; Schubert et al. 2019). Subsequently, lentivirus vector complexed with magnetic NPs delivered into different organs and cells demonstrated higher transduction efficiency than other routine techniques. This method is highly effective for gene transfer in allograft in heart transplantation or acidic stomach lumen when other physiological barriers like temperature, time of exposure, and

harsh acidic pH restrict the transduction efficiency. It also offers low immunogenicity, high reproducibility, and effective transduction of non-permissive cells (Gao et al. 2015; Dadfar et al. 2019).

17.4 Limitations

Despite numerous advantages, translation of this concept of magnetofection in translational applications has its own limitations. Firstly, in a living system, the efficiency of gene delivery is dependent on the route of administration, accessibility of target site, and the size of the animal (increase in targeted area would require stronger magnetic field). Moreover, too small particle size (less than 50 nm) renders it unsuitable for magnetic targeting, and big particle size (more than 5 μm) may impede the absorption of magnetic NPs from systemic circulation. Similarly, the regions with slow blood flow rate also affect the transfection efficacy, for example, in human aorta where the blood flow rate is 20 cm/s, effective transfection is a challenge. The external magnetic flux density and gradient decrease at a distance from the magnetic pole, which also affect the transfection efficacy. The shortcomings of this delivery system can be overcome by modifying NP formulations and generating novel magnetic field skills suitable for the relevant study. Another suggestive approach is to improvise the use of powerful magnetic bioprobes to retain a high magnetic field for efficient targeting and designing unique bioprobes for efficient drug uptake. We believe that the major challenge in magnetic drug delivery is the delivery of the magnetofectin into the deep organs. The recent strategy of magnetic or magnetizable implants looks promising but requires a further investigation and in vivo validation before being translated into clinical setting. Currently, efforts from biophysicists and engineers are being made to push this field forward to real-life applications. Regardless, magnetofection still remains the most sought localized gene delivery tool which can be adapted as per different experimental needs and therapeutic potentials.

17.5 Summary

In conclusion, magnetofection is a novel drug delivery technique with lots of pharmaceutical potential for effective drug delivery. Hopefully, future innovative work in this area will advance this field for designing of novel magnetic probes for the noninvasive targeted drug delivery of biomolecules in the human body to have efficient and targeted effect with less side effects in treating a specific ailment.

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QbD-Steered Systematic Development of Drug Delivery Nanoconstructs: Vital Precepts, Retrospect and Prospects

18

Bhupinder Singh, Teenu Sharma, Ranjot Kaur, Sumant Saini, Ripandeep Kaur, and Sarwar Beg

Abstract

Nanostructured systems provide immense advantages not only in regard to delivery and release of drugs but also in their targeting potential. Such drug nanoconstructs either can be modified for receptor-mediated and site-specific targeting or can be used to regulate release of drugs, widely ranging from insoluble to highly soluble molecules, proteins and peptides to monoclonal antibodies, genes and small RNA. Nanoscale drug delivery systems can not only be fine-tuned for desired drug release kinetics and biodistribution but can also minimize the toxic effects, thereby enhancing the therapeutic index of a given drug. The domain of developing nanostructured drug delivery formulations has lately witnessed an enormous paradigm shift towards their methodical development. Promulgation of Quality-by-Design (QbD) guidance documents by the WHO, the US-FDA and other regulatory bodies has evidently influenced the manufacturing approach of such drug products, furnishing comprehensive understanding of the consequent drug products and pharmaceutical processes. Lately, a scientific approach, christened by the authors as "Formulation by Design (FbD)", has been in vogue, devoted wholly to QbD-steered development of drug products. FbD strategy targets to harvest novel and cutting-edge systems utilizing thrifty resources of developmental time, manpower, materials and finances. A diversity of drug nanoconstructs has since been developed efficaciously as per FbD principles and testified in literature. The present manuscript tends to furnish coherent perspectives on the FbD terminology, methodology and applicability in developing several wide-ranging nanoconstructs, providing their contemporary updates from their academic, industrial and regulatory perspectives.

B. Singh (✉) · T. Sharma · R. Kaur · S. Saini · R. Kaur · S. Beg
Centre of Excellence in Nano Biomedical Applications, Panjab University, Chandigarh, India
e-mail: bsbhoop@yahoo.com; bsbhoop@pu.ac.in

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Keywords

Nanoparticles · Nanocargos · Formulation by design (FbD) · Nanomedicine · Design of experiments (DoE) · Nanoformulations

18.1 Introduction

The drug delivery domain has bequeathed a newer stance towards pharma product development and consequent patient therapeutics. The scientists have undertaken daunting strides for developing an assortment of novel delivery technologies, predominantly incorporating a plethora of drugs, excipients (whether functional or non-functional) and manufacturing processes. Drug delivery technologies, consequently, constitute different drug formulations specifically engineered to act as per the requirements and through their respective route(s) of body administration. The foremost consequences of such drug delivery developments have eventually been able to address the unmet patient needs for maximizing the clinical throughput, minimizing the possible toxic effects and surmounting the issue of patient non-compliance (Korting and Monika 2010; Momin et al. 2016).

Worth one billionth, "nano" has accomplished mammoth magnitudes today, worth billions (of currencies). Not simply an "evolution", this has recently emerged out as a "revolution" among divergent scientific realms and industrial sectors, the world over. Nanoscale systems tend to unveil astronomical benefits, including boosted surface area per unit volume, enhanced drug solubility, target specificity, biocompatibility, regulated drug release potential, stealth features, precise particle size control and increased bioavailability too (Crommelin et al. 2003; Korting and Monika 2010; Raza et al. 2013).

Nanomedicine encompasses the application of nanoscale technologies in medical practice for safe, effective and patient-compliant management of diseases. This multidisciplinary science has lately offered inimitable promise to revolutionize the therapeutic approach to detect, prevent, treat and eradicate various disorders, including cancer. Recently, nanomedicine has become the cynosure of medical investigations owing to the uniqueness of various nanoconstructs employed for the purpose. Besides their size per se, alteration in the surface properties and functional behaviour has brought a metamorphic change in the carriers and their usefulness at different levels. The key to success of nanomedicine lies in the search of apt nanocarriers and their apt blends. The ongoing battle against chronic diseases has been grossly fortified with the development of various biopolymers in nano-form, enabling the realization of the dream to use drugs as smart "magic missiles". The dynamic properties of these systems increase the selectivity of drugs towards their specific targets, leading eventually to minimization of their side effects and enhancement of their efficacy in minimal dosage.

Operation at nanoscale tends to ameliorate the ability of these drug delivery carriers to surpass cell membranes and biological barriers, including gastrointestinal, ocular, placental, skin, tumour and blood-brain barrier, as illustrated in Fig. 18.1.

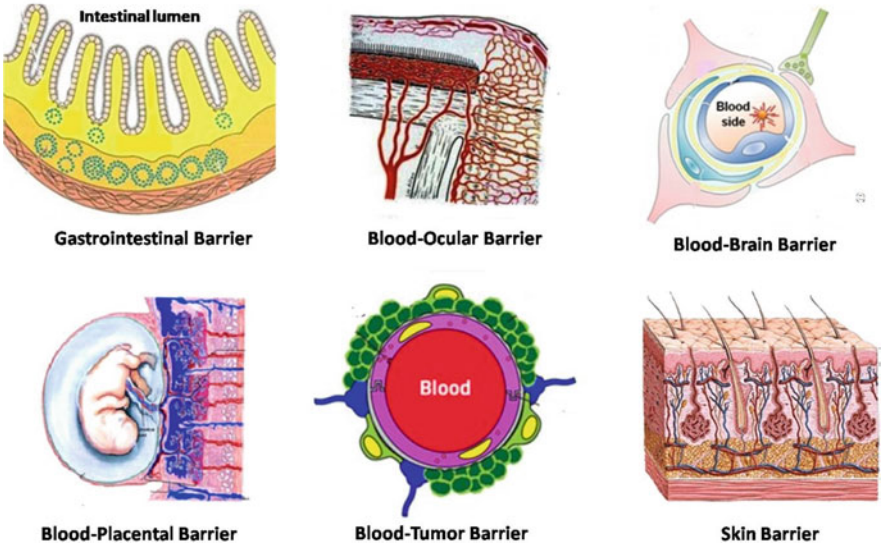

Fig. 18.1 A diversity of crucial biological barriers that a drug delivery system has to cross in the human body

Drug nanocargos establish an intimate contact with biological membranes resulting eventually into an improved membrane penetration and permeability and assigning them the ability to improve pharmacokinetic fate and biodistribution of a vast majority of drug molecules (Zahin et al. 2019). At the same time, nanoparticles, by virtue of their much greater surface area-to-volume ratios, increase drug dissolution rate too, enabling them to overcome solubility-limited bioavailability of a vast number of drug candidates (Bhatia 2017; Zahin et al. 2019).

The escalating number of approvals of nanoscale drug products by the drug regulatory agencies, with several more in various clinical trial phases, is an authoritative testament to the growing prominence of effective and safe nanopharmaceutical products (Danhier et al. 2017; Transparency Market Research 2018). Albeit such nanoformulations are estimated to fulfil the conventional compendial requirements, their strikingly different nanoscale characteristics make them amenable to high product quality inconsistencies. Design of robust nanocarriers possessing the desired quality traits, as well as their manufacturing processes, is invariably a Herculean task in this age (Prud'homme and Svenson 2012; De Crozals et al. 2016).

18.2 Drug Delivery Product Development

Drug delivery formulations have been developed since centuries by the straightforward intuitive approach of hit-and-trials. Typically, it involves investigating the effect of the respective product and process variables by choosing *one-factor-at-a-*

time (OFAT) stratagem (Lewis et al. 1998; Singh et al., 2005b, 2011a, c; Aksu et al. 2015). During such OFAT studies, the first factor is affixed at a nominated value, and the next one is scrutinized until there is no betterment observed in the response(s), further. The use of the conventional OFAT approach has verily been a unidimensional optimization plan, producing "just satisfactory" solution(s), with hardly much scope at finding errors and their plausible corrections (Lewis et al. 1998; Singh et al. 2005b, 2011a, c; Aksu et al. 2015; Singh et al. 2017a, b).

The aforesaid OFAT approach though accomplishes the solution to a particular challenging trait, yet attainment of its true optimum solution can never be warranted. This could invariably be ascribed to the prevalence of *interactions*, i.e. positive (synergistic) or negative (antagonistic) influence of one or more of input factors on the responses. The presence of such variable interactions renders the usage of OFAT methodology as unsustainable, usually fetching a solution way distant from the desired optimum (Lewis et al. 1998; Montgomery 2001; Singh et al., 2005b, 2017a, b). The eventual product accomplished using this methodology though may look to be acceptable, yet is usually sub-optimal. Not being systematic, this OFAT paradigm needs expensive and extensive experimental "pains" in order to achieve diminutive informational "gains" on the product or process getting developed (Cochran and Cox 1992; Lewis et al. 1998; Singh et al. 2005b; Aksu et al. 2015; Durakovic 2017). The OFAT approach, in a nutshell, has proved not only as untenable on account of exorbitant investments like experimental effort, time and cost but also owing to its incompetency to offer the real-time results by mending the flaws, poor predictability and many a time even attainment of successful outcomes. The erstwhile expertise, experimental know-how and experiential wisdom of the formulation scientist have been the essential requisites while developing the drug products for catering to the tailored requirements.

Despite incessant novelties brought forth by the pharmaceutical industry from time to time, recurrent incidences of product recalls, rejects and failures have been encountered, acceptably owing to their not-up-to-the-mark quality and manufacturing standards (ICH Harmonised Tripartite Guideline 2009; Singh et al. 2013; Singh 2014; Aksu et al. 2015). The conventional *Quality-by-Testing (QbT)* approach has been found to involve a great deal of expenditure of time, materials and manpower, but intermittent testing for monitoring the quality of drug delivery products is crucial throughout their development cycle (Singh 2014; Singh et al. 2017a, b).

18.3 Formulation by Design (FbD): Vital Precepts

Of late, a holistic and systematic paradigm of pharma *Quality by Design (QbD)* has been trending in drug formulation development (ICH Harmonised Tripartite Guideline 2009; Singh 2014; Aksu et al. 2015; Beg et al. 2019). As per ICH Q8 (R2), QbD is a methodical stratagem to assess, comprehend and improve the quality of product (s) and process(es) and their pertinent quality attributes (ICH Harmonised Tripartite Guideline 2009). This QbD approach has gained phenomenal popularity among

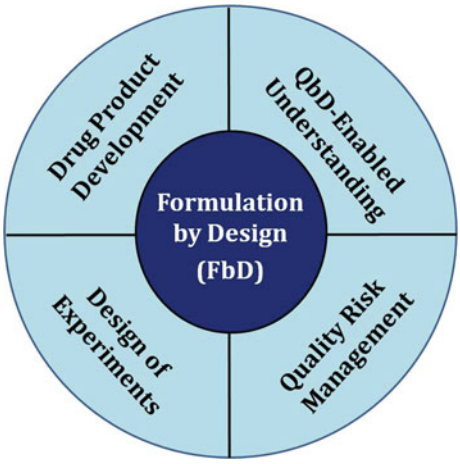

Fig. 18.2 Cardinal elements of Formulation by Design (FbD)

pharmaceutical circles, not merely due to strong persuasion from the International Council for Harmonisation (ICH), but its subsequent ratification from key regulatory agencies like the US Food and Drug Administration (US-FDA), the World Health Organization (WHO), the European Medicines Agency, and several others, testified by their corresponding regulatory guidance documents (Singh 2014; Aksu et al. 2015; Singh et al. 2017a, b).

QbD is almost an obligatory catchword today all across the pharmaceutical sector of the globe, accentuating on building quality into the system all through the product development cycle, without relying on terminal testing of the products (ICH Harmonised Tripartite Guideline 2009; Singh 2014; Aksu et al. 2015; Beg et al. 2019). Incorporating the principal elements of *Design of Experiments (DoE)* and *quality risk management (QRM),* QbD chiefly undertakes science- and risk-based approaches to hit on categorical *product* and *process comprehension* (ICH Harmonised Tripartite Guideline 2005; Singh 2014). Hence, QbD is a rational amalgam of QRM- and DoE-based pharma production while endeavouring to unravel to gain all-inclusive process and product understanding. This has lately become a routine practice in pharma industry, institutional research and regulatory compliance (Singh 2014; Djuris and Djuric 2017).

Verily, the QbD precepts are based upon J.M. Juran's quality philosophy, to produce quality products and deliver services by pre-planning of their quality and avoiding any issues at the final stages of production phase (Yu et al. 2014; Aksu et al. 2015; Montgomery 2001; Singh et al., 2005b, 2013). The benefits of QbD have not only been harvested towards unearthing the science underlying the process of product development but also of analytical development, drug substance manufacturing, dissolution testing, bioavailability studies and biologicals. Owing to much vaster purview of QbD applications today, a pithier term, viz. "Formulation by Design (FbD)", was proposed a few years ago by us, germane precisely to the practice of QbD principles in developing drug delivery products (Singh et al. 2011c). Figure 18.2 elucidates the vital implements of FbD, which comprise not only

QbD-steered product or process understanding, and usage of DoE and QRM principles, but acquiring deeper knowledge and know-how of product development paradigms too.

The exceptional feature of an archetypal FbD strategy is its precise portrayal or premonition of drug product performance and its distinct ability for detection and estimation of possible synergism or antagonism among several input variables (Singh et al. 2011c; Singh 2013; Beg et al. 2017a). Abundant shortcomings of the OFAT approach, discussed in the previous section, verily call for adoption of FbD as a much more sensible, systematic and innovative approach to engineer drug nanocarriers for catering to the specialized and customized needs of patients (Singh et al. 2011c; Beg et al. 2017a). While embarking upon the optimized nanostructured drug delivery technologies, FbD offers myriad advantages over the traditional OFAT approach (Singh et al. 2009a, b, 2011c, 2012), as outlined in Box 18.1.

Box 18.1 Key Benefits of Implementing QbD Practices During the Development of Drug Delivery Products

Meritorious visages of QbD-steered drug delivery development
• Superior quality drug delivery products
• Augmented product and process comprehension
• Perceptive planning employing synergistic team approach
• Decreased outflow of manpower, materials, money and time
• Improved access to the commercialization
• Reduced drug product rejects and recalls
• Quicker regulatory review and approval of products
• Admirable returns on corporate investments
• Negligible consumer cynicism on generic drug products
• Reduced regulatory queries and requirements
• Reduced post-approval changes
• Wider operating ranges

18.4 FbD Methodology

The entire FbD methodology can be holistically envisioned to be implemented in five steps, as illustrated in Fig. 18.3.

18.4.1 Step I: Postulation of Objective(s) of Developing Nanostructured Drug Products

A quality target product profile (QTPP) delineating various desired quality traits considers its efficacy and safety for the end user, i.e. the patient is outlined. Selection

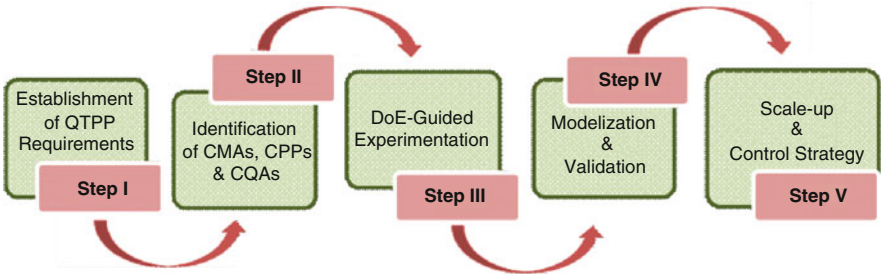

Fig. 18.3 Five-step QbD methodology for developing drug products

of *critical quality attributes (CQAs)* from the QTPP depends on the extent of their influence on the patient's health. Such CQAs are the biological, physicochemical or microbiological attributes that need to be monitored and maintained in a specific range to attain the desired product quality (Singh et al. 2005b; Yu et al. 2014). These CQAs are the key functional characteristics that must be determined at the initial development phases (Singh et al. 2005b, 2013; Aksu et al. 2015; Singh et al. 2017a, b).

18.4.2 Step II: Prioritization of Key Input Variables for Optimization

Before executing optimization studies, it is imperative to prioritize the significant input variables, i.e. factors and their appropriate levels during the initial stages, using QRM and/or factor screening studies (Singh 2014; Aksu et al. 2015). In a federally recommended approach, QRM aids in enhanced product/process understanding and mitigation of associated risk (ICH Harmonised Tripartite Guideline 2005; Singh et al. 2005b).

Material attributes (MAs) and *process parameters (PPs)* are the input product and process parameters, varying independently and impacting various CQAs, notably or mildly. An Ishikawa *fishbone diagram* is employed, *which* establishes the "cause-and-effect" relationships among multiple input factors and drug product CQAs (Singh et al. 2005b; Beg et al. 2015a). Figure 18.4 represents an Ishikawa diagram highlighting the cause-effect relationship for CQAs.

Prioritization exercise aims to identify statistically significant variables among MAs and PPs, viz. critical material attributes (CMAs) and critical process parameters (CPPs), which exert a profound impact on various CQAs. *Risk estimation matrix (REM)* is one of the most prevalent risk assessment tools. In these studies, MAs and PPs are assigned varied degrees of risk, viz. high, medium and low, based on risk severity, frequency of incidence and, at times, its detectability too. The medium- to high-risk factors from the patient's point of view, based on prior literature reports and discussions among teammates, are selected as CMAs and CPPs (Singh et al. 2005b; Aksu et al. 2015; Singh et al. 2017a, b). Figure 18.5 shows the flow layout of a QRM plan using a REM model for looking out for high-risk CMAs. Another

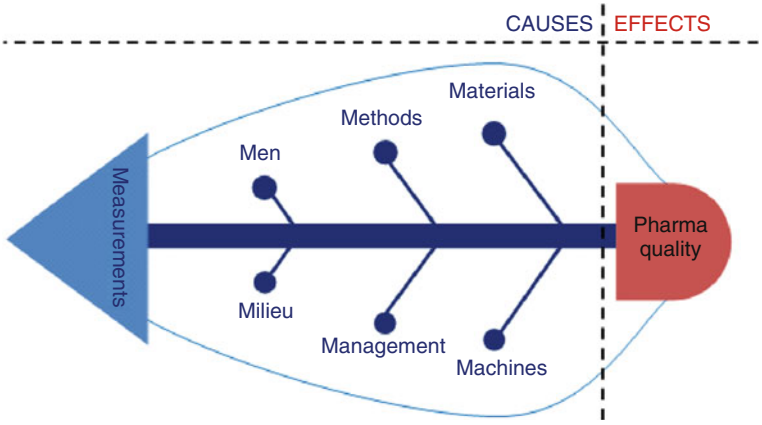

Fig. 18.4 A typical graphic of an Ishikawa fishbone diagram illustrating varied potential sources of variability which can significantly influence the drug product quality

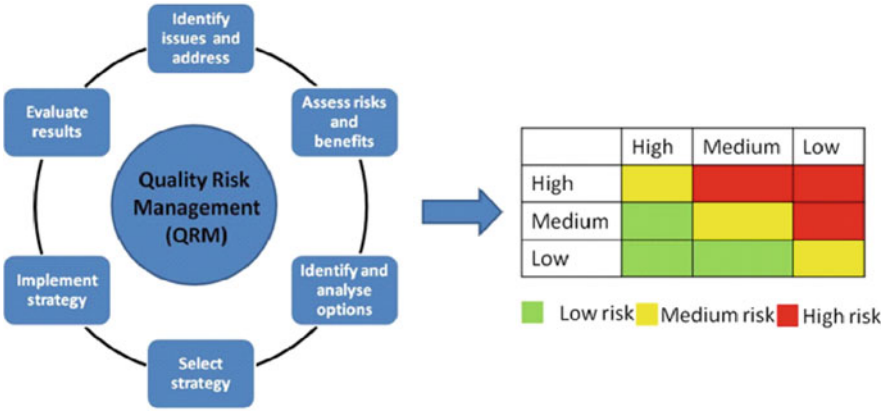

Fig. 18.5 Salient steps of a quality risk management (QRM) approach and the consequent risk estimation matrix (REM)

prioritization approach is *factor screening*, which aids in choosing statistically significant CMAs and CPPs, while ignoring the "idler" ones. Such factor screening studies and subsequent factor optimization studies employ the DoE approach, indispensably employing suitable *experimental designs* (Armstrong et al. 1991; Akesolo et al. 2004; Singh et al. 2005a, 2011a).

Generally, simpler linear experimental designs, like fractional factorial design (FFD) or Taguchi design (TgD), are apt to screen the influential factors from the "possible so many" (Lewis et al. 1998; Singh et al. 2005b, 2011a; Aksu et al. 2015). Figure 18.6 portrays a panoramic layout of various experimental designs employed in factor screening and optimization. The *experimental runs,* i.e. the experimental studies to be exercised as per a particular experimental design, are organized as a

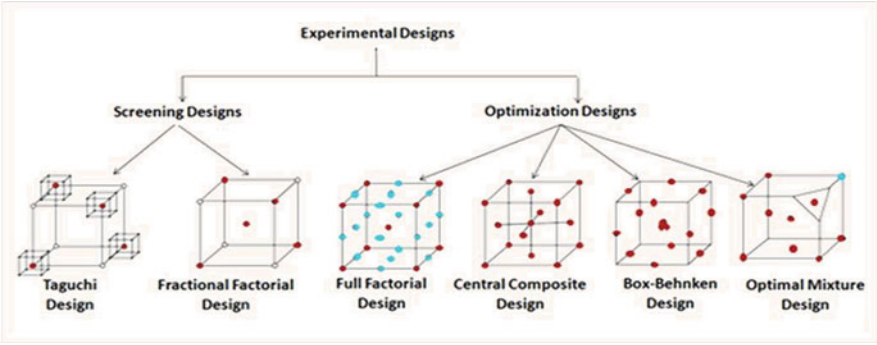

Fig. 18.6 Cubical representation of key experimental designs employed during QbD-enabled product development

table, called as *design matrix*. After screening the influential variables, experimental studies deduce the *factor levels*. These screening designs invariably operate at two levels, low (−1) and high (+1). In the course of employing FbD, QRM can be coupled up with factor screening to select high-risk CMAs and CPPs. This step is essential, as improperly selected factor may bring about unjustifiable rise in financial expenditure and experimental effort.

18.4.3 Step III: DoE-Steered Experimentation and Search for Optimum Nanoconstructs

Only after suitable prioritization of highly influential input variables, drug delivery systems are subjected to optimization. DoE trials are performed, as per the chosen experimental design taking the observed values of various CQAs, for establishing definitive relationship(s) among factors and responses. *Response surface plot* is the graphic presentation of this relationship to help in understanding the effect of each input variable along with their plausible interaction(s) on the response variable (Singh et al. 2005b, 2011a). This 3-D response surface plot is constructed between two independent variables and a CQA, with their respective 2-D slices known as the *contour plots* (Bhavsar et al. 2006; Weissman and Anderson 2015). The contour plots are graphical representations of one independent factor varying versus another, while the responses and other input factors are maintained as unaltered. For a deeper insight, Fig. 18.7 is reproduced here as 3-D and the corresponding 2-D contour plot, portraying the changes in response as the result of factor interactions.

An experimental design is imperative for response surface mapping based on the desired goals. Several second-order experimental designs, like factorial design (FD), central composite design (CCD), D-optimal mixture design (D-OD) and Box-Behnken design (BBD) (Fig. 18.6), are the most often employed for optimizing drug nanoconstructs. This is because of the fact that such designs can very well analyse various plausible nonlinear responses, interactions and mixture effects

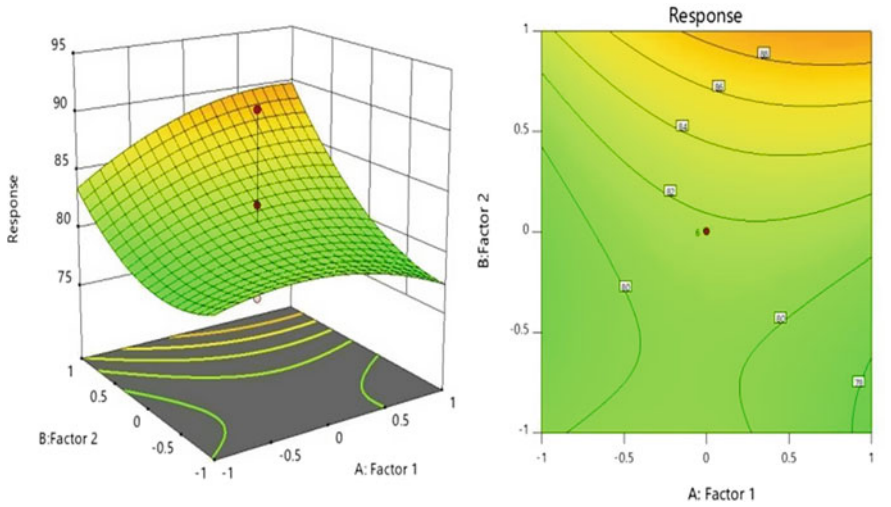

Fig. 18.7 An archetypal representation of 3-D response surface plot (left) and the corresponding 2-D contour plot (right) for any one response variable and two factors

(Cochran and Cox 1992; Lewis et al. 1998; Lewis 2002; Singh et al. 2005b; Durakovic 2017). For screening as well as optimizing the factors, an array of experimental runs, i.e. *design matrix*, is generated, and experimentation is done accordingly. *Coding* of these factor levels is accomplished by designating them as low (−1), intermediate (0) or high (+1). The nanoformulations are accordingly fabricated as per the design matrix of the chosen experimental design and analysed as per the standardized conditions determined for the formulations prepared earlier, termed commonly as *experimental runs* (Cochran and Cox 1992; Singh et al. 2005b, 2011a). The entire process of relating CQAs with the factors, i.e. CMAs and/or CPPs, for optimization is referred to as *response surface methodology (RSM)*. Search for an optimal solution is accomplished using mathematical (desirability function) and/or graphical optimum (overlay plot) (Singh et al. 2005b, 2011a; Durakovic 2017).

18.4.4 Step IV: DoE Validation and Design Space Demarcation

Modelization using data fitting into linear, quadratic and/or cubic models is imperative to obtain 3-D and 2-D plots to establish relationship(s) between the CQAs and CMAs/CPPs (Singh et al. 2011b; Beg et al. 2017b). Like other studies in pharmaceutical technology, validation of FbD methodology is also necessary to ascertain the applicability and prognostic capability of the model used.

Following this modelization and optimum search, a *design space* is demarcated as a multidimensional amalgamation of the relationship(s) between various factors (i.e. CMAs or CPPs) and the resultant response (i.e. CQA) (Araujo and Brereton

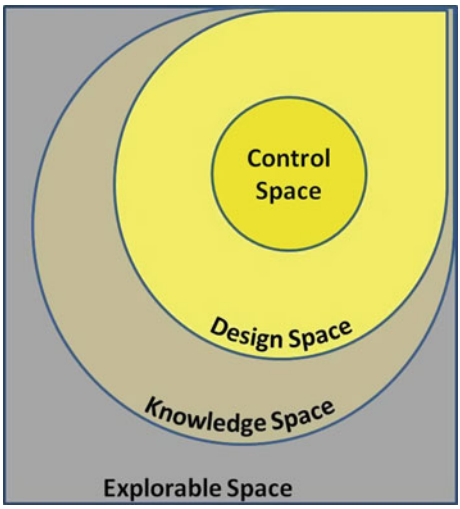

Fig. 18.8 Comprehending relationship among explorable, knowledge, design and control spaces

1996; Lewis 2002; Singh et al. 2005b, 2011b). Fig. 18.8 illustrates such interrelationship among the possible explorable, knowledge, design and control spaces. A more stringent domain is carved out of design space for further studies in an industrial setup.

For assuring the best possible as well as economical nanoformulation production, a design space is ideally generated based on the basis of experimentation at lab and pilot plant scales, which is then re-examined at an exhibit and commercial scales (Araujo and Brereton 1996; Lewis et al. 1998; Montgomery 2001; Singh et al. 2018a). From a regulatory angle, a formulation lying in the boundaries of the design space is only federally acceptable. Any movement within the design space is federally acceptable, and no further investigation is required. On the other hand, movement outside the design space calls for resubmission for its regulatory approval. On the other hand, movement outside the design space calls for resubmission to the federal agency for approval. Subsequent to the design space approval, a narrower working range, as per the customized specifications by the manufacturer called as control space, is embarked upon (Singh et al. 2013; Danhier et al. 2017). On

the whole, Box 18.2 enlists various vital technical terms usually employed during FbD and their corresponding explanations.

Box 18.2 A Holistic Overview of Key Terminology often Employed During Formulation by Design (FbD) of Various Drug Delivery Products

Term	Precise explanation
Quality target product profile (QTPP)	Ideal quality characteristics to achieve required levels of efficacy and safety
Critical quality attributes (CQAs)	Physicochemical or biological parameters of a drug product, ranging within apt limits, to ensure apt level of quality
Factors	Independent variables, notably influencing the product characteristics or process output. These can be *material attributes* or *process parameters*
Coding	Transformation of a variable into a non-dimensional coded form
Levels	Values assigned to factors usually coded as +1, 0 and −1
Critical material attributes (CMAs)	Physicochemical or biological characteristics of drug significantly impacting the quality of drug products
Critical process parameters (CPPs)	Influential independent process parameters which need to be monitored to ensure the desired quality
Interaction	Lack of additive nature of factors on their simultaneous validation
Synergism	An overall positive change owing to factor interaction(s)
Antagonism	An overall negative change owing to factor interaction(s)
Design of Experiments (DoE)	Systematic execution of a planned stratagem to establish factor-response relationship(s)
Experimental design	Systematic and statistical strategy for designing the experimental studies to maximize information to experimentation ratio
Design matrix	Strategic layout of experimental runs in a matrix form, planned as per a particular experimental design
Experimental runs	Experimental studies conducted as per an experimental design
Quality risk management (QRM)	A systematic process for identification, assessment and control of various risks to the drug product quality
Risk assessment	Process to identify and mitigate risks, find varied root causes of process failure and prevent problems to improve product quality and reliability
Response surface plot	3-D graphical representation of a response plotted between two independent variables and one response variable
Contour plot	Geometric 2-D illustration of a response by plotting one independent variable against another, holding the values of response and other variables as constant
Explorable space	Possible dimensional space, defined by different variables for various factors being investigated
Knowledge space	Scientific elements to be explored based upon previous knowledge of product attributes and/or process parameters
Design space	Multidimensional integration of varied input variables and product/process parameters, demonstrated to provide quality assurance
Control space	Part of design space selected for detailed investigations
Control strategy	Comprehensive plan to ensure the final product meets requirements

(continued)

Box 18.2 (continued)

Term	Precise explanation
Continuous improvement	Monitoring the process capability to reproduce the product quality

18.4.5 Step V: Industrial Scale-Up Studies and Production

In order to ascertain the reproducibility and validate the QbD performance demonstrated during laboratory experimentation, the drug delivery products, while in an industrial setup, have to undergo a strategic scale-up process extrapolating laboratory scale outcomes through pilot plant studies to production scale (Houson 2011; Singh et al. 2017a, b; Khurana et al. 2018).

Control strategy is a premeditated control set, attained from current product and process understanding, to ensure the anticipated process output and product performance. Incorporating various controls to ensure product quality, control strategy aims to pave the way towards *continuous improvement* in the quality of drug products (Shah et al. 2015; Singh et al. 2018a). Continuous improvement is the final component of the QbD process, serving as feedback-control mechanism of operations, integrating all the process knowledge for the purpose. This leads to improved product and process understanding at all the production levels, thereby accomplishing product quality excellence as well as requisite regulatory compliance.

18.5 FbD of Drug Nanoconstructs

Albeit QbD nowadays has pervaded to nearly all the realms of healthcare sector, sufficient scope still exists for extrapolating its varied applications in quite intricate strategies of nanomedicines. It is, for that matter, that the global pharmaceutical world, particularly since the last few decades, has been witnessing mounting adoption of QbD in the development of nanopharmaceutical products. Employing the vast rewards of implementing FbD paradigms, practically all of the foremost types of drug nanocarriers have been reported to be rationally and systematically developed (Bhavsar et al. 2006; Singh et al. 2011c, 2015, 2017a,b; Kanwar and Sinha 2019).

Earnest endeavours, accordingly, have been undertaken to deliver a categorical yet pithy overview of latest literature reports of the recent 5 years, endeavouring principally on the development of various drug nanoconstructs employing FbD. Besides, the chapter also furnishes a rational insight into various CMAs, CPPs and CQAs explored and different experimental designs used for achieving the anticipated quality targets. On the basis of the types of constitution of various drug delivery nanocarrier systems, the pertinent pictographic representations have been

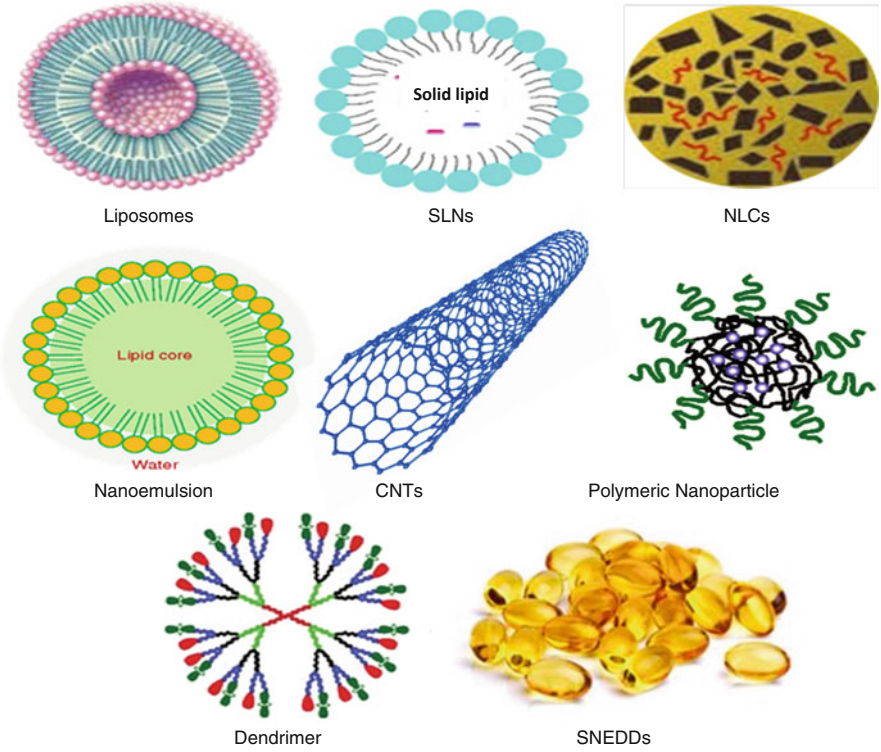

Fig. 18.9 Various important kinds of lipidic and polymeric drug delivery nanoconstructs

quite generally categorized as lipidic, polymeric and many other types of nanoconstructs, as illustrated broadly in Fig. 18.9.

18.5.1 Lipid-Based Nanostructured Systems

Table 18.1 delineates a few selected reports from the recent literature on QbD-enabled development of vesicular and non-vesicular lipid-based drug nanoconstructs of diverse types.

18.5.1.1 Vesicular Systems

Composed of spherical concentric vesicles with unilamellar, bilamellar multilamellar architectures, liposomes are often prepared from cholesterol and nature-derived biocompatible phospholipids (Khurana et al. 2017; Sharma et al. 2018). Owing to their inimitable characteristics, *viz.*, vesicle size, biocompatibility and amphiphilicity, liposomes are one of the promising carriers used to improve the therapeutic prospects of drugs at a target site (Akbarzadeh et al. 2013; Rana et al. 2018; Singh et al. 2018a, b). Besides the basic liposomes, some other important

vesicular systems, like transferosomes, niosomes, ethosomes, and bilosomes have been magnificently developed using QbD principles, with systematic optimization of various CQAs like vesicle size, poly-dispersity index (PDI), loading capacity, the skin retention and percent diffusion (Shukla et al. 2011; Garg et al. 2016a, b).

18.5.1.2 Non-Vesicular Systems

Lipidic nanocarriers are generally colloidal carriers with their particle size as 1000 nm or less (Yasir and Sara 2013; Singh et al. 2018a, b). Invariably, based upon the composition of solid lipids and different functional excipients, such systems can be classified as solid lipid nanoparticles (SLNs), nanostructured lipidic carriers (NLCs), drug-lipid conjugates and lipospheres (Patil et al. 2015; Garg et al. 2016a, b; Sharma et al. 2017; Karunanidhi et al. 2018).

A polymer or a mixture of polymers is used as stabilizer and a matrix is used to entrap lipophilic and hydrophilic drugs. NLCs are the second generation of lipid-based nanoparticles, developed by moderate addition of liquid lipid (oil) and solid lipid. The major benefits of NLCs include higher drug encapsulation efficiency, better regulation of drug release kinetics and improved drug retention within the system, upon storage (De Crozals et al. 2016; Karunanidhi et al. 2018). The most important CMAs that are taken into account during optimization of such drug delivery systems include percentage of solid and liquid lipids and of surfactant, while vital CQAs include % drug loading, particle size, PDI and % entrapment efficiency (Garg et al. 2017a).

18.5.1.3 Emulsifying Systems

These are fundamentally the biphasic systems of lipidic and aqueous phases, often along with an apt emulsifier to emulsify both of these phases. Self-emulsifying delivery systems turned out to be one of the most promising drug delivery systems for peroral administration of biopharmaceutically challenged drug molecules. These are isotropic anhydrous mixtures of drug with lipid, surfactant and/or co-surfactant. Upon administration and getting in contact with gastric fluid in the gastrointestinal tract, these systems get emulsified and tend to form micro-/nano-globules (Singh et al. 2009a, b; Korting and Monika 2010). Based upon the nanometric size of the globules, thus formed, after their dilution, these systems can be christened as self-nanoemulsifying drug delivery systems (SNEDDS). Micelles constitute another kind of emulsifying system, which tend to get formed by self-assemblage of the amphiphilic excipient(s) in the aqueous phase with their hydrophilic portion facing the outer micellar surface and the lipophilic portion remaining within the core (Singh et al. 2014; Bhatia 2017). A quicker emulsification process tends to form nano-sized micelles with higher surface area, thus resulting in accelerated drug release. During their optimization, the amounts of lipid, surfactant and co-surfactant are employed as CMAs, while the % drug release, globule size, emulsification time and zeta potential are usually assessed as the CQAs.

Table 18.1 Recent literature reports on the usage of FbD for design and development of various kinds of lipidic drug nanoconstructs

Lipid-based nanoconstructs					
Carrier	Drug	Design	IF	RV	References
Vesicular carrier systems					
Liposomes	Lamivudine	FFD	2	3	Godbole et al. (2020)
	Erlotinib	BBD	3	2	Dhoble and Patravale (2019)
	Pravastatin	D-OD	4	7	Sylvester et al. (2018)
Niosomes	Polymyxin B	BBD	3	4	Chauhan and Bhatt (2019)
	Methotrexate	BBD	3	2	Abdelbary and AbouGhaly (2015)
	Sumatriptan succinate	TgD	4	3	González-Rodríguez et al. (2012)
Ethosomes	Fisetin	BBD	3	3	Moolakkadath et al. (2018)
	Tramadol	BBD	3	3	Ahmed et al. (2016)
	Diclofenac	FD	2	4	Jain et al. (2015b)
Transferosomes	Zolmitriptan	BBD	3	3	Pitta et al. (2018)
	Risperidone	CCD	2	2	Das et al. (2017)
	Sildenafil	PBD	5	2	Ahmed (2015)
Bilosomes	Tizanidine HCl	FFD	3	3	Khalil et al. (2018)
	Tenoxicam	FFD	3	4	Al-mahallawi et al. (2015)
Elastomers	Diacerein	FD	2	5	Aziz et al. (2018)
Non-vesicular systems					
Solid lipid nanoparticles	Quetiapine fumarate	CCD	2	1	Agarwal et al. (2020)
	Carvedilol	BBD	3	5	El-Say and Hosny (2018)
	Rosuvastatin	CCD	2	3	Beg et al. (2017a)
Nanostructured lipidic carriers	Ibrutinib	CCD	3	3	Rangaraj et al. (2020)
	Isradipine	BBD	3	3	Alam et al. (2018)
	Telmisartan	BBD	3	3	Thapa et al. (2018)
Emulsifying systems					
Supersaturable SNEDDS	Sorafenib tosylate	D-OD	3	4	Sharma et al. (2020)
	Celecoxib	D-OD	4	3	Chavan et al. (2015)
	Trans-resveratrol	CCD	2	2	Singh and Pai (2015)
Liquid SNEDDS	Darunavir	IV-OD	3	5	Garg et al. (2018)
	Valsartan	CCD	4	3	Bandyopadhyay et al. (2015)
	Carvedilol	CCD	3	4	Singh et al. (2011b)
Cationic SNEDDS	Raloxifene	D-OD	3	4	Jain et al. (2018)
	Olmesartan	MD	3	3	Beg et al. (2015b)
	Candesartan cilexetil	MD	3	2	Sharma et al. (2015)

(continued)

Table 18.1 (continued)

Lipid-based nanoconstructs					
Carrier	Drug	Design	IF	RV	References
Solid SNEDDS	Polypeptide-k	BBD	3	4	Garg et al. (2017b)
	Lovastatin	CCD	2	6	Beg et al. (2015a)
	Valsartan	BBD	3	4	Beg et al. (2012)

IF input factor, *RV* response variable, *FD* factorial design, *FFD* fraction factorial design, *BBD* Box-Behnken design, *CCD* central composite design, *TgD* Taguchi design, *PBD* Plackett–Burman design, *IV-OD* IV-optimal design, *D-OD* D-optimal design, *MD* mixture design, *SNEDDS* self-nanoemulsifying drug delivery systems

18.5.2 Polymeric Nanoparticles

Nanoparticulates employing biodegradable polymers are one of the promising and effective delivery systems offering diverse biopharmaceutical roles (Korting and Monika 2010; Jain et al. 2015a, b; Jose et al. 2018). Various polymeric delivery systems fruitfully developed using FbD encompass nanocapsules, nanoparticles, nanogels, nanocomposites, nanofibres and lipid-polymer hybrids (Cun et al. 2011; Hunter et al. 2012; Cheng et al. 2015; Nagpal et al. 2019). Various CMAs and CPPs for polymeric nanoparticulate systems comprise of nature and amount of polymer and surfactant, pH, homogenization time and stirring speed, while cumulative % drug release, particle size, zeta potential, PDI and entrapment constitute the vital CQAs (Gajra et al. 2015; Li et al. 2017; Beg et al. 2019).

18.5.3 Other Nanostructured Systems

Table 18.2 enlists some recent reported studies on FbD-developed polymeric drug delivery nanoconstructs of varied kinds, along with those of nanocrystals, metallic nanoparticles, nanotubes, nanosuspensions and quantum dots.

18.5.3.1 Nanosuspensions

Nanosuspensions are submicron colloidal drug particulate dispersions stabilized using various types of surfactants or polymers. Such systems are commonly developed using wet milling techniques, that tend to reduce drug particle size, thereby enhancing the oral bioavailability of various lipophilic drugs (Attari et al. 2016; Karakucuk et al. 2016). CMAs/CPPs employed during systematic development of nanosuspensions encompass drug-to-stabilizer ratio, bead size, milling time and mill speed. These CMAs/CPPs tend to possess a significant effect on CQAs of nanosuspensions like particle size, PDI, zeta potential, etc. (Verma et al. 2009; Beg et al. 2019).

Table 18.2 Recent literature reports on the usage of FbD for design and development of various kinds of polymeric and other kinds of drug nanoconstructs

Carrier	Drug/bioactive	Design	IF	RV	References
Polymeric nanoconstructs					
Nanoparticles	Atorvastatin	BBD	3	3	Martins et al. (2020)
	Ciprofloxacin	FFD	2	3	Adebileje et al. (2018)
	Dapsone	BBD	3	3	Chaves et al. (2017)
Lipid-polymer hybrids	Gemcitabine HCl	CCD	3	3	Yalcin et al. (2018)
	Rutin	FFD	3	3	Ishak et al. (2017)
Nanocapsules	Liraglutide	BBD	3	2	Shamekhi et al. (2018)
Nanocochleates	Curcumin	BBD	3	3	Nadaf and Killedar (2018)
Nanocomposites	Sildenafil citrate	CCD	4	1	Asfaram et al. (2017)
Nanofibres	Caffeine	TgD	4	2	Dadkhah et al. (2014)
	Phenytoin sodium	D-OP	5	1	Zarandi et al. (2015)
	Doxorubicin	BBD	4	3	Nadia et al. (2015)
Nanoorganogels	Palm oil	BBD	3	4	Raviadaran et al. (2018)
	Methotrexate	BBD	3	3	Avasatthi et al. (2016)
	Novicidin	CCD	3	3	Water et al. (2015)
Dendrimers	Aceclofenac	FFD	2	1	Garala et al. (2009)
Other nanoconstructs					
Nanosuspensions	Flurbiprofen	BBD	3	3	Gajera et al. (2018)
	Ritonavir	FFD	2	3	Karakucuk et al. (2016)
Metallic nanoparticles	Dasatinib	BBD	3	3	Adena et al. (2018)
Quantum dots	Thiamine	CCD	3	1	Nemati et al. (2018)
Carbon nanotubes	Berberine	CCD	2	3	Lohan et al. (2017)
	Doxorubicin	FFD	4	2	Farahani et al. (2016)
Nanocrystals	Lurasidone hydrochloride	FFD	2	1	Shah et al. (2016)

IF input factor, *RV* response variable, *FD* factorial design, *FFD* fraction factorial design, *BBD* Box-Behnken design, *CCD* central composite design, *TgD* Taguchi design, *D-OD* D-optimal design

18.5.3.2 Nanocrystals, Metal Nanoparticles, Nanotubes and Quantum Dots

The nanocrystal approach now-a-days is considered as one of the most feasible technologies in the arena of nanopharmaceutical(s), as it imparts improved dissolution rate and oral bioavailability of various drug molecules. Nanocrystals of drug molecule(s) are usually prepared by milling techniques which comprise CPPs like time for milling and mill speed and size of beads and CMAs like amounts of surfactant and co-surfactant. The popular CQAs include particle size, PDI, zeta potential and release behaviour of drug (Junghanns and Müller 2008; Boles et al. 2016).

Lately, metallic nanoparticles have been earning vast applications in drug delivery. These nano-metallic particles can be synthesized and further engineered with various chemical moieties, enabling the conjugation(s) with varied antibodies, functional moieties, ligands and apt drugs (Mody et al. 2010; Lena Leopold et al. 2018). These nanoparticulates have immense significance in the field of biotechnology, targeted drug delivery of drugs and biologics and diagnostic imaging. Molar fraction of reactants during the synthesis of metallic nanoparticles along with the conditions thereof comprises the CMAs and CPPs, which regulate their yield, size, size distribution and drug loading as the major CQAs (Mody et al. 2010; Singh et al. 2018a, b).

Of late, carbon-based nanostructures have been explored as potential drug delivery carriers owing to their innumerable applications. Carbon nanotubes (CNTs) act as a promising carbon-based nanostructured systems owing to their higher surface area, superior electric and thermal conductivities and high mechanical strength (Baughman et al. 2002). Their hollow and nanoscale tube-shaped structure enables high loading of variegated drug molecules through their possible conjugation with the nanotube walls. Key CMAs/CPPs employed for systematic development of functionalized CNTs encompass reaction time and temperature, reacting material concentrations, which have substantial impact on yield, solubility and drug loading of the functionalized CNTs (Sun et al. 2002). Quantum dots, which constitute a vital class of delivery systems with nanometric particle size, are semiconductor particles for theranostic applications (Bera et al. 2010; Tripathi et al. 2015).

A recent literature survey of the top most journals revealed remarkably high rate of publications in this multi-, cross- and interdisciplinary field of nanomedicine. For an arena evolving as fast as this one, it is relatively challenging to acquire a comprehensive overview of the active developments, as most updated and organized information on such nano-bio interfaces lies scattered in various journals. Over the past few decades, applications of nanoparticles in biotherapeutics have become a highly distinct and dynamic area of scientific and technological development. A deluge of publications in the recent literature testifies that the scenario of science and research in drug delivery has been fast transitioning with a shift from micro- to nanonization. Drug nanoconstructs offer a more convenient alternative to the discovery of new drugs, which involve huge investments not only in terms of money but also in terms of manpower and time. In contrast, repackaging or remodelling the existing molecules of established therapeutic potential using nanostructured carrier systems can suitably address issues like solubility, stability, permeability, metabolic degradation, fast clearance and adverse effects, which tend to limit their effectiveness. In addition, the resulting product is relatively more protectable from patentability perspectives and is readily commercially available, because of the "newness" imparted to it.

Besides the development of their formulation, the constancy in terms of stability of these nanoconstructs is measured as quite critical characteristic to assess timely and assure. Based upon the chemistry and electromagnetic nature of the nanoparticles, these may occur as colloids, dispersed aerosols, suspensions or in an agglomerative state. Furthermore, as a function of the size of the agglomerates,

these might act as nano, micron or submicron particles. The size and shape of particle, surface charge and activity and nature of the material used for the development of such nanoparticles, accordingly, must be taken into wide consideration to diminish the probable lacunae associated with stability and agglomeration of particles while embarking upon the usage of novel materials (Sathigari et al. 2011; Chopra et al. 2015). Several studies report the dependence of physical stability of a nano-based drug delivery system on the kind, effectiveness and concentration of the stabilizer used for the development of such formulations (Pallagi et al. 2015; Alshweiat et al. 2018, 2019; Ismail et al. 2019). In this context, QbD paradigms are recognized as an asset to aid in categorizing the optimal type of stabilizer and concentration, i.e. basically vital to circumvent the aggregation of particles (Alshweiat et al. 2018, 2019).

Freeze-drying of the drug-loaded nanoparticles is a common practice to overcome the long-term stability issues associated with nanoparticles. For the lyophilization process, choice of apt lyoprotectants and their concentration becomes crucial factors for prohibiting the aggregation of these nanoparticles. These influential lyophilization factors have also been witnessed in various literature reports to be optimized using QbD principles (Chung et al. 2012; Niu and Panyam 2017). While nanomilling is one of the other most commonly used mechanical tools for deagglomeration of nanoparticles, influential factors including time and speed of milling, kind and amount of milling medium, size and amount of beads, drug amount and milling design exhibit maximal effect on end-product properties (Sanganwar et al. 2010; Sathigari et al. 2011; Peltonen 2018). Among many possible input variables, a few vital ones are screened during preliminary studies and later systematically optimized using QbD principles (Ghosh et al. 2013; Peltonen 2018).

18.6 Computer Usage During FbD of Drug Nanoconstructs

The salient benefits of FbD approaches are abundant and their acceptability upbeat. The implementation of this entire exercise, nevertheless, involves a great deal of logical, statistical, mathematical and graphical intricacies, making it quite cumbersome for a scientist to analyse the consequent data manually. With the comprehensive and user-interactive software, together with the powerful and economical hardware, the computational hassles of QbD have been grossly simplified and streamlined. Pertinent computer software is available not merely for DoE optimization for steering the scientists efficiently on every step during whole drug product development cycle but also for chemometric analysis through multivariate techniques, QRM execution using REM or FMEA, use of other algorithmic matrices and constructing fishbone diagrams. The computational problems get invariably surmounted through the usage of appropriate software and subsequent logical interpretations. Besides, the pertinent software package also ameliorates the presentation aesthetics of FbD outcomes in the form of response surface plots, design spaces and so on and so forth. Several dependable software are available in commercial circulation to satiate the requirements of DoE, QRM, chemometrics, etc., to

Fig. 18.10 Popular computer software employed during application of variegated QbD principles

facilitate the myriad tasks confronting a drug delivery development scientist (Singh et al. 2015; Beg et al. 2018; Singh et al. 2018a). Figure 18.10 enlists the select computer software commercially available for carrying out QbD studies in laboratory or industrial milieu.

18.7 A Holistic Retroactive Account So Far

A clairvoyant glance of over 4600 research publications reported in updated literature on FbD-enabled development of drug nanocarriers indicates an accelerated surge on the appliance of FbD paradigms, predominantly in the last couple of decades. Figure 18.11, in this perspective, chronologically portrays an authoritative testament to the escalating recognition of FbD for developing diverse nanoformulations systematically.

An extensive diversity of such nanostructured systems has been rationally developed, employing all-embracing designs and models. The pie chart in Fig. 18.12 illustratively portrays the current trends in nanomedicines.

Colossal merits have been observed while optimizing the product/process quality of such nanomedicines because of their multifaceted intricate design, vibrant material traits and stringent regulatory compliance requirements for numerous CQAs (Kumar et al. 2014; Aksu et al. 2015; Beg et al. 2017a). On the whole, the FbD studies consisting of >6 variables perpetually encompassed factor screening exercise for prioritizing "few" variables among "possible so many" ones for subsequent factor optimization. Albeit the input variables have been customarily ranging between 5 and 7 in number, yet there have been some sporadic studies with >10

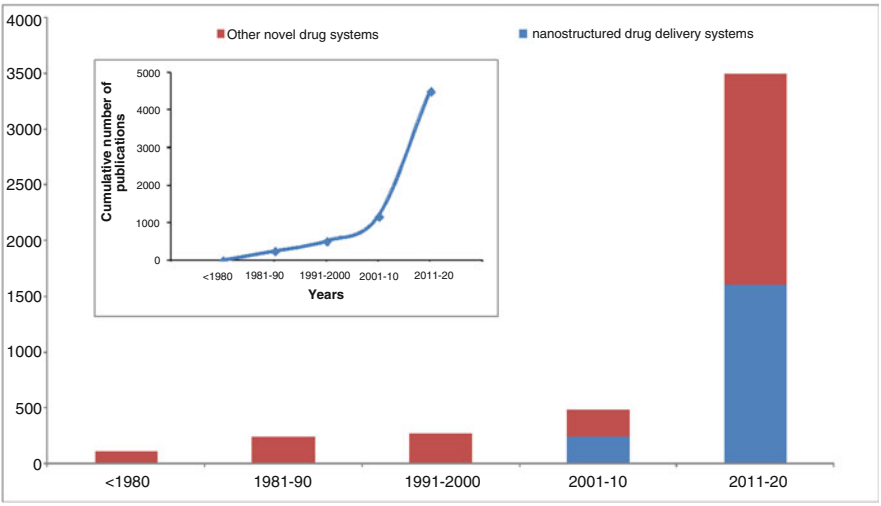

Fig. 18.11 A sequential bar chart of the mounting usage of QbD during systematic development of various drug nanoconstructs

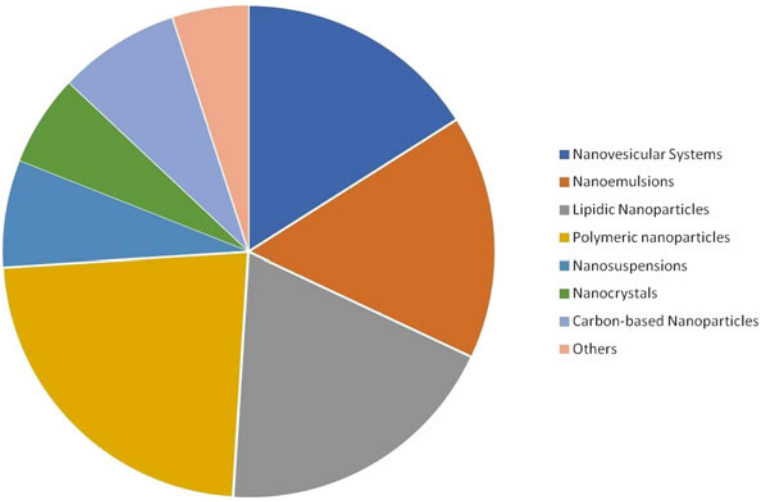

Fig. 18.12 A pie diagram depicting application of QbD on variegated kinds of drug nanoconstructs

influential variables too (Singh et al. 2011a, c; Raza et al. 2013). Major CQAs, in this context, included nanometric size, zeta potential, drug release profile, stability, drug entrapment efficiency and also organoleptic attributes (Singh et al. 2011a, c; Li et al. 2017; Singh et al. 2005b). The predictive capability of FbD optimization methods is often ratified by characterizing and evaluating a variety of checkpoint nanostructured

formulations, usually indicating high prognostic power (Schwartz et al. 1996; Singh 2014; Singh et al. 2017a, b).

18.8 Regulatory Perspectives on Drug Nanoconstructs

Reducing the size of particles to nanoscale is considered as an inimitable attribute, as the majority of inherent properties of the matter get altered remarkably at this scale. These newer attributes often significantly impact the quality, efficacy and safety of the therapeutic drug product. A nanomedicinal product, for example, may have a unique surface coating or functional moieties, including ligands, peptides, antibodies, etc., that avert its interaction with body immune cells, so that the drug molecule circulates in a bloodstream for prolonged time periods, till it reaches the targeted tumour tissues. The potential to target particular tissues of body, or circumvent many others, can markedly reduce the possible risk(s) of adverse effects and toxicity issues to non-target sites and potentially improve the success rate of the therapeutic regimen (Prud'homme and Svenson 2012; De Crozals et al. 2016). This is one of the major concerns for all the shareholders, be it regulators, scientists or the consumers. Today, nanomedicine encompasses a broad diversity of innovative nanostructured drug delivery systems as well as devices for preventive, therapeutic, diagnostic or theranostic applications. Management of nanotechnology-based therapeutics by the regulatory bodies for the past few years has, consequently, been containing different therapeutics, vaccines, biotechnological products, cosmetics, foods and medical devices (Re et al. 2012; Nanda et al. 2015; Beg et al. 2017a, b). The toxicity as well as safety concerns should be taken into account much earlier to the marketing of nanotechnology-based therapeutics for ostensible amelioration in the quality of health. As the existing know-how of the toxicological aspects of bulk ingredients may be insufficient for the reliable estimation of toxic forms of nanocarriers, an inclusive proposal to understand the nanotoxicity has been felt as an acute need of the hour, since years (Arora et al. 2012). An evolving body of studies reveals the impacts, if at all, these nanoconstructs will put forth on environment, health and safety concerns (Wang et al. 2011; Chopra et al. 2015).

In 2013, a categorization system was introduced for describing the toxicological aspects of nanostructures, on the basis of their particle size and biodegradability. The Class I nanostructures (biodegradable systems with the size range of 100–1000 nm) are designated with low risk and are demarcated with green colour. Class II nanostructures are non-biodegradable and are greater than 100 nm, and Class III particles are biodegradable having their sizes less than 100 nm. As both of the Class II and III nanostructures tend to exhibit medium risk, these are demarcated with yellow colour. Class IV nanostructures (non-biodegradable with size less than 100 nm), on the contrary, are related with high-risk toxicity and are marked with red colour.

In spite of these specific characteristics of nanoconstructs, the regulatory procedures entailed in approval of nanopharmaceutical drug products by the federal agencies, like the US-FDA, have generally been quite analogous to those suggested

for the conventional drug therapeutics. The usage of prevailing guidelines and standards to regulate nanotechnology-enabled therapeutics has been fraught with numerous difficulties. Afterwards, the nanotechnology-enabled therapeutics started flooding in the marketplace, the US-FDA in 2006 started to determine and develop regulatory methods that aid in the continuous development of advanced, effective and safe FDA-managed drug products, made out of nanoscale materials (Nanda et al. 2015). In 2011, the European Medicines Agency (EMA) published on the necessity of regulatory requirements on nanomedicines for their approval, related to key issues associated with their development, characterization and challenges with the chemistry, manufacturing as well as regulation of the nanotechnology-based therapeutics (European Medicines Agency 2014a, b). Subsequently, the EMA came up with the guidance for the pharmaceutical manufacturing sector on the genuine provisions for liposomal products in 2014 (European Medicines Agency 2014a), surface modification on nanomedicines in 2013 (European Medicines Agency 2013), development of block-copolymer-micellar drug products in 2014 (European Medicines Agency 2014b) and colloidal intravenous iron nanoparticles in 2015 (European Medicines Agency 2015). Quite lately, in December 2017, the US-FDA provided a draft guidance for industries associated with nanotechnology-based applications on "Drug Products, Including Biological Products, That Contain Nanomaterials" (FDA 2017; Department of Biotechnology Indian Society of Nanomedicine 2019). In fact, the FDA does not unequivocally denounce all the nanotech-enabled therapeutics as fundamentally benign or toxic. Besides the US-FDA guidance on liposomal drug product, the regulatory agencies of Canada, Australia, Taiwan, India and Japan have also issued their identical guidance for managing the applications pertaining to intravenous liposomal preparations (Ministry of Health Labour and Welfare Japan 2016; Therapeutic Goods Administration Australia 2016; Centre for Drug Evaluation Taiwan 2017; Health Canada 2017; Department of Biotechnology Indian Society of Nanomedicine 2019). Figure 18.13 outlines an overview of varied federal agencies involved in the regulation of nanomedicines.

Implementation of systematic as well as rational principles of QbD into the preparation of nanoparticulates, to a great extent, has been appreciably valued across the pharma world for varied aspects of pharmaceutical drug products, processes and drug substance manufacturing too (ICH Harmonised Tripartite Guideline 2005, 2008, 2009). Nonetheless, hardly any guidance(s) specific to the applicability of QbD/FbD approaches in the preparation of nanotech-based therapeutics has been issued yet. A distinct number of formulations as well as process factors have now been identified that could impact the CQAs like nanoparticle size, drug loading, drug release behaviour and biopharmaceutical attributes (De Crozals et al. 2016; Khurana et al. 2017; Li et al. 2017; Singh et al. 2018a, b). Accessibility of pharmaceutical product-related guidance on QbD-steered development of drug nanopharmaceuticals is, nevertheless, highly coveted, not only to control and assure the quality, safety, efficacy and robustness of these multifaceted pharmaceuticals, but also to support and motivate diverse pharmaceutical companies to produce safer and more therapeutically effective nanostructured systems for addressing the patients' unmet needs.

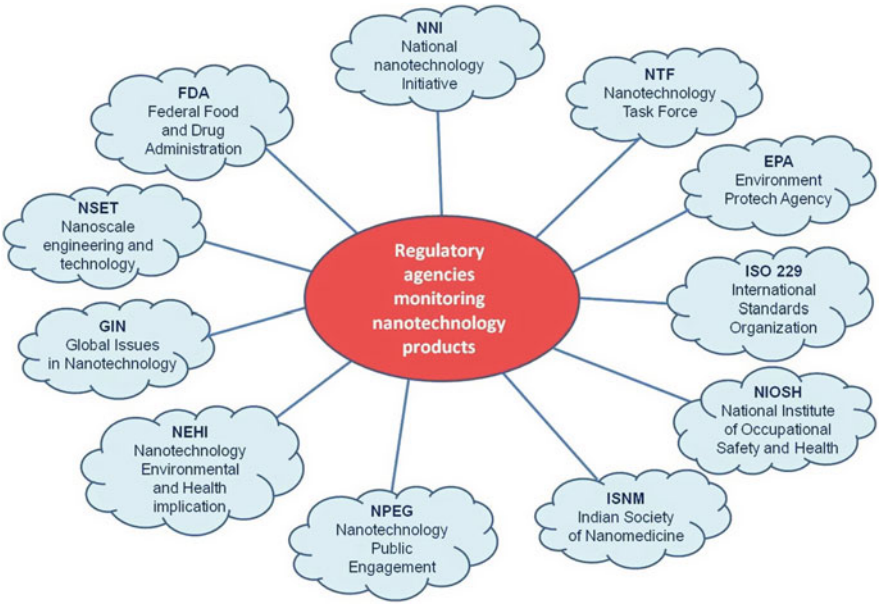

Fig. 18.13 Vital governmental bodies across the globe regulating the nanotechnology-oriented drug products

18.9 QbD-Steered Nanomedicinal Work From Our Laboratories

The sojourn of implementing systematic approaches started at the University Institute of Pharmaceutical Sciences (UIPS), Panjab University, way back since the early 1990s somewhat quite intuitively, when the entire pharmaceutical world was banking on the traditional OFAT approach of developing drug products. Since then, adoption of such systematic approaches has been a regular phenomenon at the Institute for developing novel and nanostructured DDS of diverse types employing diverse experimental designs, multivariate techniques, QRM and chemometric tools. A platitude focussed to systematic, modern and rational drug product development, as christened by us in 2001, viz. *Formulation by Design (FbD)*, has been in vogue all across the pharma world today (Singh 2013, 2014). Over the period of time, more than 250 publications, including 15 books and 45 book chapters, have been churned out exclusively on QbD-enabled development as the globally sought-after repertoire of information (Beg et al. 2017a; Khurana et al. 2017; Garg et al. 2018; Sharma et al. 2020), bringing out six patents and a couple of synergistic technology transfers of nanostructured drug delivery technologies too to the pharma industry. Systematic QbD-steered development of the "best plausible" nanoformulations has invariably resulted in tangible amelioration in the pharmacokinetic and/or pharmacodynamic attributes over the conventional drug bioactives or their conventional formulations, construing markedly superior therapeutic and safety potential of the nanoconstructs

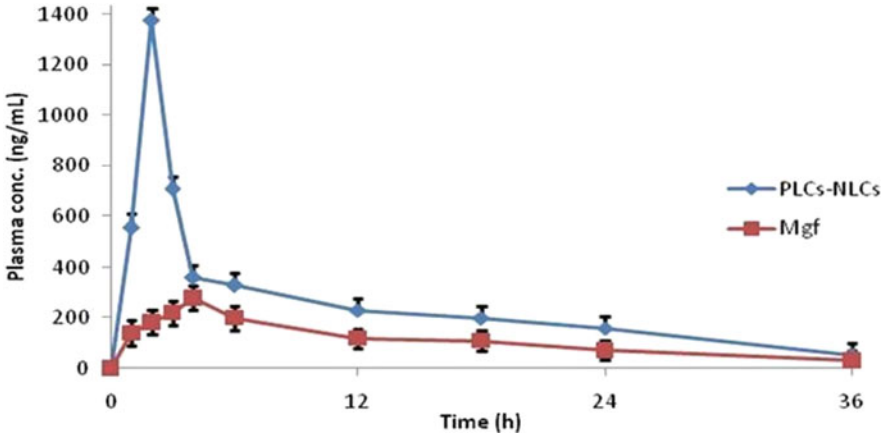

Fig. 18.14 Plasma concentration-time profile of mangiferin (Mgf) and its phospholipid-based nanostructured lipidic carriers (PLCs-NLCs) in rat (mean ± SEM; $n = 6$)

in the correspondingly lower doses required. Figure 18.14 illustrates an instance demonstrating the distinct pharmacokinetic superiority in rate as well as extent of its systemic bioavailability of the phospholipid-based NLC formulation of a plant bioactive, mangiferin, developed at our end as per FbD approach (Khurana et al. 2017).

Several hundreds of industrial and academic scientists of India, Canada, Taiwan and Bangladesh have been trained on such industrially pragmatic and federally essential paradigms of QbD and nanomedicines through on-site training programmes, seminars and conference workshops. The pictorial collage in Fig. 18.15 portrays some of the logos of industrial houses with which industry-academia interactions have taken place, primarily for their industrial training on QbD paradigms. Concerted contributions as a pioneering torchbearer and persistent crusader entirely in the domain of QbD-enabled drug delivery work conducted at the Institute have fetched a number of highly prestigious global recognitions and awards, including:

- *Pharma QbD Excellence Award 2012* by CPhI-Asia, Ahmadabad, India
- *AAPS QbD & Product Performance Award 2012*, Chicago, USA
- *AAPS QbD & Product Performance Award 2013*, San Antonio, USA
- *Outstanding QbD Scientist Award 2014* by Select Bio, Mumbai, India
- *Pharma QbD Performance Award 2015* by Stat-Ease, Minneapolis, USA
- *Scientist Par Excellence Award 2016* by Minitab, Coventry, UK
- *Global QbD Excellence Award 2019* by Shengjie, Shanghai, China

The unquenchable quest for knowledge has been continuing to reinforce our humble contribution in this scientific domain and to serve the society thereof.

Fig. 18.15 A pictorial collage of the company logos of varied pharma houses which have been involved in industry-institute liaison and consultancies, including updated training to their scientists on QbD precepts

18.10 Conclusion and Future Prospects

Having proved their enormous worth in improving the product quality, therapeutic efficacy, patient compliance and safety, a diversity of nanostructured drug delivery systems have progressively been sought-after today. To facilitate their smooth approvals and launches, accordingly, rational formulation development of such drug nanoconstructs is judiciously called for. FbD, being a science-, risk- and design-steered paradigm, extends unique benefits to a drug delivery scientist in the rational development of robust, efficacious, safe and stable nanopharmaceuticals in an effectual, time-effectual and cost-effectual fashion. Of late, the drug control agencies accentuate for demonstration of "patient-centric" quality as "built-into" the system, instead of testing the same in the final end products. Concerted initiatives embarked upon to ingather immense utility of such rational approaches would, therefore, be crucial in realizing the "win-win" state for patients, pharma houses and drug regulators.

For a drug delivery researcher, the principal benefits of switching over to these systematic and rational FbD approaches are unearthing potential of the cause-effect minutiae during the formulation development path, leading eventually to comprehensive product and process understanding. Its thriving implementation relies upon

the accuracy and immensity of the input data, as well as on the befitting selection of designs and models. The phenomenal merits of FbD aside, it cannot be considered as a panacea for all the issues pertaining to product development. Hence, appliance of FbD needs to be coupled with the experiential prudence of an efficient scientist.

The practice of efficient enactment of QbD principles for developing novel and nanostructured drug delivery technologies has certainly spiced up in the last few decades, yet much needs to be done to make it a routine practice. As variability tends to be omnipresent during the entire drug delivery product development life cycle, application of QbD principles needs to be implemented at each and every stage. Accordingly, a battery of endeavours needs to be undertaken to inculcate consistent use of diverse systematic approaches in the holistic domain. With the widely growing popularity and acceptance of the QbD paradigms, nowadays, these are also required to be implemented for generic nanopharmaceuticals (or *nanosimilars*), biopharmaceuticals (or *biosimilars*) and/or newer innovative brand products, analytical development, development of pure drug substances and even beyond, in order to meet the unmet needs of patients. QbD is rationally prophesized to be a quality-stimulator boon to speed up the pharma development issues, expending insignificant resources for attaining the most significant performance.

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Nanoemulsions: A Potential Advanced Nanocarrier Platform for Herbal Drug Delivery

19

Sumit Sharma, Sonali Batra, and V. R. Sinha

Abstract

Nanotechnology is an emerging science in pharmaceutical technology with numerous opportunities to address the various challenges in drug delivery. Despite the scientifically proved therapeutic activities and good safety and efficacy, the use of herbals is limited. This is due to challenges like higher molecular size, low aqueous solubility, lipid permeability, and elevated degradation profile in vitro and in vivo which lead to ineffective pharmacodynamic and pharmacokinetic profile. Amid the advancement in novel drug delivery systems for herbal bioactives, nanoemulsion technology is highly popular in the pharma industry. Nanoemulsion technology is the nanocarrier system which has the ability to overcome all aforesaid challenges and potentiate the biological efficacy of herbal bioactives. This chapter highlights the concept and state of the art for the nanoemulsion and describes the applications of nanoemulsions for herbal drug delivery.

Keywords

Nanotechnology · Herbals · Nanoemulsion · Microemulsion · Drug delivery application

S. Sharma
Delhi Pharmaceutical Sciences and Research University, New Delhi, India

S. Batra
Department of Pharmaceutical Sciences, Guru Jambheshwar University of Science and Technology, Hisar, Haryana, India

V. R. Sinha (✉)
University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh, India
e-mail: vrsinha@pu.ac.in; vr_sinha@yahoo.com

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19.1 Introduction

Nanoemulsions are classified as nanoscale emulsions which are specifically regarded as kinetically stable systems. The nanoemulsions are optically isotropic mixtures and can be translucent or transparent. However, microemulsions are also nanoscale emulsion but are both thermodynamically and kinetically stable as compared to nanoemulsions with a droplet size of less than 100 nm. These emulsions are liquid-liquid colloidal dispersion with amphiphilic surface-active agents (Barkat et al. 2020; McClements 2012). Due to its nano-size, these novel drug delivery systems offer enhanced solubility and permeability of poorly aqueous soluble compounds (Ghai and Sinha 2012), optical clarity (de Oca-Ávalos et al. 2017), better bioavailability (Li et al. 2017), and increased shelf life (de Oca-Ávalos et al. 2017; Sharma and Sinha 2018; Parveen et al. 2015). Nanoemulsion mainly comprises three components, i.e., oleaginous phase, surface-active agents, and aqueous phase. The oleaginous phase involves the lipophilic components such as free fatty acids, mono-, di-, or triacylglycerols, essential oils, etc. The role of surface-active agents is to stabilize the nanoemulsion by preventing Ostwald ripening, coalescence, and flocculation. These surface-active molecules also prevent collision of small droplets and provide kinetic stability to nanoemulsions. Surface-active agents form a layer around the dispersed phase or droplet which can be monolayer or multilayer and reduce the interfacial tension between two immiscible liquids. Selection of surface-active agents with an appropriate hydrophilic-lipophilic balance (HLB) value is necessary as this is an important parameter which determines the type of emulsion (oil in water or water in oil). Moreover, the surface-active agents are important components of nanoemulsion which determine as well as maintain the droplet size throughout the shelf life. These surface-active molecules may be used as stabilizers, emulsifiers, wetting agents, and viscosifiers. Finally, the third component is aqueous phase which influences polarity, ionic strength, and phase behavior of nanoemulsion. Sometimes apart from the aforesaid components, cosolvents are also utilized in nanoemulsion formulation so as to increase the emulsification attribute and provide stability to the nanoemulsion. Short-chain alcohols, proteins, and carbohydrates are some of the examples that are utilized as cosolvents in nanoemulsion (Saxena et al. 2017). Amid the advancement in novel drug delivery systems for herbal bioactives, nanoemulsion technology is highly popular throughout the globe. Herbal-derived molecules or extracts have several therapeutic benefits and are scientifically proven also. But challenges like higher molecular size, low aqueous solubility, lipid permeability, and elevated degradation profile in vitro and in vivo lead to ineffective pharmacodynamic and pharmacokinetic profile. Nanoemulsion technology is the nanocarrier system which has the ability to overcome all aforesaid challenges and potentiate the biological efficacy of herbal bioactives. Also, scientific studies have meticulously provided the evidences which demonstrate dose minimization (Kazemi et al. 2020), facilitate targeting (Shobo et al. 2018; Ahmad et al. 2018), improving bioavailability (Zhao et al. 2013), release behavior (Macedo et al. 2014), and reducing side effects (Maghbool et al. 2020). In this chapter, we highlight the

concept and state of the art for the nanoemulsion and describes the applications of nanoemulsions for herbal drug delivery.

19.2 Nanoemulsion: The State of the Art

Among the formulation considerations, nanoemulsions formulated with low-energy methods relatively require high concentration of surface-active agents as compared to the high-energy methods. A drawback of destabilization of micelles is associated with higher concentration of surfactants utilized for the fabrication of nanoemulsions by low-energy method. Such methods utilize only environmental changes and form a spontaneous nanoemulsification system. On the other hand, high-energy methods utilize intensive mechanical forces such as high-pressure homogenization/microfluidization and ultrasonic waves. These intensive disruptive forces break down the large droplets of dispersed phase into smaller droplets. Therefore, the high-energy methods require relatively less concentration of surfactants as intensive mechanical forces facilitate the size reduction of micelles (Salvia-Trujillo et al. 2016). Also, microemulsions due to its thermodynamic stability generally require low-energy methods for preparation (Nastiti et al. 2017). The surface-active agents can be ionic (anionic or cationic or zwitterionic) or nonionic (neutral) in nature and depending on the nature these agents impart stability to nanoemulsions. For instance, ionic emulsifiers induce electrostatic repulsion and prevent dispersed phase aggregation. On the other hand, nonionic emulsifiers predominantly induce steric hindrance and reduce droplet aggregation (Aswathanarayan and Vittal 2019). Nanoemulsions are nonequilibrium type of system which cannot be formed spontaneously, and external energy input is required to process the formulation. This external energy facilitates to circumvent the challenge of interfacial tension between the immiscible liquids and form kinetically stable nanoscale emulsified droplets for a long time (Aboofazeli 2010). The importance of additional shear is to break the micron size droplets into nano-range. The high shear required for formulating nanoemulsions is due to the inverse relation of Laplace pressure with the curvature radius (nonplanar surface), and mathematically it is denoted as $\Pi_L = 2\sigma/r$ (Bhattacharjee 2019; Mason et al. 2006). The Π_L refers as Laplace pressure, σ as interfacial tension between two immiscible liquids, and r as droplet radius. Laplace pressure is defined as the pressure exerted by curved interface on the molecules inside the droplet. This also signifies that smaller droplets have relatively high Laplace pressure than larger droplets. Furthermore, nanoemulsions are thermodynamically unstable and have positive value of Gibbs free energy on the formation of nano-droplets. This is due to significant increase in surface area of while conversion from large size droplets (micron/submicron) to small droplets. Consequently, the magnitude for the change in the surface area of dispersed phase becomes positive, and entropy of formed nanoemulsion is also more than zero or positive. Hence, referring the following mathematical equation (Eq. 19.1), Gibbs free energy for formed nanoemulsion becomes positive which signifies that the formation of nanoemulsion is a non-spontaneous process (Barkat et al. 2020):

$$\Delta G = \Delta Ar\gamma + T\Delta S \tag{19.1}$$

where
ΔG is the Gibbs free energy or total free energy
γ is the interfacial tension
ΔAr is the change in the surface area of the interface
T is the temperature of the system
ΔS is the change in entropy

19.3 Types of Nanoemulsion

Nanoemulsions are mainly classified as oil in water (o/w), water in oil (w/o), and bicontinuous phases. In principle, among the two immiscible liquids, one component will be dispersed phase and the other will be regarded as continuous phase. By convention, the phase with higher volume fraction becomes the continuous phase and the other will become dispersed phase, but it is dependent on the type of the emulsifier. Accordingly, the surfactant molecules will orient themselves around the dispersed phase and impart kinetic stability. The nonpolar tail part of the surfactant orients towards the hydrophobic phase, whereas the polar head towards the hydrophilic phase. Hydrophilic-lipophilic balance (HLB) is a parameter which aids in the selection of appropriate surfactant. HLB of a surfactant signifies a ratio of hydrophilic segment to the lipophilic segment. This means the lower the HLB value (around 3–8), the surfactant is more suited for the preparation of w/o type nanoemulsion, and higher HLB (around 8–18) forms o/w nanoemulsion (Che Marzuki et al. 2019). In the case of bicontinuous phase, both immiscible liquids do not form globules in the dispersion; instead, they form irregular structures or birefringence. This state is also known as liquid crystalline state.

19.4 Components of Nanoemulsion

19.4.1 Oil

Oil is one of the components of nanoemulsion and is employed to solubilize hydrophobic drug molecules. Also, oil phase facilitates drug permeation through the biological membrane of the gastrointestinal tract or skin and improves pharmacokinetic behavior. In association with surfactants, oil phase tends to emulsify and form nano-micelles with hydrophobic drug in solubilized form. In general, for pharmaceutical use, hydrolyzed vegetable oils, chemically modified triglycerides, essential oils, and medium-chain fatty acids are preferred. The screening of oil is done on the basis of drug solubility in oil phase, and also miscibility with surfactant is also evaluated before the final selection of all excipients.

19.4.2 Surfactants and Cosurfactants

The surfactants or cosurfactants are also known as surface-active agents, and they can be nonionic, cationic, anionic, and zwitterionic. The role of surfactant is to determine the type of emulsion, size of dispersed droplet, and stability and sometimes also impart toxicity to the nanoemulsion. Among the toxicity issues, the excess amount of surfactants may cause gastric irritancy if taken orally, skin hypersensitivity in topical use, and renal toxicity in parenteral use. Therefore, it is necessary to critically monitor the type and concentration of surfactants while preparing nanoemulsions for pharmaceutical use. Surfactant alone is not sufficient to reduce interfacial tension and stabilize nanoemulsion. Therefore, an additional cosurfactant is usually required for the preparation of nanoemulsion. Preferably, C3–C8 chain alcohols (ethanol, glycerin, propylene glycol, polyethylene glycol 400, Transcutol P) are employed as cosurfactant and are supposed to increase the fluidity as well as synergistically reduce the interfacial tension (Lawrence 1996). Generally, cosurfactants are needed in lower concentrations as compared to the surfactant. For instance, in the preparation of o/w nanoemulsion with one surfactant, a small area signifying the nanoemulsion region has been observed. However, in combination with suitable cosurfactant, an increase in the nanoemulsion region towards the water-rich apex has been observed. Also, more oil can be transformed into nanoemulsified droplets. Therefore, a proper optimization of surfactant mixture has to be validated so that surfactant and cosurfactant mixture can provide an optimum reduction in interfacial tension (Sharma 2018) (Table 19.1).

19.5 Technology Involved for Preparing Nanoemulsion

Nanoemulsions are a non-spontaneous and non-equilibrated system, which means they require some extra energy to form. Nanoemulsion comprises numerous nanodroplets which cause an increase in the surface area, and to increase the surface area, additional energy input is required. Therefore, to fabricate nanoemulsion, the type of constituents, processing methods (high-energy or low-energy method), and processing conditions are the critical factors among the nanoemulsion formulation considerations. The primary aim is to achieve the minimum interfacial tension with maximum stabilizing capacity and small size. In some cases, the mixture formed spontaneously by mixing components all together is coarse dispersion, and hence such premixtures are then subjected to high-energy processes like high-pressure homogenization, microfluidization, and ultrasonication. These methods have different mechanisms to reduce the size from coarse to significantly small size range as shown in Fig. 19.1. High-energy methods utilize mechanical devices to forcefully break down the bigger droplets to ultrasmall size. On the other hand, low-energy processes comparatively require low energy input, and size reduction is carried out by phase inversion composition, phase inversion temperature, and solvent diffusion method. Low-energy processes generally utilize the intrinsic properties of the components.

Table 19.1 Some examples of components of microemulsion/nanoemulsion used for herbal drug delivery

Oils	Surfactant/ cosurfactant	Concentration	Application	References
Cassia oil	Tween 20, ethanol	Cassia oil/ethanol/ Tween 20 (1: 3:6)	Antifungal activity	Xu et al. (2012)
Clove oil	Tween 20	Clove oil/Tween 20/water (5: 30:65)	Antiparasitic activity	Gupta et al. (2005)
Neem oil	Tween 80, sodium dodecyl benzene sulfonate, and hexyl alcohol	Neem oil/emulsifiers/ water (1:3.5: 5.5)	Antiparasitic activity	Xu et al. (2010)
Origanum vulgare essential oil	Tween 60, butylene glycol	Oil/Tween 60/butylene glycol/water (5:25:25:45)	Anti-inflammatory property	Laothaweerungsawat et al. (2020)
Artemisinin loaded in a eutectic oil mixture of lidocaine and ibuprofen	Tween 80, Span 20, and ethanol in a ratio of 1:1:1	1% of artemisinin loaded in oil phase; oil/surfactant/ water (1:4.5: 4.5)	Transdermal delivery system	Zhang et al. (2020)
Curcumin in vitamin E oil	Tween 20 and ethanol	Vitamin E/Tween 20/ethanol/ water (3.3: 53.8:6.6:36.3)	Improved solubility, stability, and oral availability	Bergonzi et al. (2014)
Piperine solubilized in Capryol 90	Cremophor RH 40, Tween 80, and Transcutol HP	o/w emulsion: Capryol 90/S_{mix}/water (32:34:34) w/o emulsion: Capryol 90/S_{mix}/water (64:27:9)	Oral delivery of piperine for the treatment of Alzheimer's disease	Etman et al. (2018)
Berberine in oleic acid	Tween 80, PEG 400	Oleic acid/ Tween 80/PEG 400/water (15: 17:17:51)	Oral drug delivery system	Gui et al. (2008)
Capsaicin solubilized in medium-chain triglycerides (MCT)	Cremophor EL, ethanol	MCT/ Cremophor EL/ethanol/ water (1.0:7.2: 1.8:20.5)	Enhanced oral bioavailability	Zhu et al. (2015)

(continued)

Table 19.1 (continued)

Oils	Surfactant/ cosurfactant	Concentration	Application	References
Tea tree oil	Polysorbate 80, isopropyl myristate, isopropyl alcohol	Tea tree oil (5%)	Anti-psoriatic activity	Khokhra Sonia (2011)

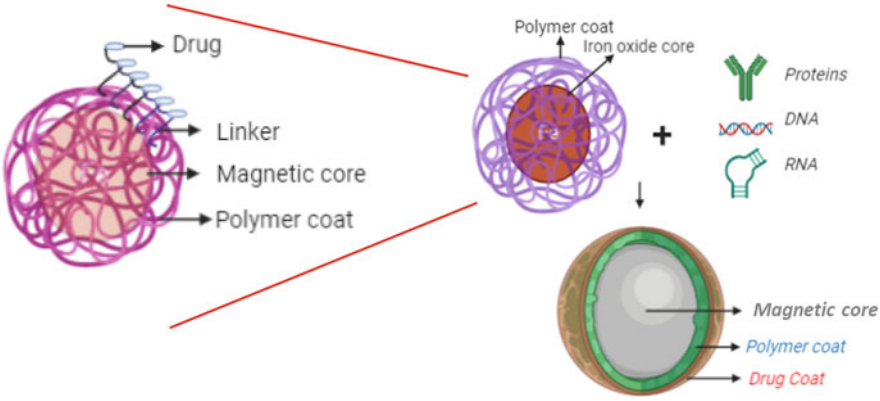

Fig. 19.1 Overview of method of preparation for nanoemulsions

19.5.1 High-Energy Processes

These methods mainly involve mechanical energy in the form of pressure, wave, or mechanical stirring. After disruption of coarse dispersion into very small droplets and increasing the surface area, the process allows adsorption of surfactants at the interface of and enables steric stabilization. The magnitude of mechanical energy must be significantly greater than the interfacial energy so as to achieve a nano-range of droplets. The following are the generally employed high-energy processes for the fabrication of nanoemulsions.

19.5.1.1 High-Pressure Homogenization

The principle of nanoemulsification through high-pressure homogenization involves passing the fluid through micro-orifice under positive pressure through the homogenizer valve. In this process, shear, impact, and cavitation are the principal mechanisms producing mechanical energy to disrupt the droplets into smaller size (Shen 2012; Villalobos-Castillejos et al. 2018). The method involves mainly a two-step process where in the first step, coarse droplets are reduced to ultrafine droplets with an increased surface area. After size reduction to nano-range, the droplets tend to undergo coalescence. Hence, the second step involves the role of the emulsifier wherein the emulsifier adsorbs on the interface and reduces the interfacial tension. An increase in emulsifier concentration and faster adsorption retard the coalescence

process and impart stability to nanoemulsion. The process of homogenization is performed in cycles and optimized. During homogenization, sometimes heat is generated which may have a detrimental effect on heat-sensitive bioactives. However, this situation can be countered through ice or cold water jacketing and reducing the homogenization time of each cycle.

19.5.1.2 Microfluidization

The microfluidization process utilizes a static and mechanical mixer which involves driving a fluid through microchannels under high pressure which results in ultrafine droplets of dispersed phase. The positive pressure applied has a direct impact on size reduction, which means an increase in pressure may result in a decrease in droplet size. The principle of microfluidization is almost similar to high-pressure homogenization except the passage through microchannels whose pore size ranges from 50 to 300 μm. Generally, a pressure of around 270 mPa is applied in microfluidization, and the fluid is allowed to move downstream through microchannels at a velocity of approximately 400 m/s. Through the inlet, the fluid passes through the Y junction where it splits into two branches and then reconnects at the interaction chamber at high velocity and high shear rate. After size reduction, the surface area of dispersed phase increased significantly, and the surfactant has to adsorb on the interface rapidly to avoid coalescence. Therefore, fast-adsorbing surfactants are often selected for fabricating nanoemulsions through microfluidization. Also, increasing the viscosity of continuous phase also retards the coalescence of ultrafine droplets. These forces result in mechanical energy with high magnitude sufficient to counter the interfacial energy and significantly reduce the droplet size (Che Marzuki et al. 2019; Villalobos-Castillejos et al. 2018).

19.5.1.3 Ultrasonication

Among other high-energy methods, ultrasonication is the simplest, is easy to use, and requires low-end mechanical instruments. It is the ultrasound waves that are responsible to produce shock waves, resulting in disruption of mainly oil droplets into smaller size in water. These intensive ultrasonic waves generate vibrations and acoustic cavitation which creates high pressure in dispersed phase and turbulence that collapse the droplets. The frequency of sonic waves and time of sonication play an important role for appropriate size reduction. An optimum frequency is necessary to produce shock waves with sufficient high energy input that can disrupt the droplet. Generally, frequency with more than 20 KHz is suitable for droplet size reduction. Also, the more the time of ultrasonication, the more efficiently size reduction takes place. This is because an increase in the time of ultrasonication produces higher energy input capable to reduce the interfacial tension (Behrend et al. 2000).

19.5.2 Low-Energy Processes

Low-energy processes are of great interest for those bioactives which are heat sensitive as in the case of high-energy processes, some of the heat energy is

produced. On the other hand, low-energy processes, particularly the phase inversion composition and solvent diffusion method, show spontaneous emulsification with slight agitation. All methods of low-energy processes depend on inherent physicochemical properties of all components to form nanoemulsions such as solubility, concentration, and impact of temperature. Unlike the high-pressure energy process, the low-energy process does not utilize high energy input for reducing interfacial tension which may generate additional heat in the system. This approach for the fabrication of nanoemulsion recommends the use of medium-chain triglycerides for oily phase, whereas these methods are not suitable for long-chain triglycerides. Hence, this limits the use of several oils in which the herbal bioactive shows good solubility. But this situation can be resolved in some of the cases by using a mixture of medium-chain and long-chain triglycerides (Pathania et al. 2018).

19.5.2.1 Phase Inversion Temperature

In this method, a key role is played by the emulsifier which tends to change their hydrophilic and lipophilic character with respect to temperature at fixed concentration. With an increase in temperature, the emulsifier changes their curvature at the interface, and the process of phase inversion begins. For instance, o/w nanoemulsion is subjected to higher temperature, and with an increase in temperature, the solubility of the emulsifier tends to alter. Particularly, the solubility of nonionic surfactant decreases in aqueous solution with an increase in temperature due to the dehydration of the hydrophilic component of nonionic surfactant. Therefore, at a specific temperature, the type of nanoemulsion reversed, i.e., from o/w to w/o nanoemulsion, due to the change in the solubility of the emulsifier as a function of time. This temperature is known as phase inversion temperature. A continuous stirring is generally required in this technique for the uniform distribution of temperature in the system and ultimately the uniform influence of temperature on size reduction. Comparatively, higher concentration of the emulsifier (such as ceteareth-12, cetostearyl alcohol, and tetra-ethylene glycol dodecyl ether) is required in this method as it is mainly the inherent property of the emulsifier which is influenced by temperature that plays a critical role in phase inversion, droplet size, type of emulsion, and stability (Pathak 2017; Anton and Vandamme 2009; Förster et al. 1990).

19.5.2.2 Phase Inversion Composition

This is a type of low-energy method wherein the change in fraction of oil to water or vice versa at fixed temperature leads to phase inversion. The mechanism involved a change in emulsifier orientation with an increase in dispersed phase volume. Due to the change in emulsifier orientation, the micelle transformed from one type to another, i.e., o/w to w/o or vice versa. The process involves slow addition of one component with slight stirring. In between the complete transition, a phase comes where the content of both oil and water reaches almost in equal fractions. Bicontinuous structures are formed in this phase and are also known as liquid crystalline phase. After this phase further addition of oil or water forms the opposite type of micelle and with an increase in dilution results a further droplet size reduction (Sharma and Sinha 2018; Che Marzuki et al. 2019; Sharma 2018).

19.5.2.3 Solvent Diffusion Method

In solvent diffusion method, oily components are mixed into organic solvents like ethanol, acetone, ethyl acetate, isopropyl acetate, etc. The prepared oily phase is then mixed in aqueous phase consisting of surfactant or surfactant mixture with continuous stirring. The agitation caused by the stirring in the system forms nano-droplets. The organic solvent is then removed from the system using rotary vacuum evaporator. The safety consideration of organic solvent is important in this method as the solvent has to be safe in terms of toxicity. Also, the solvent should have miscibility with both the oil and aqueous phases. The concentration of organic solvent along with the concentration of oil, surfactant, and water plays an influential role in the determining the formation of nanoemulsion and its stability (Porras et al. 2008; Bouchemal et al. 2004).

19.6 Application of Nanoemulsion for Herbal Bioactives

Herbal bioactives have been pharmacologically utilized for the treatment of various diseases. Despite the huge potential of phytopharmaceuticals (herbal extracts and bioactives), their pharmaceutical application is limited due to poor water solubility, membrane permeability, and limited bioavailability. All these issues can be resolved through nanoemulsion technology wherein the active compound is encapsulated in nano-droplets dispersed in continuous medium. Also, self-nanoemulsifying system has the property to transform into nano-droplets when comes in contact with gastric environment and additional energy required for the formation of nanoemulsion is provided by the gastrointestinal motility itself. Such type of nanoemulsions is also known as self-emulsifying nanoemulsion. The dispersed phase rapidly diffuses into the continuous phase and instantaneously forms nanoscale emulsions in vivo. Such nanoscale emulsions usually carry low oil content and high concentration of surfactant or surfactant mixture which enables to form o/w nanoemulsion spontaneously (Kumar et al. 2019). Nanoemulsion also provides stability to phytopharmaceuticals from gastric degradation and can also impart controlled release pharmacokinetics. Several herbal phytopharmaceuticals (quercetin, camptothecin, rutin, genistein, resveratrol) have been reported to be delivered using nanoemulsion technology primarily with an aim to improve their permeability through the gastrointestinal membrane and skin (Salvia-Trujillo et al. 2016; McClements and Xiao 2017; Aboalnaja et al. 2016; Rahman et al. 2020).

The important challenge associated with phytopharmaceuticals is their variable bioavailability that may produce nonuniform therapeutic effects. This is the reason phytopharmaceuticals are unable to produce favorable therapeutic action consistently and sometimes cannot satisfy the medical community. This nonuniformity causes uncertainty in treating disease and reduced level of significance in statistical clinical data. There are mainly three major factors that influence the oral bioavailability of phytopharmaceuticals, i.e., (1) the dose of the herbal extract or bioactive to be ingested and fraction available for dissolution in gastric environment (this factor can be a rate-limiting factor for most of the herbal bioactives because of their

hydrophobicity that limits their solubilization in vivo); (2) fraction of the solubilized concentration which is supposed to be absorbed through the gastrointestinal membrane and enter into the systemic circulation (in this factor, the permeability factor plays a key role that determines the level of absorption); and (3) after absorption through oral route, the absorbed fraction undergoes biotransformation and then transferred to the site of action.

Therefore, several novel drug delivery approaches have been concurrently performed and developed to maximize the therapeutic efficacy of herbal bioactives. Apart from solubility, permeability, and stability issues of phytopharmaceuticals, improvising the biological half-life is of great concern among the formulation scientists. As biological half-life directly determines the bioavailability and potency of herbal bioactives at the site of action. Low biological half-life signifies low bioavailability and high biological half-life signifies high bioavailability (Mukherjee et al. 2015).

To curb all aforesaid problems associated with herbal bioactives, nano-sized emulsions are pioneering among the novel drug delivery systems (NDDS) in context to industrial viability and providing promising uniform therapeutic results to clinicians. The herbal bioactives are usually lipophilic or have poor aqueous solubility which generally limits their bioavailability. Hence, nanoemulsions are the best suitable drug carrier system as the hydrophobic bioactive substance is dissolved in oil phase, whereas the hydrophilic bioactive is dissolved in aqueous phase. After solubilizing the bioactive in selective phase (oil/water) along with appropriate surfactant and cosurfactant are mixed into the continuous phase. The colloidal dispersion formed is subjected to either high-energy processes or low-energy processes for nanoemulsification or size reduction. In some cases co-solvents are also added as an adjuvant as they have been shown to increase the solubility of compounds and penetration into biological tissues as well. In such cases, transforming the bioactive substance into a crystalline form can resolve the issue (Chen et al. 2011; Shegokar and Müller 2010). It has been observed that most of the herbal bioactives are usually poorly soluble in oil and water as discussed in Table 19.2. Under such circumstances, the route of administration plays a key role in defining the formulation considerations. For instance, if the hydrophobic bioactive is intended to deliver through oral route, then the oily phase is preferred as it tends to digest rapidly in the gastrointestinal medium and forms mixed micelles also known as swollen micelles (Rana et al. 2017). In the case of topical or transdermal route, certain percutaneous absorption enhancers like ethanol or essential oils are reported as an adjuvant to nanoemulsion (Shen et al. 2011). Also, for topical applications, the increased concentration of surfactants or cationic surfactants can be employed for improvising the low solubility and permeability issues with clinical safety (Dario et al. 2016).

Selection of oil also plays a major role particularly for drug loading and ultimately bioavailability of lipophilic herbal actives. The oils with long-chain triglycerides comprise comparatively more lipophilic character due to long nonpolar chains. This property allows accommodation of more drug into the mixed micelle formed and nanoemulsification technology facilitates the dissolution and permeability through

Table 19.2 Some of the examples of herbal bioactives formulated as nanoemulsion

Herbal extract/ bioactive	Therapeutic activity	Route of administration	Pharmaceutical challenge	NDDS technology	Method of preparation	Outcomes	References
Mangiferin (xanthone glycoside)	Anti-inflammatory	Topical	Low aqueous solubility (experimentally approximately 2 mg/mL at 30°C)	Hyaluronic acid (low molecular weight) assisted o/w nanoemulsion gel with globule size ~290 nm	High-energy emulsification method using homogenization followed by ultrasonication	Presence of low molecular weight HA in combination with Transcutol P has significantly increased in vitro permeation of mangiferin	Pleguezuelos-Villa et al. (2019), Acosta et al. (2016)
Silymarin (flavonoid)	Hepatoprotective drug, free radical scavenger, and oxidase enzyme inhibitor	Oral	Low oral bioavailability (<47%)	o/w nanoemulsion using Capryol 90 (oil), Solutol HS 15 (surfactant), and Transcutol HP (cosurfactant) with globule size ~50 nm	High-pressure homogenization followed by ultrasonication	Enhanced bioavailability of silymarin was observed with nanoemulsion as compared to oral suspension	Nagi et al. (2017), de Groot and Rauen (1998)
Quercetin (flavonoid)	Antioxidant	Topical as hair conditioner	Low water solubility (~50 μM)	Cationic o/w nanoemulsion with average globule size ~20 nm	Low-energy process using phase inversion temperature	High drug loading up to 0.5% w/w and stable at room temperature and ~5°C	Dario et al. (2016)
Myricetin (flavonoid)	Antioxidant	Oral	Low oral bioavailability (<10%) and aqueous solubility (~16.60 g/mL)	Self-nanoemulsifying drug delivery system using Capryol 90 (oil), Cremophor RH 40 (surfactant), and polyethylene glycol	Low-energy process	Improved solubility and permeability in duodenum and jejunum as compared to free drug	Qian et al. (2017)

				400, 1,2-propanediol, and Transcutol HP (cosurfactant) with globule size ranging between 20 and 140 nm			
Zedoary turmeric oil (essential oil comprising mainly sesquiterpenes)	Antibacterial, antithrombotic, hepatoprotective, and increase gastrointestinal motility	Oral	Poor water solubility, irritant, and unstable in the gastrointestinal environment; also high volatility as a consequence reduced drug loading in solid dosage forms	Self-nanoemulsifying drug delivery system using ethyl oleate (oil), Tween 80 (surfactant), Transcutol P (cosurfactant) with ~30% drug loading and a globule size of ~68 nm	Low-energy process	Oral bioavailability of bioactive marker (germacrone) of zedoary turmeric oil increased significantly in rats as compared to unformulated zedoary turmeric oil	Song and Sun (2016), Zhao et al. (2010)
Colchicine (alkaloid)	Potent anti-inflammatory	Oral	Low aqueous solubility and membrane permeability	Eugenol (absorption enhancer) assisted o/w nanoemulsion using isopropyl myristate and eugenol (oil), Tween 80 (surfactant), and ethanol with average globule size ~40 nm	Low-energy process	Increased bioavailability due to eugenol-based nanoemulsion as compared to free drug solution	Shen et al. (2011)
Ferulic acid (4-hydroxy-3-methoxycinnamic acid)	Potent antioxidant, anti-inflammatory, antiaging, free	Topical	Limited solubility in water and oils	o/w nanoemulgel with isostearyl isostearate (oil), Labrasol (surfactant), and	Spontaneous emulsification followed by ultrasonication	Improved solubility, permeability, and UV protection	Harwansh et al. (2015), Batra et al.

(continued)

Table 19.2 (continued)

Herbal extract/ bioactive	Therapeutic activity	Route of administration	Pharmaceutical challenge	NDDS technology	Method of preparation	Outcomes	References
	radical scavenging activity, antiatherogenic, antianxiety, neuroprotective, and cardioprotective			Plurol isostearique (cosurfactant) with globule size ranging between 100 and 200 nm, resulting in sustained release, better permeability, and antioxidant activity		activity and showed sustained release profile	(2018), Sonali et al. (2020)
β-Carotene (carotenoid)	Singlet oxygen scavenger, precursor of vitamin A	Oral	Low aqueous solubility, low bioavailability, high melting point, and highly susceptible to autoxidation due to the presence of conjugated polyunsaturated hydrocarbon chain	o/w nanoemulsion using corn oil, β-lactoglobulin (surfactant), and EDTA	High-pressure microfluidization	Increased stability against the color fading of carotenoids and reduced rate of degradation	Qian et al. (2012b), Padmanabhan et al. (2016)
Lycopene (carotenoid)	Potent antioxidant, singlet oxygen, and free radical quencher	Oral	Sensitive to chemical degradation and undergo oxidation on storage	o/w nanoemulsions using sesame oil, linseed oil, or walnut oil as the oil phase and lactoferrin (emulsifier) with globule size ~200 nm	High-pressure homogenization	Reduced oxidative degradation during digestive process, thereby resulting in increased bioaccessibility and improved efficacy	Zhao et al. (2020)

the biological membrane (Qian et al. 2012a; Yang and McClements 2013). Such micelles with comparatively large dimension of nonpolar chains also facilitate loading of large hydrophobic bioactives and capable to transport these large hydrphobic molecules across the biological membrane. Some of the oils like tocopherols have antioxidant property which can be utilized for imparting the stability to bioactives which are sensitive to oxidation such as carotenoids. Ethylenediaminetetraacetic acid and ascorbic acid are some other water-soluble examples of antioxidants which are commonly utilized in nanoemulsions for providing chemical stability to sensitive bioactives (Qian et al. 2012b). Herbal bioactives like resveratrol with short half-life in water and susceptible to aqueous degradation can be formulated as nanoemulsion (Francioso et al. 2014). Nanoemulsions by encapsulating the bioactive in oil phase not only increase the shelf life but also slow down the degradation kinetics of resveratrol when administered orally and hence improve the bioavailability (Davidov-Pardo and McClements 2015). In conclusion, nanoemulsions as a drug delivery system for herbal bioactives are the ideal approach for hydrophobic herbal bioactives as it addresses major challenges of poor water solubility, low permeability, chemical degradation, and reduced bioaccessibility. Moreover, nanoemulsions are suitable for oral, topical, and pulmonary route of administration and hence prove its wide acceptance. Oil solubility, water solubility, miscibility of oil and surfactants, polarity, viscosity, optical clarity, stability, crystal form of bioactive, and partition coefficient are some of the important formulation considerations that should be taken into account while formulating nanoemulsions for bioactives.

19.7 Future Prospects

The amalgamation of nanotechnology with herbal bioactives is quintessential in the current scenario. Nanoemulsions, among all the nanocarrier systems, use industrially viable and comparatively low-end mechanical instruments for its fabrication; hence, it is widely accepted and used. The benefits of herbal bioactives are effectively delivered with the help of nanoemulsions, especially dealing with low-solubility criteria which most of the natural products have. Nanoemulsions offer great advantages as they can formulate herbal bioactives and the route of administration can vary from oral, topical, as well as parenteral. Improved targeting, reduced size, increased solubility, as well as better bioavailability are some of the major criteria for a formulation to be efficient. Nanoemulsions inculcate all these features and effectively deliver herbal bioactives without altering their health benefits. With the upcoming renewal of the use of herbal moieties, it becomes the need of the situation to effectively deliver the bioactives. This is thus possible with the exploration of the systematic and effective approach for the formulation of such bioactives. Nanoemulsions have been widely used in many such cases and have shown tremendous results in nanoresearch. Thus, this collaboration of nanoemulsions with herbal bioactives holds immense potential for future, providing formulation experts a pathway to enhance in the field of nanoresearch.

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siRNA-Encapsulated Nanoparticles for Targeting Dorsal Root Ganglion (DRG) in Diabetic Neuropathic Pain

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Ranjana Bhandari, Priya Badyal, Garima Khanna, and Anurag Kuhad

Abstract

Diabetic neuropathy is the most established complication of diabetes. Typically, diabetic neuropathy involves the distal foot and toes but eventually advances to include the lower part of the legs. The toxic effects of hyperglycemia are accepted to be a major factor in the emergence of this complication. In the dorsal root ganglion, upregulation of voltage-gated sodium channels due to hyperglycemia was commonly seen in models of neuropathic pain. To increase intracellular sodium ion levels, DRG increases its opening frequency, which in turn may lead to increased calcium channel opening that further triggers other pathways that lead to DPN. Relief from pain due to diabetic neuropathy has been seen with the use of antidepressants, GABA analogues, opioids, and topical agents that are recommended in clinical guidelines. Currently available medications provide adequate pain relief for approximately half of the affected patients, and their use is also restricted due to unwanted adverse reactions like somnolence, dizziness, and multiple daily doses reduce patient compliance. siRNA showed behavior-associated inhibition in allodynia as well as hyperalgesia which was correlated with the downregulated P2X3 receptor in the dorsal root ganglion and spinal cord. siRNA is very unstable under normal physiology in the blood wherein it undergoes digestion by nuclease enzymes. Thus, the development of drug delivery systems that can enhance site-specific delivery of siRNA therapeutics for aiding relief from disease is important. siRNA encapsulated in nanoparticle delivery devices can be utilized as a plausible therapeutic in relieving neuropathic pain.

R. Bhandari (✉) · P. Badyal · G. Khanna · A. Kuhad
University Institute of Pharmaceutical Sciences, UGC-Centre of Advanced Study, Panjab University, Chandigarh, India
e-mail: akb10in@yahoo.co.uk

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Diabetic neuropathic pain (DNP) · Dorsal root ganglion (DRG) · siRNA

20.1 Introduction

Diabetic neuropathy is a well-established outcome of diabetes that affects nearly 90% of diabetic patients (Boulton et al. 2005). Diabetes has now become an epidemic globally; nearly 463 million adults in the age groups of 20–79 years had diabetes in 2019, and this number is projected to rise to 700 million by 2045 (International Diabetes Foundation 2019). Distal symmetric polyneuropathy (DSPN) is the most well-known diabetic neuropathy (Tesfaye et al. 2013). The distal part of the foot and toes is typically affected by DSPN but progresses gradually towards the legs. The progressive loss of nerve fibers is one of the most common consequences of it that leads to diabetic retinopathy or nephropathy, affecting both the autonomic nervous system and somatic divisions (Tesfaye et al. 2013). Ulcers on the foot and debilitating neuropathy are the main clinical complications with greater morbidity and mortality (Boulton et al. 2004). Diabetic neuropathic pain is characterized by prickling or burning sensations (Cobos-Palacios et al. 2020). It usually gets worse during nights that lead to poor sleep. This pain can be persistent and followed by epidermic allodynia, which majorly affects the quality of life of patients and impairs their capabilities to perform daily activities (Quattrini and Tesfaye 2003). Several hypotheses are suggested to justify the pain in diabetic neuropathy like changes in the nerves present peripherally, changes in the expression of sodium and calcium channel, and metabolic and autoimmune disorders caused by glial cell activation (Tesfaye et al. 2013). More recently, core pain pathways have been observed, such as facilitator/inhibitor descending pathway imbalance and an increase in thalamic vascularity and (Tesfaye et al. 2013). It has been known from many studies that dorsal root ganglion (DRG) neurons are one of the specific targets and may contribute to major complications of diabetic neuropathy, as chronic hyperglycemia results in radiculopathy and distal sensory neuropathy associated with vacuolar ganglionopathy (Kishi et al. 2002). There is a major clinical application of the dorsal root ganglion (DRG), especially in its connection with diabetic neuropathic pain. DRG neurons arise from the dorsal root of the spinal nerves, bringing sensory signals to the central nervous system for a response from different receptors, including those for pain and temperature (Ahimsadasan and Kumar 2018). Until recently, the dorsal root ganglion has been regarded as a passive organ that serves functions and pathways between the PNS and CNS metabolically. However, recent findings indicate that DRG is an active participant in peripheral processes, including PAF damage, inflammation, and the production of neuropathic pain (Ahimsadasan and Kumar 2018). A lesion or disorder of peripheral neuropathic pain is peripherally originated by the somatic nervous system. Peripheral damage to the nerves in neuropathic pain leads to overexpression of the P2X3 receptor in the DRG. The P2X3 receptor is expressed primarily in DRG neurons and is seen to be

involved in the transmission of pain signals (Burnstock 2006, 2007; Tsuda et al. 2010; Liang et al. 2010; Zhang et al. 2010). Pain management in PDN is done via the antidepressant agents such as duloxetine, GABA analogues, opioids, and topical drugs such as capsaicin. The FDA has approved the use of duloxetine and pregabalin for the treatment of PDN in 2004, and tapentadol which is formulated as the extended-release drug was approved for DNP in 2012 for long-term treatment of this disorder where other drugs cannot be preferred (Javed et al. 2015). Currently, available therapies do not offer to relieve pain to all the patients suffering from DNP and are also seen to be restricted by unexpected adverse effects, such as somnolence and dizziness, and the requirement for numerous daily doses that further reduces the patient's compliance. It is reported that P2X3 receptor activation leads to allodynia in rat models of diabetes (Xu et al. 2011). DM rats when treated with NONRATT021972 siRNA have shown that the expression of the DRG P2X3 receptor is significantly decreased as compared to T2DM rats in which no treatment is given. Ribonucleic acid interference (RNAi) is emerging as a gene-silencing tool that inhibits the expression of the gene after transcription that inhibits the particular protein synthesis by activating the RNA-induced silencing complex (RISC). It is now possible for physicians to treat a disease with the help of genes by administering RNAi therapeutics such as siRNA to a patient to inhibit the expression of a particular gene rather than using complex treatment strategies (Setten et al. 2019). siRNA's short half-life, nonfunctioning of administered siRNA due to degradation by circulatory RNase, as well as rapid clearance by the renal route are some of the key difficulties complicating the clinical translation of siRNA therapeutics (Youngren et al. 2013; Tekade et al. 2015). Efficient nanocarriers should ensure evasion from immunogenic recognition and clearance through our reticuloendothelial system. Serum proteins like albumin and IgG tend to interact with siRNA cationic bodies, leading to the enhanced size of the complex. This ultimately lessens the targeted siRNA fraction that reaches the target site (Zhao and Feng 2015). Attaching a ligand entity like an antibody, aptamer, or peptide provides specificity to the siRNA molecule and ensures release at the desired site of action. Lipid nanoparticles possessing a positive charge attributed through cation lipidic formulations are efficient in condensing the genes and ensuring uptake by the cell (Ozpolat et al. 2014). In the following chapter, siRNA-encapsulated nanoparticles for targeting dorsal root ganglion with the paradigm of diabetic neuropathic pain are discussed.

20.2 Diabetic Neuropathic Pain: An Unmet Medical Need

20.2.1 Epidemiology

Diabetic neuropathy, with various anatomic characteristics, clinical courses, and phenotypes, includes a series of clinical complex disorders that affect the central nervous system (Martin et al. 2014). Diabetic peripheral neuropathy (DPN) and its incidence rise with the duration of diabetes. Diabetes has now become an epidemic globally; nearly 463 million adults in the age groups of 20–79 years had diabetes in

2019, and this number is projected to grow to 700 million by 2045 (International Diabetes Foundation 2019). This rise shall be followed by an increased incidence of diabetes-related complications (Thibault et al. 2016). In various studies across India, peripheral neuropathy prevalence ranges from about 10.5% to 32.2% in diabetic patients (Maser et al. 1989). Compared to the West, it has a higher prevalence of DM in India (Forouhi and Wareham 2014). Many studies have noted that glucose tolerance test findings are abnormal in idiopathic PN patients. Sample of 107 patients who were suffering from neuropathy due to some unknown reason were tested for glucose tolerance, 13 suffered from diabetes mellitus and 36 had shown less glucose tolerance (IGT) when compared to control (Singleton et al. 2001). A study conducted in Sothern India indicated that the group in which glucose tolerance is reduced displayed a substantially alleviated mean nerve conduction velocity (NCV) compared to normal individuals (Viswanathan et al. 2004). In Indian epidemiological studies from different areas, the average prevalence of PN in different community studies ranged from 5 to 2400 per 10,000 populations (Trivedi et al. 2017). DPN is considered the most common cause of neuropathy globally which affects more than half of the people suffering from diabetes (Young et al. 1993). Indeed, about a quarter portion of US diabetes health care spending is spent on DPN (Gordois et al. 2003). Due to various patient groups, concepts of neuropathy used, and methods of evaluation, epidemiological experiments of diabetic neuropathy have produced complex results (Stino and Smith 2017). Cardiovascular researchers found that the incidence of neuropathic pain was 13.3% among patients who have diabetes versus 4.2% and 1.2% in subjects who possessed impairment in fasting glucose and controls, respectively (Ziegler et al. 2009). About 66% of patients with type 1 diabetes and 59% of patients suffering from type 2 diabetes were diagnosed with DPN (Dyck et al. 1993). A community-based study found that 34% had signs of painful neuropathy in 15,000 diabetes patients (Abbott et al. 2011). More recently, DPN's prevalence has been re-evaluated in young people with shorter durations of diabetes. In a youth study (Hamman et al. 2014), a group of young people who are below 20 years of age with diabetes duration around 5 years was diagnosed for DPN, indicating an extreme DPN burden even in teenagers (Jaiswal et al. 2017).

20.2.2 Pathogenesis of Diabetic Neuropathic Pain

A logical explanation is not yet available to explain why certain patients with diabetes develop diabetic neuropathic pain while some do not. The mechanisms leading to DNP are not well known, although the pathological effects of hyperglycemia are a significant factor that leads to this complication (Dobretsov et al. 2003; Oyibo et al. 2002). Studies have shown partial peripheral sensory nerve degeneration of the plantar hind paw skin area in diabetic neuropathic pain syndromes (Siau et al. 2006). Partial nerve fiber loss leads to hyper-excitability, spontaneous discharge, and mechanosensitivity of nerve fibers with degenerated terminal arbors (Devor and Seltzer 1999). Neuropathy condition causes cold and heat allodynia due to a decrease in Aδ fibers which are cold specific and C fibers which are heat specific

from the epidermis, respectively (McCarthy et al. 1995). A mitochondrial abnormal function has a crucial role to play in the development of many disorders of the nervous system, including neuropathic pain which is a peripheral nervous system disorder (Bouillot et al. 2002). There are various interrelated mitochondrial-based pathways, such as intracellular Ca^{2+} regulation (Shishkin et al. 2002), reactive oxygen production (Chung 2004), and apoptotic signaling pathways (Joseph and Levine 2004), which play a significant role in the development of neuropathic pain. Neuropathic pain is not due to one independent pathway but many pathways interrelated to one other.

Hyperglycemia seems to trigger changes in the sodium channel expression. Upregulated sodium channels (voltage-gated) were widely seen in pain models of neuropathy in dorsal root ganglion (DRG) (Black et al. 1999). Hyperglycemia affects $Na^{(+)}$ currents, possibly due to polyol pathway activation, and impairs $Na^{(+)}$-$K^{(+)}$ pump (Misawa et al. 2009). These channels influence the processing of action potential along with their transmission and can be categorized as tetrodotoxin sensitive (TTX-S) (Roy and Narahashi 1992). In the animal models of diabetes, many findings suggest upregulation of Nav1.3 channels which are tetrodotoxin sensitive and have shown a role in the embryonic development stage (Felts et al. 1997) and Nav1.7 in the dorsal root ganglion (DRG) (Ogata and Ohishi 2002; Galloway and Chattopadhyay 2013; Hong et al. 2004). DRG neurons and sensory neurons of the dorsal horn show a rise in the frequency of opening of sodium channels and their opening duration has also been seen to be prolonged to elevate the levels of intracellular sodium ion. The polarization of the neuron further causes increased opening of calcium channels and causes hyperpolarization (Misawa et al. 2009). Overexpression of α2δ-1 subunit of the calcium channel has been reported to play a part in the oversensitivity of nociceptive responses to harmless mechanical stimulation that is a characteristic feature of allodynia in rats in which nerves of the spinal area are injured (Luo et al. 2002). Due to the overexpression of this subunit, more extracellularly present calcium enters the cell that further leads to various signaling cascades (Luo et al. 2002). Also, glutamate is released in the presynaptic zone which causes NMDA receptors to activate. NMDA receptor activation will again elevate the entry of extracellular calcium inside the cell (Zhou et al. 2011). Mitochondria in response to hyperpolarization of cells start releasing more calcium in the cytoplasm from its intercellular stores. As calcium concentration elevates inside the cell, it leads to activation of various signaling cascades, and phosphorylation of protein kinase C is one of them (Lipp and Reither 2011), leading to upregulation of transient receptor potential vanilloid (TRPV) (Bhave et al. 2003), which directly causes alterations in the sensory neurons which results in hyperresponsive state. Due to TRPV upregulation, nitric oxide and oxygen free radicals are also produced and ultimately cause neuronal cytotoxicity (Fernandes et al. 2012).

TRPV1 co-reside with transient receptor potential ankyrin 1 (TRPA1) in particular neurons of DRG, and they are proven to have a role in the generation of the pain signals and in inflammation that may occur due to various irritants like chemical agents, ROS, or nitrogen radicals (Ta et al. 2010). Cytochrome *C* may be released from the mitochondria, while permeability of mPTP pores increased due to

hyperpolarization inside the neuronal body that further begins apoptotic cascades. Caspases are activated in the apoptotic pathways and cause damage to neuronal bodies which ultimately cause cytotoxicity (Areti et al. 2016). Ultimately, A fibers which are sensitive to cold and C fibers which are warm specific start decreasing from the epidermis which is known as loss of intra-epidermal nerve fibers. Loss of nociceptors have also been observed which will consequently result in the hyperresponsive state of the remaining nociceptors (Bennett et al. 2014). TNF-α, interleukin-1, and interleukin-6 are the inflammatory markers that are secreted from the glial cells and macrophages present in DRG also seen to be involved in this signaling cascade. Cytokines act on their receptors and further cause changes that include activation of PKC (protein kinase C) and MAP (mitogen-activated protein) kinase that further contribute to the development of neuropathic pain (Gonçalves dos Santos et al. 2020). Also, inflammatory cytokines often increase the level of expression of various ion channels, such as sodium ion channels, that cause neuronal excitotoxicity and also cause an increase in the response of nociceptors to noxious and even to the non-noxious stimuli and make a significant contribution to neuropathic pain pathogenesis (Mamet et al. 2002). The pathogenesis of DNP interlinking different pathways is represented in Fig. 20.1.

20.2.3 Present Treatment Strategies for Diabetic Neuropathic Pain

Diabetic neuropathy is an emerging outcome of diabetes that is seen to affect one out of every five diabetic patients. PDN is difficult to assess objectively, making its diagnoses a bit difficult (Javed et al. 2015). Treatment strategies recommended by clinical guidelines are antidepressants like duloxetine, GABA analogues like pregabalin, opioids, and topical agents like capsaicin to relieve PDN pain. The US Food and Drug Administration (FDA) approved duloxetine and pregabalin for the treatment of PDN in 2004, and extended release formulation of tapentadol was approved in 2012 (Javed et al. 2015). Lowering HbA1c levels can increase the activity of nerves present peripherally and conduction (Bertelsmann et al. 1987). The HbA1c target for most DPN patients is less than 6.5% (American Diabetes Association 2014). There also is clinical evidence that those who have had intensive glycemic regulation in the past have a "metabolic memory" that may help avoid DPN (Albers et al. 2010). Antidepressants, anticonvulsants, analgesics, and topical drugs are among these remedies (Tan et al. 2010).

20.2.3.1 Antidepressants

The disturbance in the balance of release of norepinephrine and serotonin in neurons has been linked to DPN in various studies (Sultan et al. 2008). Serotonin-norepinephrine reuptake inhibitors (SNRIs) such as duloxetine, which comes under antidepressants also used for treating DPN (Lindsay et al. 2010). TCAs (tricyclic antidepressants) are typically less well received than SNRIs (Lindsay et al. 2010).

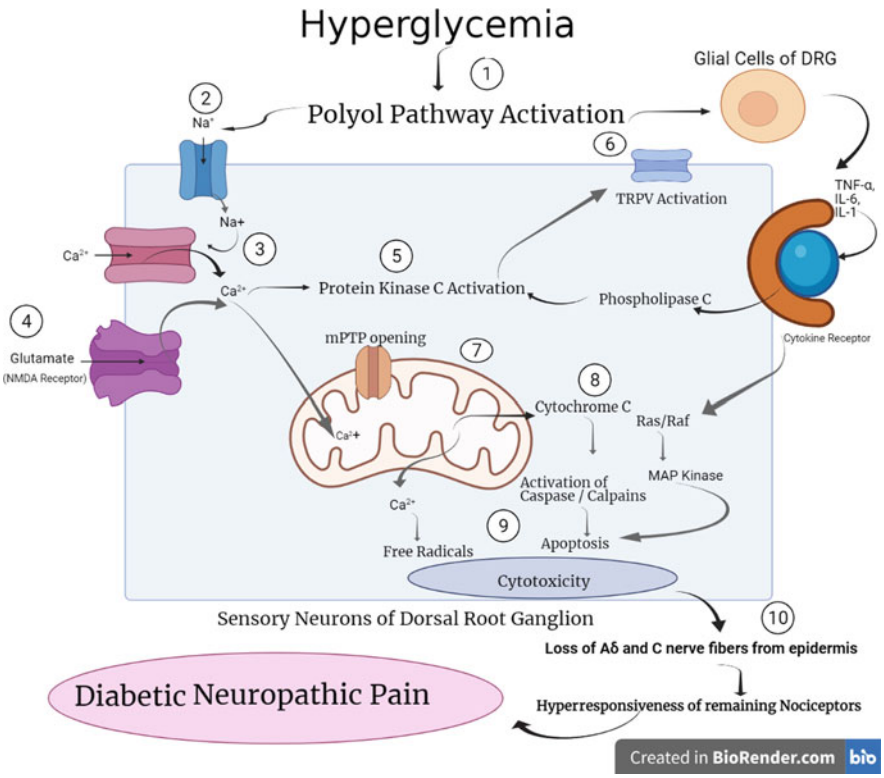

Fig. 20.1 Pathogenesis of diabetic neuropathic pain (DNP). (1) Hyperglycemia leads to the activation of the polyol pathway that further leads to impairment of Na^+ currents. (2) Sensory neurons of DRG increase the opening frequency of Na^+ channels and also prolonged duration of opening to increase intracellular sodium ion levels. (3) Polarizations will further cause the opening of calcium channels. (4) Furthermore, pronounced NMDA receptor activation due to glutamate presence in the presynaptic zone causes more influx of calcium. Increased cytosolic calcium causes more calcium to be released from intercellular stores, particularly mitochondria. (5) Increased calcium can cause many secondary changes like activation of protein kinase C. (6) Protein kinase C phosphorylates and activates transient receptor potential vanilloid (TRPV), which causes sensory neurons to become hyperresponsive, as well as the production of ROS and nitrogen radicals, which causes cytotoxicity in neuronal cell bodies. (7) Cytochrome *C* released by mitochondrial mPTP pores after its opening to start (8) apoptotic cascades with activation of caspases to cause sensory neuronal damage (9) and cause apoptosis. (10) The epidermis may lose A fibers and C fibers and some nociceptors resulting in a hyperresponsive state of remaining nociceptors. Inflammatory mediators like TNF-α, IL-1, and IL-6, which are produced by glial cells of DRG, are also involved in this cascade, acting on cytokine receptors present on the sensory neurons to induce changes such as PKC (protein kinase C) and MAP (mitogen-activated protein) kinase activation, which play an important role in the development of neuropathic pain

20.2.3.2 Anticonvulsants

Pregabalin is a GABA structural derivative (McClelland et al. 2004). It's binding to α2δ-1 subunit of voltage-gated calcium channels linked to the antinociceptive

activity (Taylor et al. 2007). Alpha2δ-1 is an auxiliary subunit of voltage-gated calcium channels, and pregabalin binding to this subunit is responsible for pain attenuation. When this drug binds to the alpha2δ-1 subunits, it leads to inhibition of nerve injury-induced accumulation of alpha1 pore that forms calcium channel units from the cytoplasm to the plasma membrane of sensory neurons of the dorsal root ganglion (DRG) (Kukkar et al. 2013). Gabapentin administration leads to inhibition of axoplasmic transport of alpha2δ-1 subunits from DRG to dorsal horn neurons (Kukkar et al. 2013).

20.2.3.3 Opioids

The use of opioids for the treatment of pain has increased significantly in recent years. However, long-term use can lead to abuse and hyperalgesia (Bril et al. 2011). As a result, using opioids in the DPN setting is controversial. These drugs can be used as monotherapy only in situations where other medications have failed to offer pain relief (Lindsay et al. 2010). In the spinal cord as well as in the brain, unique opioid receptors for opioid-like endogenous compounds were identified. Prescription for oxycodone and morphine sulfate is popular among other opioids (Cohen et al. 2015).

20.2.3.4 Topical Medications

Capsaicin is known to decrease the sensitivity of sensory nerves and has shown pain relief effects in DPN (Chong and Hester 2007). But it still offers some adverse effects in some patients like erythema (Zin et al. 2008). In a study, a single capsaicin formulation of 8% for patch therapy in patients with DPN provided relief from severe pain throughout 12 weeks, and no relief is seen in patients treated with a placebo patch (Simpson et al. 2017). In July 2020, the FDA approval is given to a new capsaicin formulation for the treatment of neuropathy pain in diabetes (Cohen et al. 2015). Studies to determine the most potent drugs or formulations for PDN treatment are increasingly required to optimize pain relief and to enhance the quality of life of patients.

20.2.4 Challenges With Current Treatment of Diabetic Neuropathic Pain

With recent progress in identifying pain-generating processes and adopting evidence-based treatments, patients suffering from DPN are still difficult to cure (Tölle 2010). The latest treatments do not provide adequate pain relief for about half of the patients and also offer many non-desired side effects such as somnolence and dizziness, as well as the requirement of a complex dose regimen to reduce patient compliance. Standard agents for topical administration are there for the treatment of DNP, such as capsaicin cream, without any side effects but have low efficiency, and complex multiple administration is required, which can cause discomfort, and also the chances of contamination of sensitive body areas are also there, both of which can lead to poor patient compliance (Tölle 2010). Ultimately, more understanding of

the basic pathophysiological processes that lead to this complication should make it possible to devise optimal therapies for individual patients suffering from neuropathic pain (Tölle 2010).

20.3 siRNA Nanocarrier as Plausible Therapeutic Recourse

20.3.1 siRNA

The discovery of siRNA in 1999 was guided by the sequence-influenced endonuclease-based cleavage of mRNA in mammal cells. Further, in 2001, synthesized siRNA was utilized for silencing. Henceforth, the principle and structure of siRNA were elucidated, which paved a way for future implications of RNAi in therapeutics (Dana et al. 2017). RNA interference is a natural process occurring in multicellular organisms involving the silencing of genes. The complementary RNA is degraded in this post-transcription event originating through the double-stranded RNA. siRNA possesses merely 21 nucleotide sequences which are utilized as a tool for gene silencing specifically in mammal cells facilitating specificity of interferon activity (Kurreck 2006). siRNA offers an innovative recourse to the available therapeutic alternatives. They offer a safer option as they act on the post-translational stages of DNA expression. As a result, they do not influence the genetic material directly and hence evading mutagenic risks. With its impactful efficacy, siRNA causes potent suppression of gene expression with the use of one cell and associated few copies. Another advantage is offered by the specificity of complementary bases as compared to chemical therapeutics. However, many limitations are presented in the delivery of siRNA to the targeted cell site. siRNA is very unstable under normal physiology in the blood wherein it undergoes digestion by nuclease enzymes (Subhan and Torchilin 2019). Thus, the development of drug delivery systems that can enhance site-specific delivery of siRNA therapeutics for aiding relief from disease is required.

20.3.2 siRNA Nanocarrier as Drug Delivery System

siRNA are prone to degradation by nucleases and require attention to protect it from blood enzymes. Transfection agents are needed to facilitate their movement across the membrane since siRNA possesses an overall negative charge (Zhang et al. 2018). Efficient nanocarriers should ensure evasion from immunogenic recognition and clearance through our reticuloendothelial system. Serum proteins like albumin and IgG tend to interact with siRNA cationic bodies, leading to the enhanced size of the complex. This ultimately lessens the targeted siRNA fraction that reaches the target site (Meng et al. 2013). Attaching a ligand entity like an antibody, aptamer, or peptide provides specificity to the siRNA molecule and ensures release at the desired site of action. Lipid nanoparticles possessing a positive charge attributed through cation lipidic formulations are efficient in condensing the genes and ensuring uptake

by the cell (Zhang et al. 2018). Many natural polysaccharides, namely, chitosan and hyaluronic acid, offer a biocompatible and non-immunogenic approach in formulating a siRNA delivery system. Since chitosan possesses a positive charge, it is capable of reacting with siRNA, leading to the formation of polyplex complexes which show good stability. Thus, this electrostatic interaction ensures the protection of the therapeutic siRNA from getting degraded. Hyaluronic acid is anionic and requires an additional cation to ensure electrostatic reaction with the siRNA molecule. It makes the entire complex stable and improves the targeting efficiency of siRNA therapeutic (Serrano-Sevilla et al. 2019). Solid lipid nanoparticles contain a lipid bilayer with a cation and fusogenic lipid mix that ensure uptake by the cell and endosome-based releasing of siRNA. These nanocarriers can be made stable through coatings of polyethylene glycol which are successful in providing a hydrophilic outer shell (Ozpolat et al. 2014). Because cation-based polymers are highly stable and easy to prepare, they are a preferable coating agent. Many synthetic polymers have been reported for delivery, namely, polyamino acid, polyethyleneimine, and polyamidoamine. Inorganic nanocarriers lack amino-containing functional moieties which favor siRNA delivery. These include gold nanoparticles, nanorods, and apatite nanocarriers (Meng et al. 2013).

20.3.3 Molecular Mechanisms and Biological Function of siRNA Nanocarriers in Diabetic Neuropathic Pain

Diabetic neuropathic pain involves a sharp burning and lancinating sensation which may even be perceived as a shock. This pain starts with moderate intensity and worsens during night hours which leads to alteration of the sleep cycle and even insomnia. The constant pain alters the quality of life of the patient. As a result, the patient undergoes withdrawal from social activity giving rise to depressive symptoms (Schreiber 2015). siRNA is a double-stranded RNA molecule that causes interference in the genetic expression of complementary base pairs of mRNA and results in a knockdown of expression. It contains only 20–25 nucleotide sequences situated at both ends. These ends undergo binding to cause degradation of the mRNA molecule. The genetic component involved in pathogenesis is silenced and inhibition of neuropathic progression prevails. When a siRNA molecule lies independently without conjugated associations, it is called a naked siRNA molecule (Shende and Patel 2019).

Dorsal root ganglion accepts pain signaling from peripherally situated nerves and transmits them to the central nervous system. Receptors, namely, $P2X_3$ and $P2X_7$, have been linked with the pathogenesis of central neuropathic pain. While $P2X_3$ resides majorly in the primary sensory neuron, $P2X_7$ is mainly situated in the satellite glial cells in the dorsal root ganglion. $P2X_3$ elevates nociceptive action by sensitization of pain fiber, $P2X_7$ is involved in the purinergic signal pathway of chronic pains. In a study performed on $P2X_7$ receptor-mediated diabetic neuropathy in rats, BC1 68687 siRNA intrathecal injection was successful in inhibiting the expression of the causative receptor $P2X_7$. As a result, the pathology of diabetic neuropathy was

directly influenced. BC168687 siRNA caused upregulation of glial fibrillary acidic protein on the satellite glial cells present in the dorsal root ganglion. As a result, the upregulated nitric oxide associated with the nociception of rats was also inhibited (Liu et al. 2017).

In another pain model, the siRNA showed behavior-associated inhibition in allodynia as well as hyperalgesia which was correlated with the downregulated $P2X_3$ receptor in the dorsal root ganglion and spinal cord. This effect was then attempted for reproducibility in another model for neuropathic chronic pain (Dorn et al. 2004). Microglia homing peptide molecules are promising delivery candidates for siRNA because of their potent knockdown efficacy. Interferon regulatory factor-1 is situated in the microglia. MG1 is the most commonly utilized homing peptide for the siRNA-interferon regulatory factor-1 complex. This delivery system is successful in suppressing hyperalgesia-associated spinal nerve injury in comparison with other peptide molecules or even naked siRNA. The study highlights siRNA delivery devices as a plausible therapeutic in relieving neuropathic pain (Terashima et al. 2018). Sensory neurons which show expression of calcitonin gene-related peptide situated in the trigeminal ganglion or dorsal root ganglion are known to influence nociception in afferent input of transmission. The activation of the primary afferent neuron preceded by the sensory axon reflex releases the calcitonin gene-related peptide in the spinal cord. There is an enhancement in the glutamate release in the presynaptic membrane. Activated NMDA receptors increase the entry of calcium in the cell which acts as a trigger for the intercellular calcium stores. Enhanced calcium concentration activates many protein kinases which contribute to the pathology of neuropathic pain (Lipp and Reither 2011). Inflammatory pain response prevails. siRNA delivery device alleviates neuropathic pain by inhibition of excitation transmission due to the $P2X_3$ receptor in the dorsal root ganglion as well as inhibition of expressed calcitonin gene-related peptide in the spinal cord (Xiong et al. 2017). Glial cells induce the release of various inflammatory cytokines which activate the kinase-activated cascade situated in the sensory neuron cytokine receptors. siRNA therapeutics inhibit this activation and hence eradicate the manifestation of neuropathic pain (Gonçalves dos Santos et al. 2020).

A rat chronic constriction injury model investigated the importance of TLR4 and the plausible implication of siRNA-associated inhibition through TLR4 mRNA block. On injecting siRNA-TLR4, inhibition of allodynia, hyperalgesia, TNF-α, and IL-1β was seen. Moreover, these observations were seen to be isochronous and efficient in neuropathy pain (Wu et al. 2010). In a rat model of chronic constriction injury, lentivirus containing siRNA was administered into the spinal cord through the intrathecal route. The results indicated a reduction in nociception due to the consequent inhibition of mRNA and expressed protein GluN2B. The lentiviral delivery device showed success in transfecting to the dorsal horn where the GluN2B resides and thus reducing pain (Wu et al. 2014). Figure 20.2 shows the molecular mechanism of siRNA-based nanocarriers in relieving neuropathic pain.

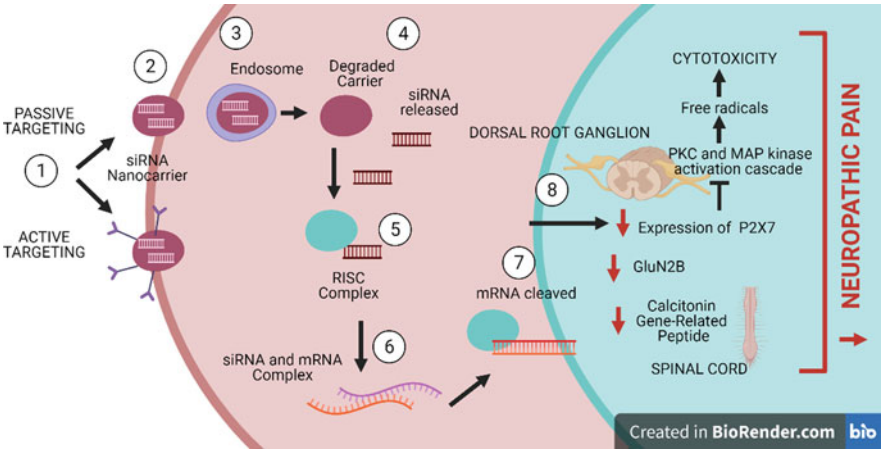

Fig. 20.2 Molecular mechanism of siRNA-based nanocarriers in relieving neuropathic pain. (1) siRNA delivery device enters the cell either through passive or active targeting. Active targeting is facilitated by the attachment of antibodies or aptamers which enhance the specificity of the device. (2) The siRNA nanocarrier then enters the cell. (3) The endosome engulfs the delivery device. (4) Consequently, the outer carrier is degraded to release free siRNA therapeutics. (5) The siRNA leads to the formation of an RNA-induced silencing complex (RISC). (6) The mRNA and siRNA interact with each other to progress the knockdown of the desired mRNA. (7) The mRNA is cleaved through the RISC to silence proteins involved in the pathology of neuropathic pain. (8) The expression of the P2X7 receptor in the dorsal root ganglion, GluN2B peptide, and the calcitonin gene-related peptide residing in the spinal cord is inhibited, all of which contribute to alleviating neuropathic pain and show promise of siRNA nanocarriers as novel therapeutics. siRNA delivery device alleviates neuropathic pain by inhibition of excitation transmission due to the P2X3 receptor in the dorsal root ganglion as well as inhibition of expressed calcitonin gene-related peptide housed in the spinal cord which alters the calcium-augmented pathways in neuropathy pain

20.4 Conclusion

Diabetic neuropathy is a well-established outcome of both types of diabetes. Typically, diabetic neuropathy affects the toes and distal foot but eventually advances to include the legs. The toxic effects of hyperglycemia are accepted to be a major factor in the creation of this complication. Usage of antidepressants, GABA analogues, opioids, and topical agents to treat pain in PDN is recommended. Currently available systemic medications provide adequate pain relief for approximately half of affected patients and are limited by unwanted adverse reactions and multiple-dose regimens. So, other treatment options need to be explored in need to treat this widespread complication of diabetes. siRNA demonstrated symptomatic relief in allodynia and hyperalgesia associated with the downregulation of the P2X3 receptor in the dorsal root ganglion and many other models. siRNA can be used as potential therapeutics to treat DPN but are limited by its unstable nature under normal physiology in the blood wherein it undergoes digestion by nuclease enzymes. So, its encapsulation in novel

drug delivery systems such as nanoparticles might be able to enhance its efficacy, and it can be utilized as a therapeutic for diabetic neuropathic pain.

20.5 Future Perspectives

While numerous formulations have recently been developed for the effective delivery of siRNA to be used as therapeutics in various disorders, the biological stability, specificity, and protection of nanocarriers, which can be easily translated from bench to bedside, should remain the priority. For siRNA delivery, lipids form the core components of several forms of nanocarriers. New therapeutic modalities may become combination therapies with multiple siRNAs targeting various survival pathways or a combination of specific siRNAs that may sensitize the treatment of diabetic neuropathic pain with other pain-relieving drugs. The future of personalized medicine will inevitably materialize with the continuing advances in molecular and next-generation omics technology as well as the interdisciplinary partnerships between geneticists, material scientists, immunologists, and biochemists. The positive effects of many undertrial RNAi therapeutics would also improve the drug industry's confidence in investing in this avenue with a special focus on various disorders including neuropathy pain.

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EGFR-Targeted Quinazoline Clubbed Heterocycles as Anticancer Agents

21

Vivek Panwar, Kritika Mukherji, Manjunath Ghate, Deepak K. Jindal, and Deepak Kumar

Abstract

Cancer is a multifactorial chronic disease cohorted with different protein kinases. Epidermal growth factor receptor (EGFR) is the ligand-gated ion channel receptor or protein kinase implicated in the regulation of various biochemical incidents occurring in cell. Although EGFR has a variety of physiological roles, its over-activation or mutational modifications at different exons result in oncogenesis. Out of 52 Food and Drug Administration (FDA)-approved EGFR inhibitors for different cancers, seven are quinazoline clubbed heterocyclic molecules. Quinazolines are the medicinally active scaffolds in which nitrogen atoms are present as a part of their heterocyclic ring system. Therefore, this chapter is focussed on the EGFR-targeted quinazoline-based heterocycles.

Keywords

Cancer · Epidermal growth factor receptor (EGFR) · Quinazolines · Heterocycles · Food and Drug Administration (FDA)

V. Panwar · K. Mukherji · D. Kumar (✉)
Department of Pharmaceutical Chemistry, School of Pharmaceutical Sciences, Shoolini University, Solan, Himachal Pradesh, India
e-mail: guptadeepak002@gmail.com

M. Ghate
Department of Pharmaceutical Chemistry, Institute of Pharmacy, Nirma University, Ahmedabad, Gujarat, India

D. K. Jindal
Department of Pharmaceutical Sciences, Guru Jambheshwar University of Science and Technology, Hisar, Haryana, India

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21.1 Introduction

EGFR stands for epidermal growth factor receptor. It was discovered in 1968 as the first member of the ErbB/HER family (Bhatia et al. 2020). Cohen was awarded the Nobel Prize in Medicine for the discovery of growth factor known as epidermal growth factor (EGF) and its receptor in 1986 (Mendelsohn 2001). EGFR is a cytomembrane ligand-gated protein channel that is prompted when specific ligands bind to the catalytic domain of the protein. The specific ligands include EGF and TGFα (Sternlicht and Sunnarborg 2008). Binding of specific ligands results in the transition of inactive monomeric form to the active homodimeric form. Upon dimerization, the congenital intracellular tyrosine kinase activity of EGFR is stimulated. This acceleration of kinase activity upshot in autophosphorylation of several tyrosine (Y) residues in the C-terminal domain. The tyrosine residues autophosphorylated were Y992, Y1045, Y1068, Y1148 and Y1173. All these events finally activate the downstream signalling responses of the cell through the SH2 domains of the phosphorylated domains.

Several signal communication streams or networks like MAPK, Akt and JNK pathways are prompted, which urges to DNA synthesis and cell escalation. The ability of the kinase domain of EGFR to cross-phosphorylate the tyrosine residue of other receptors also results in self-activation of the protein and thus may cause oncogenesis (Kumar et al. 2021; Kenney et al. 2003). As a growth factor receptor, the EGFR was the first protein to be identified in malignant cells (Carpenter et al. 1975). The recognition of EGFR as an oncogene has led to the expansion of different strategies to overcome over-activation and resistance issues. Different strategies adapted include:

1. Conjugated immune toxin: The receptor ligands or antibodies are exposed to the environment of immune toxins or recombinant molecules. Example is diphtheria toxin-human epidermal growth factor fusion protein (DAB (389) EGF), in this the toxin used is diphtheria toxin and the receptor ligand is EGF.
2. Monoclonal antibodies: Monoclonal antibodies bind to the ectodomain of EGFR that leads to the repression of downhill signalling. Cetuximab, panitumumab, necitumumab and nimotuzumab are the anti-EGFR monoclonal antibodies (Paez et al. 2004).
3. Adaptor protein inhibition: Adaptor proteins are the molecules that are essential to influence the downstream signalling and thus regulate cellular proliferation. To inhibit cellular proliferation, the adaptor proteins need to be inhibited. Cbl ubiquitin ligase, Grb-7/2 and APPL1 are the examples of EGFR-associated adaptor proteins that lead to oncogenesis (Bhatia et al. 2020).
4. Kinase inhibition: Kinases are the protein molecules that are reported to be overexpressed in different malignancies. To date, there are 52 FDA-approved kinase inhibitors. Among these, the quinazoline-based anti-EGFR kinases are gefitinib (Rawluk and Waller 2018), erlotinib, afatinib, brigatinib, icotinib and osimertinib (Yarden and Schlessinger 1987).

21.2 Quinazoline-Based EGFR Kinase Inhibitors

Quinazolines are the heterocyclic scaffolds in which nitrogen atoms are present as a part of their heterocyclic ring system. Chemically they are basic in nature and possess a wide range of medicinal properties such as anticancer (Kumar et al. 2017; Molina-Pinelo et al. 2014), antitubercular, anti-inflammatory and antimicrobial activities. The quinazoline derivatives were found to inhibit the tyrosine kinase domain of EGFR (Stuckey et al. 2015).

In the first decade of the century, gefitinib and erlotinib were discovered as anticancer drugs and were approved by the FDA in the years 2003 and 2004, respectively, for NSCLC. These molecules bind either reversibly to the intracellular catalytic domain of the receptor and competitively inhibit the binding of adenosine triphosphate. Occupation of the catalytic domain of EGFR by the inhibitors reversibly/irreversibly inhibits phosphorylation and blocks the downstream signalling and oncogenic effects associated with EGFR activation. Both drugs were quinazoline derivatives in which substitution at different anilo derivatives was done at position 4. The discovery of these compounds unfolded new and hopeful 4-anilinoquinazoline compounds in later years such as lapatinib, vandetanib, afatinib and dacomitinib. The structures of these compounds are given in Fig. 21.1. As a molecular targeting approach, EGFR is an auspicious target for cancer therapy. Substitution of amino acid in exon 21 (i.e. L858R) and an in-frame deletion in exon 19 are two typically perceived driver mutations in EGFR (Bhatia et al. 2020).

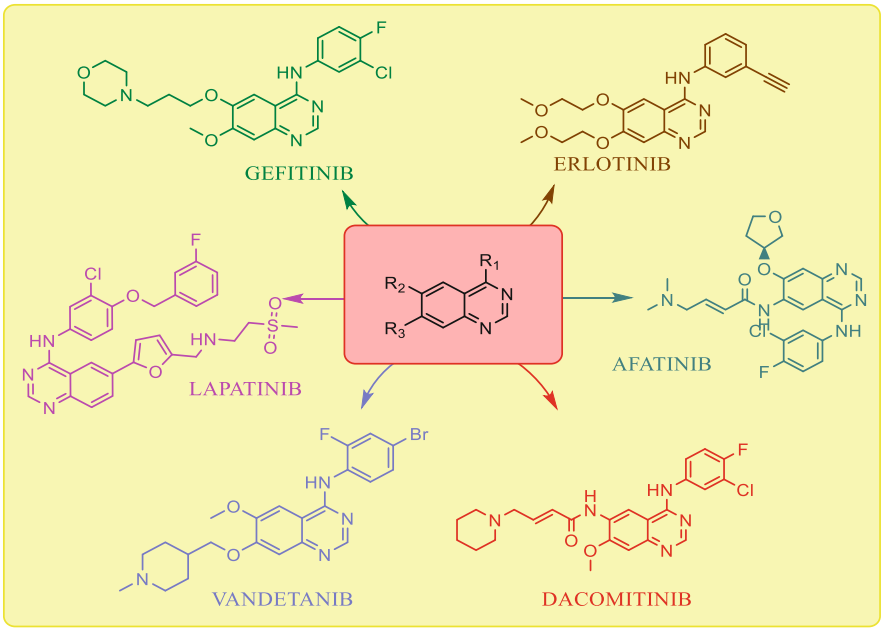
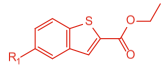
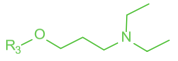
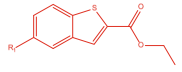
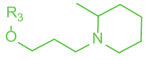
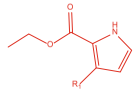
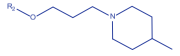
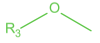
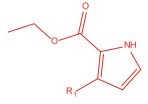
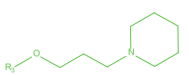
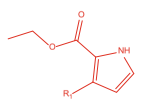
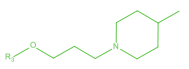
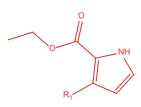
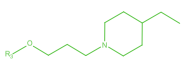
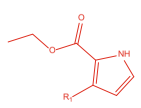
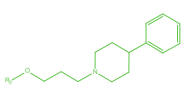
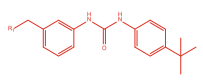
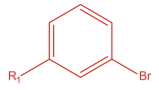
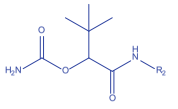
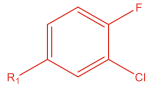
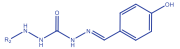
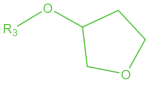
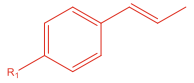
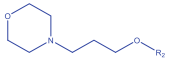
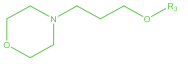
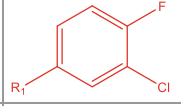
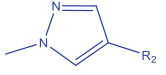
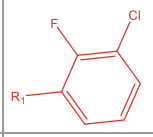
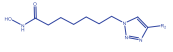
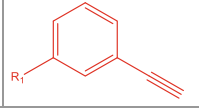
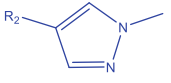


Fig. 21.1 FDA-approved quinazoline-based EGFR inhibitors as anticancer agents

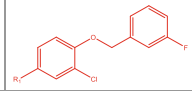
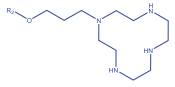
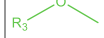
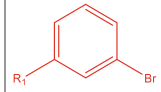
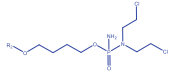
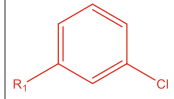
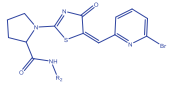
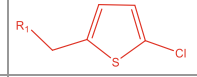
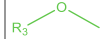
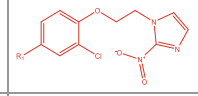
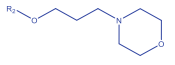
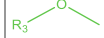
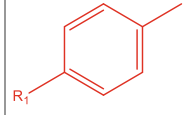
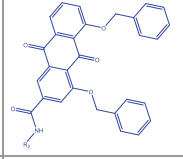
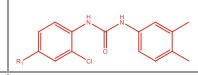
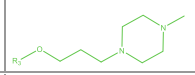
Table 21.1 Representation of the structures of 25 quinazoline-based anti-EGFR compounds

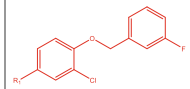
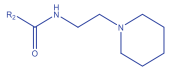
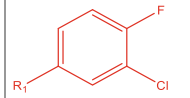
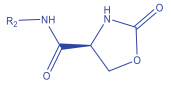
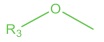
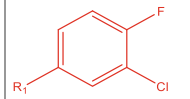
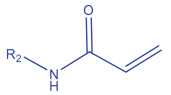
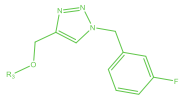
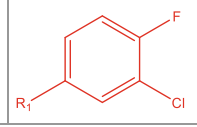
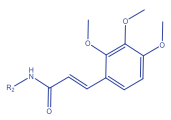
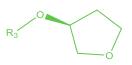
Sr. no.	Substitution at R1	Substitution at R2	Substitution at R3	Comments	References
1.				Higher selectivity and enhanced anti-EGFR activity as compared to gefitinib	Wu et al. (2010)
2.					
3.				The anticancer potential of the compounds was increased by replacing benzene ring with a pyrrole ring and when position 6 or 7 of the quinazoline nucleus was substituted or branched with basic side chain	Ahmad (2017)
4.					
5.					
6.					
7.					

8.				The morpholin-3-one-fused quinazoline derivatives have efficient binding with the c-Raf (active site)	Palumbo et al. (2016)
9.				First-generation EGFR inhibitor having six times more EGFR inhibitory potential	Zheng et al. (2017)
10.				Second-generation EGFR inhibitor with comparable anticancer activity	Tu et al. (2017)
11.				First-generation EGFR inhibitor. It is more potent than lapatinib for EGFR inhibition	Chen et al. (2017)
12.				First-generation inhibitor. Lesser potent than gefitinib and is moderately cytotoxic	Hou et al. (2016)
13.				First-generation inhibitor. Produces synergistic effect and is a potent anticancer drug	Ding et al. (2017)
14.				First-generation inhibitor. Lesser potent than gefitinib and is moderately cytotoxic	Hou et al. (2016)

(continued)

Table 21.1 (continued)

Sr. no.	Substitution at R1	Substitution at R2	Substitution at R3	Comments	References
15.				Second-generation inhibitor. Better activity as compared to gefitinib as well as lapatinib	Ju et al. (2018)
16.				Promising EGFR inhibitory activity with non-specific DNA damage	Lin et al. (2017)
17.				First-generation inhibitor having better potency but lesser activity as compared to gefitinib	Li et al. (2015)
18.				First-generation inhibitor having poor inhibitory activity but good anticancer activity	Zou et al. (2019)
19.				First-generation inhibitor having lesser potency than lapatinib but better anti-proliferative activity	Cheng et al. (2019)
20.				First-generation inhibitor having remarkable anti-proliferative activity	Liang et al. (2020)
21.				First-generation dual inhibitor for EGFR/VEGFR-2. More potency than vandetanib	Zhang et al. (2017)

22.				Third-generation inhibitor having higher potency in resistant cell lines than gefitinib	Mowafy et al. (2016)
23.				First-generation inhibitor and also inhibits mutated cell lines reversibly and non-selectively	Shao et al. (2016)
24.				Third-generation inhibitor. Highly selective activity for mutated strains of EGFR as compared to wild type	Song et al. (2019)
25.				Second-generation inhibitor. Lower potency but better activity as compared to afatinib	Tu et al. (2016)

However, till 2010, there was no defined approach to combat EGFR resistance. Therefore, steady and extended efforts were being undertaken for designing and developing more potent and selective EGFR inhibitors. X Wu et al. designed and synthesized two series of 4-benzothienyl amino quinazoline derivatives as new analogues of gefitinib in 2010. A series of compounds were synthesized and evaluated in comparison to parental gefitinib and the compounds with higher selectivity and enhanced anti-EGFR activities were selected. The selected compounds were compound 1 and compound 2 and are given in Table 21.1 (Wu et al. 2010; Ravez et al. 2015). Different series of quinazoline-based compounds were designed using gefitinib as a reference. In the designed series, benzene ring was substituted with a pyrrole ring. Taking gefitinib as a standard, the synthesized compounds were evaluated for kinase inhibitory and antitumour activities. Among a series of compounds, five compounds were significantly potent. These five compounds are given in Table 21.1 as compounds 3–7. The structure-activity analysis proposed that the anticancer activity of the compounds was raised by replacing benzene ring attached to the 4-anilo nitrogen. Also, the anticancer potential of the compounds was increased when position 6 or 7 was substituted or branched with basic side chain (Ahmad 2017; Bhatia et al. 2020).

A novel series of quinazoline-based compounds was designed by X Qin et al. in 2016. The morpholin-3-one-fused quinazolines were synthesized by intramolecular cyclization, and their anti-EGFR potential was evaluated. A compound with a tert-butyl substituent on the lateral phenyl ring, a dimethoxyquinazolinyl moiety at positions 6 and 7 and a benzylamino linker at position 3 showed the maximum activity. Additionally, in silico studies revealed that the compound also had efficient binding with the c-Raf (active site). The structural details of the compound are given in Table 21.1 as compound 8 (Palumbo et al. 2016).

21.3 FDA-Approved Quinazoline-Based EGFR Inhibitors

21.3.1 Gefitinib

Gefitinib is a quinazoline-based small molecule EGFR inhibitor. It was approved by the FDA in 2003 for the treatment of NSCLC. It was the first FDA-approved quinazoline-based EGFR inhibitor. Gefitinib acts by binding to the active conformation of EGFR. The chemical structure of the drug is given in Fig. 21.2. Gefitinib is administered through oral route and is absorbed with a mean bioavailability of 60%. The volume of distribution of the drug is very high (1400:1) and thus is distributed through the body tissues such as the kidney, liver, lungs and tumours. The drug reaches to its peak level from 3 to 7 h with a mean elimination half-life of 48 h. After getting absorbed in the blood, 90% of the drug binds to the serum albumin and α1-acid glycoproteins. CYP3A4 is involved in the hepatic metabolism of the drug. This enzyme biotransforms the drug by demethylating the methoxy substituent, metabolizing the *N*-propoxymorpholino group and by oxidative defluorination of

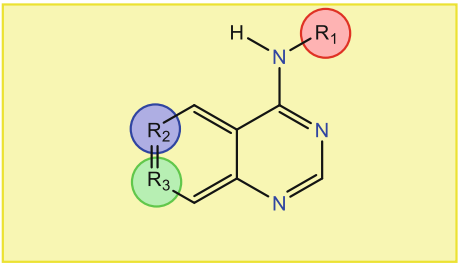


Fig. 21.2 Quinazoline-based nucleus as a parent moiety substituted at R1, R2 and R3

the halogenated phenyl group. About 86% of the drug is removed via faeces whereas 4–5% is metabolized via renal route (Rawluk and Waller 2018).

21.3.2 Erlotinib

Erlotinib is a quinazoline-based small molecule EGFR inhibitor. It was approved by the FDA in 2004 for the treatment of pancreatic and NSCLC. Erlotinib acts by binding to the active conformation of EGFR. The chemical structure of the drug is given in Fig. 21.2. The drug is administered through oral route with 60% oral bioavailability. After about 4 h, the drug reaches to its peak plasma concentration with an apparent volume of distribution of 232 L. After reaching to systemic circulation, 92–94% of the drug gets bound to the albumin and glycoprotein. CYP3A4 is the hepatic enzyme involved in the biotransformation of the drug. After biotransformation, 80–82% of the drug is eliminated through faeces and 8–10% of the drug is eliminated via renal route (Abdelgalil et al. 2019).

21.3.3 Lapatinib

Lapatinib is a quinazoline-based small molecule EGFR inhibitor. It was approved by the FDA in 2007 for the treatment of HER2-positive breast cancer and lung cancer. The chemical structure of the drug is given in Fig. 21.2. Gefitinib and erlotinib act by binding to the active site or conformation of the EGFR, lapatinib acts by binding the inactive conformation of the receptor. Lapatinib is a dual inhibitor and inhibits two oncogenes, HER2 and EGFR. The drug is given through oral route with varying rates of intestinal reabsorption. After 3–6 h of administration, the drug reaches to its peak plasma concentration. The drug attains the protein binding of >99% after reaching to systemic circulation. Biotransformation of the drug is done by the hepatic enzyme CYP3A4. About 14% of the drug is eliminated by anal route and 10% via urine (Voigtlaender et al. 2018).

21.3.4 Vandetanib

Vandetanib is a quinazoline-based small molecule EGFR inhibitors. It was approved by the FDA in 2011 for the treatment of medullary thyroid cancer. The chemical structure of the drug is given in Fig. 21.2. The drug is administered through oral route and the peak plasma concentration is achieved after 6 h of ingestion. The volume of distribution of the drug is about 7450 L. The drug attains the protein binding of about 90% after reaching to systemic circulation. The drug is metabolized by the hepatic enzyme CYP3A4 and FMO1/3 into *N*-desmethyl vandetanib and vandetanib N-oxide, respectively. About 44% of the drug is eliminated via faeces and 25% via urine (Karras et al. 2014).

21.3.5 Afatinib

Afatinib is a quinazoline-based small molecule EGFR inhibitor. It was approved by the FDA in 2013 for the treatment of NSCLC. The chemical structure of the drug is given in Fig. 21.2. The drug acts by binding irreversibly or covalently with the catalytic domain of the receptor. The drug is administered through oral route and reaches to peak plasma concentration after 2–5 h of administration. The volume of distribution of the drug is 4500 L. The drug attains the protein binding of about 95% after reaching to systemic circulation. The drug is metabolized by the enzyme-catalysed process to a negligible extent, and the major circulating enzymes are the covalent adducts. About 86% of the drug is eliminated via faeces and 5% via urine (Wecker and Waller 2018).

21.3.6 Dacomitinib

Dacomitinib is a quinazoline-based small molecule EGFR inhibitor. It was approved by the FDA in 2018 for the treatment of EGFR-mutated NSCLC. The chemical structure of the drug is given in Fig. 21.2. The drug acts by binding covalently to the catalytic domain of the kinases. It is the selective and irreversible inhibitor of EGFR. The drug is administered through oral route and reaches to peak plasma concentration after 5–6 h of administration. The volume of distribution of the drug is 2415 L. The drug attains the protein binding of about 98% after reaching to systemic circulation. The drug is metabolized by the enzyme-catalysed process to a negligible extent, and the major circulating enzymes are the covalent adducts. About 79% of the drug is eliminated via faeces and 3% via urine (Shirley 2018).

21.4 Conclusions

EGFR is a ligand-gated protein channel or biomolecule having a variety of cellular functions in maintaining physiological processes. Alterations in the level of growth factors or mutational modifications in the protein end up with many pathological states including cancer. Although different EGFR inhibitors are available, quinazoline-based anti-EGFR agents are of prime importance in current scenario. Quinazoline is a medicinally active scaffold having a broad spectrum of pharmacological potential. Gefitinib and erlotinib are the first two quinazoline-based anti-EGFR agents approved by the FDA in the years 2003 and 2004, respectively. After the discovery of gefitinib and erlotinib as effective anti-EGFR agents, quinazoline-based molecules or derivatives have attained a great interest in the field of anticancer drug development. With steady efforts, four more quinazoline-based anti-EGFR drugs were approved in the past few years. So till date, among 52 FDA-approved molecules, 6 of them are quinazoline-based anti-EGFR inhibitors. Many quinazoline-based novel and effective EGFR inhibitors designed and synthesized in the past few years with a good inhibitory potential are represented in Table 21.1. So, in this chapter, the different strategies to inhibit overexpressed or altered EGFR, different compounds with good inhibitory potential developed in the past few years and FDA-approved quinazoline-based anti-EGFR molecules are discussed in brief.

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Therapeutic Human Monoclonal Antibodies 22

Satish Kumar Gupta and Piyush Chaudhary

Abstract

Since the discovery of obtaining mouse monoclonal antibodies (MAbs) in 1975 by somatic cell hybridization, there have been rapid developments to use antibodies as therapeutics. To minimize human anti-murine antibody immune response, initially mouse-human chimeric antibodies (constant region of mouse MAb replaced with human antibody) have been developed. Subsequently using recombinant DNA technologies, humanized antibodies wherein only the complementarity-determining regions of the mouse MAb have been grafted onto the human antibody backbone followed by the development of fully human MAbs from phage-display technology, humanized mouse, or single-B cell polymerase chain reaction from immunized/infected human subjects have also been generated. Based on clinical applications, various formats of human antibodies such as single-chain variable fragment (scFv), bispecific antibody, antibody-drug conjugate, fragment antigen-binding (Fab), etc. have been developed. As of 2018, 64 antibodies have been approved by the US Food and Drug Administration for clinical use. The majority of these antibodies are used for the treatment of cancers, transplant rejection, rheumatoid arthritis, Crohn's disease, psoriasis, viral infections, macular degeneration, anthrax, etc. In future, it is anticipated that therapeutic antibodies will be developed against other diseases and their use increases substantially and will constitute as one of the major portfolios of the pharmaceutical/biotech industries.

S. K. Gupta (✉) · P. Chaudhary
Present address: Basic Medical Sciences Division, Indian Council of Medical Research, V. Ramalingaswami Bhawan, Ansari Nagar, New Delhi, India
e-mail: skgupta@nii.ac.in

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Keywords

Mouse monoclonal antibody · Chimeric antibody · Humanized antibody · Human antibody · Therapeutic applications of monoclonal antibody

22.1 Introduction

Production of monoclonal antibodies (MAbs) has evolved since the first demonstration by Köhler and Milstein at Medical Research Council (MRC), Cambridge, UK, about the feasibility to obtain hybrid cell clones capable of growing in culture and secreting antibodies of predefined specificity by somatic cell hybridization (Köhler and Milstein 1975). For this discovery, Cesar Milstein and George J. F. Köhler won the Noble Prize in Physiology or Medicine in 1984 along with Niels K. Jerne. The MRC, UK, awarded its first Millennium Medal to Cesar Milstein in 2000 in recognition of his groundbreaking work, and its Chief Executive Professor Sir George Radda commented that "the discovery of monoclonal antibodies revolutionized biomedical research and sparked an international billion-pound biotechnology industry. No other MRC scientist has made such an outstanding contribution to Britain's science, health and wealth creation" (*The Times*, London, 26 March 2002, Obituaries-Cesar Milstein). MAbs are homogeneous in nature and can be produced in large amounts. Due to the high specificity of MAbs, these are useful in targeting tumor cells and microbial pathogens. These can be used as either stand-alone or adjunct therapy in conjunction with the conventional therapy. In cancer patients as compared to radiation and chemotherapy, immunotherapy by MAbs may have less side effects. Furthermore, therapeutic MAbs are also useful for immunocompromised hosts, elderly persons, and pregnant women. The first murine MAb OKT3™ that recognizes a nonpolymorphic subunit of human T cell receptor, CD3, was approved by the US Food and Drug Administration (FDA) for use in human subjects to prevent rejection of renal allografts (Chatenoud 2003). In addition, mouse MAb generated against CD20 on B cell was also used in patients suffering from non-Hodgkin's lymphoma. Though murine antibodies have exquisite specificity for therapeutic targets, they do not always trigger appropriate human effector functions of complement and Fc receptor. Further, murine antibodies are recognized as foreign by the human immune system evoking human anti-murine antibody immune response, thus cutting short their therapeutic window (Shawler et al. 1985). However, limitations in the use of murine MAbs for therapeutic applications can be overcome by using newer protein engineering and molecular biology tools and advances in cell biology to develop more human-like MAbs with lesser immunogenicity. Evolution of the therapeutic MAbs is schematically shown in Fig. 22.1. In this chapter, we will briefly describe the strategies to produce chimeric, humanized, and human MAbs and their therapeutic applications. In addition, novel MAb variants and their potential application will also be discussed. The current and forecasted market of therapeutic MAbs will also be presented.

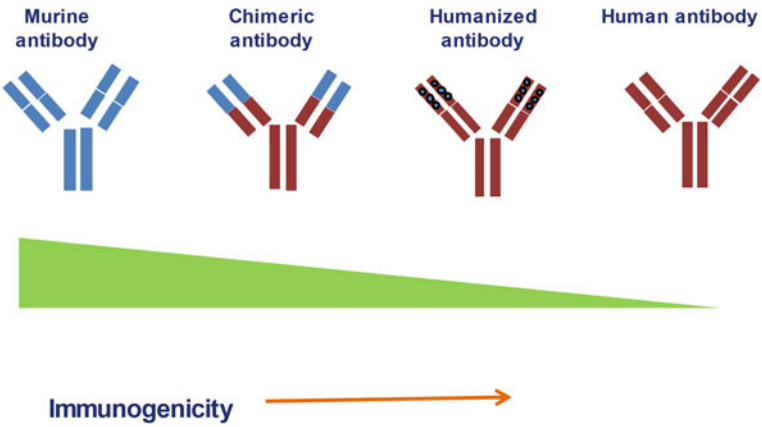

Fig. 22.1 Schematic diagram to show the progression of therapeutic antibodies: Initially murine MAbs have been proposed for therapeutic application in humans. Repeated use of murine antibodies in humans leads to the generation of the human anti-mouse antibodies. To reduce the immunogenicity of mouse antibodies, subsequently chimeric, humanized, and finally full human antibodies have been developed for their clinical use in humans

22.2 Mouse-Human Chimeric MAbs

Chimeric antibodies are made by genetic engineering that involves grafting of the variable domains of light and heavy chains of the murine MAb to human constant domains of light and heavy chains (Morrison et al. 1984; Fig. 22.1). It took a decade for the first chimeric MAb, abciximab for homeostasis, to be approved by the FDA in 1994 (Faulds and Sorkin 1994). Abciximab is made from the Fab fragments of an immunoglobulin that targets the glycoprotein IIb/IIIa receptor on the platelet membrane (Table 22.1). It is a platelet aggregation inhibitor and mainly used during and after coronary artery procedures like angioplasty to prevent platelets from sticking together and causing thrombus formation within the coronary artery. Rituximab, another chimeric MAb against CD20, was developed to treat non-Hodgkin's lymphoma, chronic lymphocytic leukemia, transplant rejection, and rheumatoid arthritis (Table 22.1). It destroys both normal and malignant B cells that have CD20 on their surface. Cetuximab is an epidermal growth factor receptor (EGFR) inhibitor, given by intravenous infusion for the treatment of metastatic colorectal and head and neck cancer. Table 22.1 also summarizes additional chimeric MAbs that have been approved by the FDA for clinical applications.

22.3 Humanized MAbs

To further reduce the murine content of mouse-human chimeric antibodies, only complementarity-determining regions (CDRs) have been grafted onto the human framework regions (FR) giving rise to "humanized" antibodies (Kettleborough et al.

Table 22.1 Partial list of chimeric MAbs approved for therapeutic applications in humans

Product	Trade name	Indication	Target	Company (FDA approval)
Abciximab	ReoPro®	High-risk angioplasty (prevention of blood clots)	Glycoprotein IIb/IIIa receptor on platelet	Centocor Ortho Biotech (Johnson & Johnson), Eli Lilly (1994)
Rituximab	Rituxan®	Non-Hodgkin's lymphoma, chronic lymphocytic leukemia, rheumatoid arthritis	CD20	Genentech (Roche) (1997)
Basiliximab	Simulect®	Transplant rejection	CD25 (a chain of IL-2 receptor)	Novartis (1998)
Infliximab	Remicade®	Rheumatoid arthritis, Crohn's disease, psoriasis, ulcerative colitis	TNF-α	Centocor Ortho Biotech (Johnson & Johnson) (1998)
Cetuximab	Erbitux®	Colorectal cancer, head and neck cancer	EGFR	Merck Serono/ Bristol Myers Squibb/ImClone (Eli Lilly) (2004)
Siltuximab	Sylvant®	Giant lymph node hyperplasia	cCLB8	Janssen-Cilag International (2014)
Obiltoxaximab	Anthim®	Anthrax infection	PA component of *B. anthracis* toxin	Elusys Therapeutics, Inc. (2016)
Infliximab	Inflectra®	Ankylosing spondylitis, rheumatoid arthritis, ulcerative colitis, psoriatic arthritis, Crohn's disease, psoriasis	TNF-α	Hospira UK Limited (2016)

1991; Fig. 22.1). The basic design issues involved in the CDR-grafting methodology are (1) defining CDRs of the donor antibody that needs to be grafted, (2) source of human light- and heavy-chain sequences to be used for CDR grafting, and (3) careful selection of the residues outside CDRs that are critical for antibody specificity and binding to the target antigen and their back mutation in human sequence to restore or improve the affinity of the humanized antibody. The experimental structure of the murine antibody in complex with the antigen can provide a detailed map of amino acid residues in contact with the antigen and thus facilitate determining the regions to be grafted. However, at the beginning of the humanization protocol, only on rare occasions the experimental structure of the antibody is known. In the absence of such precise definition of antibody residues responsible for specificity, CDRs have been

employed as regions determining the specificity (Riechmann et al. 1988; Foote and Winter 1992). CDRs have been identified as the regions with highest variability values in multiple alignment of antibody sequences as per Kabat definition (Wu and Kabat 1970). Another definition for regions determining specificity based on the structure of antibody has been used (Chothia et al. 1989). The advantage of using the latter definition is that the CDRs are shorter; therefore, the humanized antibody will have less xenogeneic component. However, the use of Kabat definition generally leads to less iteration in the humanization design (Presta et al. 1993).

Either mature or germline gene sequences of human antibody are used for grafting the identified CDRs of the mouse antibody. Mature sequences carry somatic mutations which are not under species selection, resulting in potential immunogenic residues. Thus, human germline genes have increasingly been utilized as source of FR donors (Neuberger and Milstein 1995; Tan et al. 2002; Hwang et al. 2005). There are certain advantages in using germline gene sequences as human FR acceptor. Primary advantage can be attributed to the fact that due to the absence of somatic mutation, it may be less immunogenic. Further, the physical maps of the germline human H and L chain loci and the functional germline gene repertoire they encode have been thoroughly characterized. In addition, the use of human germline genes encoding light and heavy chains has more plasticity to accommodate diverse CDRs with fewer back mutations (Wedemayer et al. 1997; Zimmermann et al. 2006). In fact, "superhumanization" protocol takes into account the homology of CDRs of nonhuman and germline human template regardless of FR homology (Tan et al. 2002).

In general, the affinity of humanized antibody decreases after CDR grafting as a consequence of incompatibilities between nonhuman CDRs and human FRs. Hence, it is critical to identify amino acid residues that must be retained during grafting of CDRs to prevent affinity loss of the humanized antibody. There are some residues underlying the CDRs in variable part of both light and heavy chains of immunoglobulins that are responsible for stabilizing the hypervariable loop structure. Since these residues fine-tune the antibody affinity, this region is designated as vernier zone (Foote and Winter 1992; Makabe et al. 2008). Another important class of residues is interchain packing residues that lie at the interface between variable light and heavy chains (Chothia et al. 1985, 1989). Further, canonical residues and presence of additional unusual residues close to the antigen binding site should also be retained during humanization of the antibody (Shearman et al. 1991; Graziano et al. 1995). The first humanized Mab Daclizumab for kidney transplant rejection was approved for clinical use by the FDA in 1997 (Vincenti et al. 1998; Table 22.2). After this, till 2017, several humanized antibodies got license for clinical use for a variety of disorders that has been summarized in Table 22.2. For example, bevacizumab (Avastin®) inhibits angiogenesis by neutralizing vascular endothelial growth factor (VEGF). It has been licensed to treat various cancers including colorectal, lung, breast, and glioblastoma. Another humanized IgG1 neutralizing MAb, palivizumab (Synagis®), that binds to the fusion protein of respiratory syncytial virus (RSV) inhibits the virus entry into the cell. It has been used to prevent RSV infection in infants.

Table 22.2 Partial list of humanized MAbs approved for therapeutic applications in humans

Product	Trade name	Indication	Target	Company (FDA approval)
Necitumumab	Daclizumab	Transplantation rejection	CD25 (a chain of IL-2 receptor)	Roche (1997)
Palivizumab	Synagis®	Respiratory syncytial virus (RSV)	Fusion protein of RSV	MedImmune, Abbott (1998)
Trastuzumab	Herceptin®	Breast cancer, metastatic gastric or gastroesophageal junction adenocarcinoma	HER2	Genentech (Roche) (1998)
Alemtuzumab	Campath®	B cell chronic lymphocytic leukemia	CD52	Millennium Pharmaceuticals and Genzyme (2001)
Efalizumab	Raptiva®	Psoriasis	CD11a	Merck Serono, Genentech (Roche) (2003)
Omalizumab	Xolair®	Asthma	IgE	Genentech (Roche), Novartis (2003)
Bevacizumab	Avastin®	Metastatic colorectal cancer; non-small cell lung cancer, metastatic breast cancer	VEGF	Genentech (Roche) (2004)
Natalizumab	Tysabri®	Multiple sclerosis, Crohn's disease	VLA-4	Biogen Idec and Elan (2004)
Catumaxomab	Proxinium®	Head and neck cancer	EpCAM	Viventia (Eleven Biotherapeutics) (2005)
Eculizumab	Soliris®	Paroxysmal nocturnal hemoglobinuria	Complement C5	Alexion Pharmaceuticals (2007)
Tocilizumab	RoActemra®	Rheumatoid arthritis	IL-6 receptor	Chugai (Roche) (2010)
Pertuzumab	Perjeta®	Breast cancer	HER2	Roche (2012)
Obinutuzumab	Gazyvaro®	CLL	CD20	Roche (2013)
Trastuzumab emtansine	Kadcyla®	Breast cancer	HER2	Roche (2013)
Alemtuzumab	Lemtrada®	Multiple sclerosis	CD52	Sanofi (2014)
Vedolizumab	Entyvio®	Ulcerative colitis, Crohn's disease	Integrin-α 4β7	Takeda Pharma (2014)
Ocrelizumab	Ocrevus™	Multiple sclerosis	CD20	Genentech (Roche) (2017)

22.4 Human MAbs

Taking a clue from production of mouse MAbs, initially to produce human MAbs, various investigators made attempts to fuse human B cell with mouse myeloma cells. However, mouse-human interspecies hybridomas preferentially segregate human chromosomes. However, loss of human chromosome from mouse-human hybrids is not random as human chromosomes 14 (encoding heavy chain) and 22 (encoding λ-light chain) are preferentially retained. However, chromosome 2 encoding κ-light chain is preferentially lost. In parallel, attempts have been made to develop suitable human plasmacytoma and lymphoblastoid cell lines as fusion partner with human B cell. Lymphocytes isolated from peripheral blood, bone marrow, spleen, tonsil, or lymph node have been used for fusion. The isolated lymphocytes are stimulated in vitro with pokeweed mitogen (PWM) or antigen or a combination of both. Alternatively, Epstein-Bar virus (EBV) has also been used to transform human B cell. EBV transformed human B cells divide, which can be grown in-vitro to produce human antibodies. The main limitation of the EBV-transformed human B cell clones is low amount of antibody produced and relative instability of these clones. The relative instability and low amount of antibody produced by EBV-transformed cell line can be overcome by their fusion with human plasmacytomas/lymphoblastoid cell line. However, MAbs produced by using EBV-transformed human B cells have safety concerns due to the possibility of minor contaminant of EBV nucleic acid in the antibody formulation. Primarily, three different approaches are being used currently to produce therapeutic human MAbs, which are briefly described below:

22.4.1 Production of Human MAbs Using Transgenic Humanized Mice

Basically, this approach uses transgenic mice expressing heavy and light chains of human instead of mouse antibody. In this direction, to begin with, transgenic mouse lines have been developed in which the endogenous mouse heavy- and κ-light-chain genes are inactivated and human transgenes encoding the heavy chain and κ-light chain are introduced (Lonberg et al. 1994; Green et al. 1994). However, in these transgenic mice, endogenous λ-light-chain locus of mouse has not been inactivated. However, both IgG (Lonberg et al. 1994) and IgM (Green et al. 1994) human MAbs recognizing specific target antigens have been produced using these transgenic mice. In these transgenic mice, VDJ joining and somatic mutations as a function of antibody class switch and their affinity maturation have also been documented (Taylor et al. 1994). Subsequently, double "trans-chromosomic" mice have been developed harboring human chromosome 2 and 14 fragments encompassing Ig heavy chain locus and kappa light chain locus, whose endogenous IgH and Igkappa loci were inactivated (Tomizuka et al. 2000). These mice were capable of producing every subtype of fully human immunoglobulin, and on active immunization, antibody affinity maturation was also observed. Most of the transgenic mouse-derived human MAbs have high binding affinity, which is comparable to mouse MAbs

Table 22.3 Partial list of therapeutic human MAbs produced by using transgenic mice

Product	Trade name	Indication	Target	Company (FDA approval)
Panitumumab	Vectibix™	Metastatic colorectal cancer	EGFR	Amgen (2006)
Canakinumab	Ilaris™	Cryopyrin-associated periodic syndromes including familial cold, autoinflammatory syndrome, and Muckle-Wells syndrome	IL-1β	Novartis (2009)
Golimumab	Simponi™	Rheumatoid and psoriatic arthritis, active ankylosing spondylitis	TNF-α	Centocor, Ortho Biotech (Johnson & Johnson) (2009)
Ustekinumab	Stelara™	Plaque psoriasis	IL-12/ IL-23	Centocor, Ortho Biotech (Johnson & Johnson) (2009)
Ofatumumab	Arzerra™	Chronic lymphocytic leukemia	CD20	Genmab and GSK (2009)
Denosumab	Prolia™	Treatment of postmenopausal osteoporosis	RANKL	Amgen (2010)
Ipilimumab	Yervoy™	Melanoma	CTLA-4/ CD152	BMS (2011)

derived by immunization of non-transgenic mice. Panitumumab (Vectibix™) against EGFR was the first human MAb obtained using transgenic mice that got FDA approval in 2006 for clinical use in colorectal, non-small cell lung cancer, and renal cell carcinoma (Chua and Cunningham 2006). Table 22.3 lists some of the human therapeutic MAbs produced using transgenic mice.

22.4.2 Phage-Display-Derived Therapeutic Human Antibodies

Transgenic humanized mice led to successful development of several therapeutic human MAbs; however, to generate antibodies against toxin and unstable antigens required alternate technology. In vitro selection technologies such as antibody phage display do not depend on the in vivo immune response and thus can be used to make antibodies to almost every type of antigen. This approach is based on the groundbreaking work of George P. Smith who demonstrated the feasibility of expression of peptides on filamentous *E. coli* phage M13 (Smith 1985). Antibody phage-display technology has been further facilitated by the discovery of small recombinant antibody formats such as single-chain variable fragment (scFv) and their ability for periplasmic expression and secretion in *E. coli* (Bird et al. 1988; Skerra and Pluckthun 1988). Subsequently, phage-display antibody libraries have been developed expressing either scFv (Vaughan et al. 1996) or fragment antigen-binding (Fab)

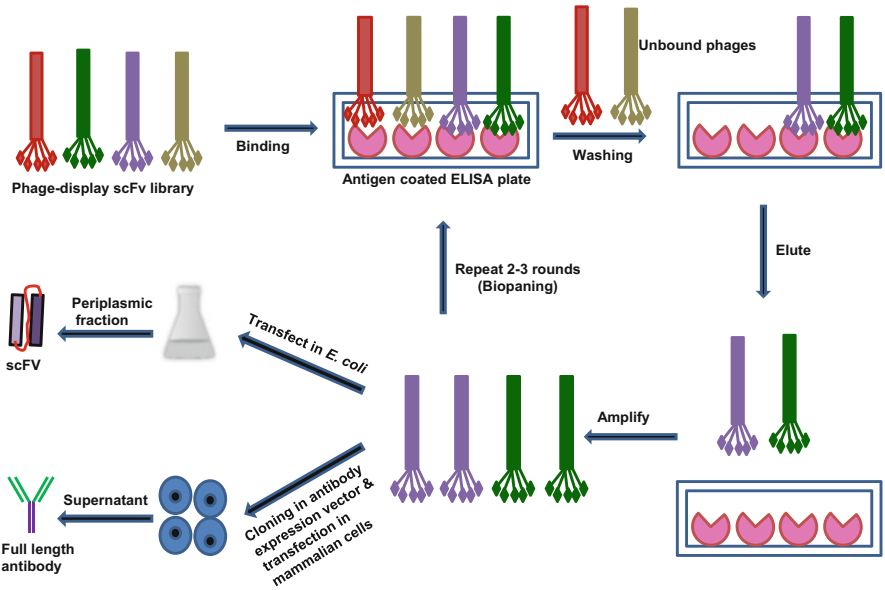

Fig. 22.2 Outline of the procedure to generate human antibodies by using phage-display library: The phage-display scFv library is incubated with antigen-coated microtitration plate, followed by washing to remove the unbound phages. The antigen-binding phages are eluted, followed by their amplification, and this process called biopanning is repeated two to three times using increasing stringent conditions for washing to enrich phages expressing antigen-specific antibodies. The specific antibody-expressing phages are amplified and the target antibody could be expressed either in *E. coli* or the specific antibody variable regions excised, cloned into whole antibody expression vectors and antibody expressed in mammalian cells

(de Haard et al. 1999). Phage-display antibody libraries have been constructed by using blood samples from immunized/vaccinated human subjects or those suffering from infections or other diseases. In addition, naive human antibody gene repertoires, which are close to the human germline with low risk of immunogenicity, have also been developed. Further, synthetic human antibody phage-display libraries have also been constructed. From the phage-display library, antigen-specific binders are selected by "panning" using antigen-coated solid surface such as ELISA plates. Antigen-nonbinding phages are removed by stringent washing. Subsequently, the bound specific antibody-expressing phages are eluted and re-amplified by transfection of *E. coli* and packaging with helper phage, which are again used for next round of panning under higher stringent conditions of washing. Usually, two to three rounds of panning are required to enrich specific antibody-expressing phage. The selected antibodies can be either expressed as soluble proteins in the bacterial expression system or the specific antibody variable regions can be excised and cloned into whole human antibody expression vector and antibody can be expressed using a mammalian expression system. Figure 22.2 schematically depicts the outline of the procedure to make human antibody by phage-display library. Adalimumab

Table 22.4 Partial list of therapeutic human MAbs produced by using phage-display technology

Product	Trade name	Indication	Target	Company (FDA approval)
Adalimumab	Humira™	Rheumatoid arthritis	Tumor necrosis factor (TNF)	Abbott (2002)
Ranibizumab	Lucentis™	Macular degeneration	Vascular endothelial growth factor A (VEGFA)	Genentech (2006)
Belimumab	Benlysta™	Systemic lupus erythematosus (SLE)	B lymphocyte stimulator (BLyS)	GSK (2011)
Raxibacumab	Abthrax™	Anthrax	Protective antigen (PA)	GSK (2012)
Ramucirumab	Cyramza™	Gastric, colorectal, and non-small cell lung cancers	Vascular endothelial growth factor receptor 2 (VEGFR2)	ImClone/ Lilly (2014)
Necitumumab	Portrazza™	Squamous non-small cell lung cancer	Epidermal growth factor receptor (EGFR)	ImClone/ Lilly (2015)

(trade name Humira™) generated against tumor necrosis factor (TNF) was the first phage-display-derived MAb approved by the FDA for the treatment of moderate-to-severe forms of rheumatoid arthritis (Weinblatt et al. 2003). Subsequently, it has also been approved in various countries for the treatment of polyarticular juvenile idiopathic arthritis in children, psoriasis and psoriatic arthritis, pediatric and adult Crohn's disease, ulcerative colitis, etc. Belimumab (marketed as Benlysta™) generated against B lymphocyte stimulator (BLyS) has been approved by the FDA for the treatment of systemic lupus erythematosus (SLE) (Navarra et al. 2011). Table 22.4 lists some other therapeutic antibodies that have been obtained by using phage-display technology and approved by the US FDA.

22.4.3 Single-B Cell Polymerase Chain Reaction-Derived Therapeutic Human Antibodies

Rapid development of immunotherapies for newly emerging pathogens such as influenza virus, Ebola virus, and lately COVID-19, or analysis of antibody repertoires, depends on highly sophisticated technology platforms such as single-B cell sorting. In acute cases, it is important to utilize the most rapid direct method for the isolation of potent human antibodies to generate therapeutic molecules for clinical applications—even within a few weeks. Immunological response against a pathogen is enforced by the cellular (CD4 and CD8 T cells) and humoral (B cells) immune components. Antibody-secreting cells (ASCs) and memory B cells (MBCs) constitute the primary cellular components of T cell-dependent antibody response to a variety of viral pathogens. Upon re-exposure to virus, activated MBCs rapidly differentiate into ASCs that produce high-affinity antibodies and in the steady state

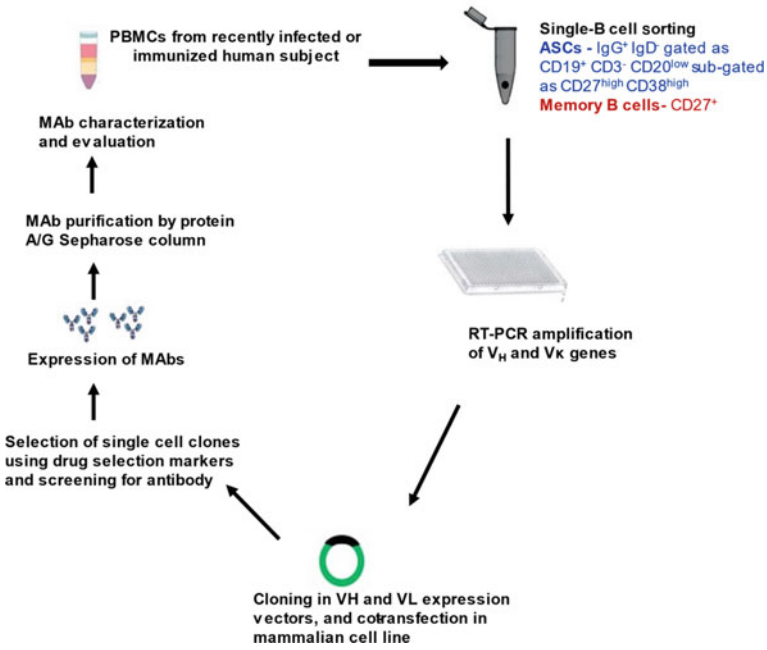

Fig. 22.3 Outline of the procedure to generate fully human antibody using single-B cell PCR: Peripheral blood mononuclear cells (PBMCs) are isolated from the blood of either naturally infected or immunized human subjects, followed by purification of single antibody-secreting cells (ASCs)/memory B cells by flow cytometer into 96-well PCR plates. Subsequently, light and heavy chains of the antibody from single cells are amplified by RT-PCR using sense primers specific for the leader region of heavy and light chains and antisense primers to the Cγ constant regions for heavy chains and Cκ for the light chain. The amplified light and heavy chain fragments are cloned into VH and VL expression vectors. The mammalian cells are co-transfected with the VH and VL expression vectors. Single-cell clones are isolated by using appropriate drugs as selection markers and antibody-secreting clones are identified by high-throughput screening assays, followed by antibody expression, purification, and characterization

could also replenish long-lived ASCs throughout life. Virus-specific ASCs transiently circulate in the peripheral tissues at the acute phase of an infection and survive in the bone marrow from months to years.

The outline of various steps involved in the production of human antibodies using single-cell PCR is summarized in Fig. 22.3. In brief, it involves isolation of peripheral blood mononuclear cells (PBMCs) from naturally infected or vaccinated human subjects. The PBMCs are sorted by flow cytometer into ASCs ($IgG^{+}IgD^{-}$; $CD19^{+}$ $CD3^{-}$ $CD20^{low}$ and then sub-gated as $CD27^{high}$ $CD38^{high}$) and memory B cells ($CD27^{+}$) into 96-well PCR plates. In humans actively immunized with influenza vaccine, ASCs peaked around day 7, whereas the peak of memory B cells has been observed from days 14 to 21 after vaccination (Wrammert et al. 2008). VH and Vκ genes from each cell are amplified in a one-step RT-PCR reaction using a cocktail of sense primers specific for the leader regions and antisense primers to the Cγ constant regions for heavy chains and Cκ constant region for the light chain. Subsequently,

VH or Vκ genes amplified from each single cell are cloned into IgG1 or Igκ expression vectors. Heavy- and light-chain plasmids are subsequently co-transfected into the mammalian cell line such as HEK 293A cells for expression of the antibody. Expression of specific human antibody in the culture supernatant is confirmed by using high-throughput screening assays. Subsequently, antibody can be purified by using protein A/G Sepharose column. A large number of human antibodies against different neutralization-relevant epitopes of the glycoprotein of Ebola virus have been isolated from the survivor of the 2014 Ebola outbreak (Bornholdt et al. 2016). Similarly from influenza vaccinated human subjects, using single-B cell PCR, approximately 50 human MAbs binding to 3 influenza vaccine strains with high affinity have been generated (Wrammert et al. 2008).

22.5 Various Formats of Therapeutic Antibodies

Depending on the clinical application of the therapeutic antibodies, beside full-length antibodies, these have been expressed in various formats (Fig. 22.4), which are briefly described below:

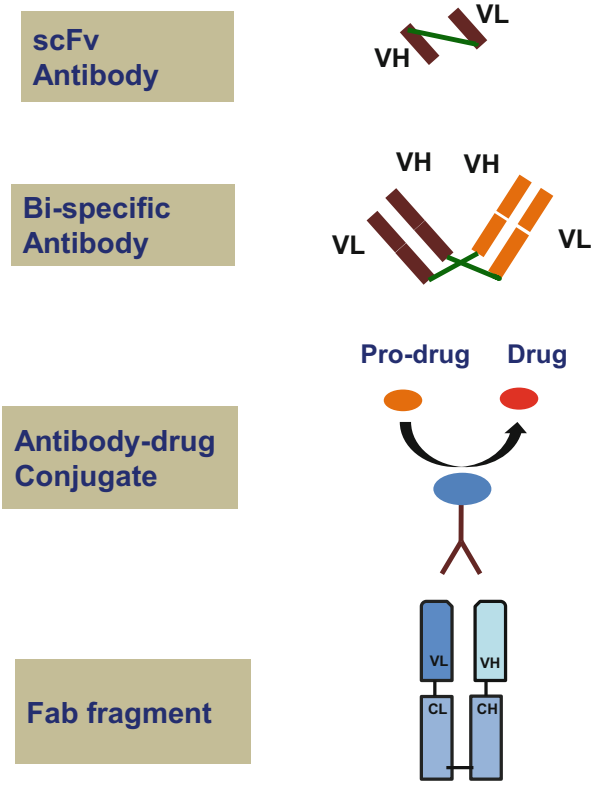

Fig. 22.4 Various derivatives of antibodies: Depending on the clinical application, antibodies have been made in different formats using genetic engineering. Schematic representation of scFv comprising the variable region of heavy and light chains joined by a peptide linker, bispecific antibody capable of binding to two different targets, antibody-drug conjugate to deliver the drug to specific cells, and Fab fragments is shown

22.5.1 Single-Chain Variable Fragment (scFv)

Antibody Fv fragment is the smallest unit responsible for antigen-binding activity, and single-chain variable fragment (scFv) format consists of variable region of heavy (V_H) and light (V_L) chains, which are joined together by a flexible peptide linker. The commonly used peptide linkers comprise streches of glycine and serine residues which provides scFv flexibility. Inclusion of glutamic acid and lysine residues in the linker enhances the solubility of scFv. The scFv format of therapeutic antibodies is a good delivery vehicle for radionucleotides as these can rapidly penetrate tissues as compared to whole antibodies and thus used as reagents for radio-imaging and radioimmunotherapy (Hudson and Souriau 2009). These can also be used to deliver a range of toxins or drugs to the specific cells. The physiological disadvantage of scFv format is rapid elimination from the body. Intrabodies which can penetrate the cells to target various viruses or cancers by neutralizing a range of oncogene or signaling molecules have been developed as variant of scFv (Strube and Chen 2002).

22.5.2 Bispecific and Tri-Specific MAbs

Bispecific antibodies with ability to engage two different antigens have been produced with clinical applications. Bispecific antibodies are made in two formats: (1) IgG-like bispecific antibodies which carry Fc region and (2) non-IgG format. IgG-like bispecific antibodies have Fc-mediated effector functions such as antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), and complement-dependent cytotoxicity, whereas non-IgG format primarily mediates its action by binding to two different antigens. The first bispecific antibody catumaxomab (Removab®) approved by the European Union has been used for malignant ascites. It is based on IgG format with Fc region. It recognizes CD3 antigen on cytotoxic T cells and epithelial cell adhesion molecule (EpCAM) which is a type 1 transmembrane glycoprotein associated with malignant ascites (Seimetz 2011). Another bispecific antibody, blinatumomab, approved by the US FDA in December 2014 comprised two scFv connected by peptide linker (Newman and Benani 2016). Bilnatumomab recognized simultaneously CD19 antigen expressed on all stages of B cell lineage and CD3 T cell receptor complex and approved for treatment of relapsed or refractory Ph-negative acute lymphoblastic leukemia in adults. The first full-length bispecific MAb engineered on the structure of a humanized IgG4 (emicizumab, Hemlibra®) was approved in 2017 by the US FDA. It has been used to reduce the frequency of bleeding episodes in hemophilia A patients. In addition to bispecific, a tri-specific antibody recognizing a tumor antigen, CD16 on NK cells, and IL15 has also been made. Such a tri-specific antibody directly triggers NK cell activation through CD16, amplifying NK cell cytolytic activity and cytokine production against various tumor cell antigen targets (Tay et al. 2016). Even a tetra-specific antibody simultaneously directed against HER1, HER3, C-MET (hepatocyte growth factor receptor), and insulin-like growth factor 1 (IGFIR) with enhanced antitumor effect has also been made (Castoldi et al. 2016).

22.5.3 MAb-Drug Conjugate

With an aim to antibody-mediated tumor-selective drug delivery, MAb-drug conjugates have been developed. It relies on antibody-induced receptor internalization, followed by trafficking of the MAb-drug conjugate to the lysosomes where the cytotoxic drug is released, thereby initiating its antitumor activity. The linker joining MAb and drug should be stable in the circulation to prevent premature release of the cytotoxic drug. The most common linkers are either cleavable peptides by proteases or disulfide linkers which undergo reduction in lysosomes to release the drug. Brentuximab vedotin (trade name Adcetris®) against CD30-positive Hodgkin's lymphoma and anaplastic large cell lymphoma has been approved for therapeutic application. Another MAb-drug conjugate, trastuzumab emtansine (Kadcyla®), has also been used for metastatic breast tumors overexpressing HER2.

22.5.4 Fab Fragment and Other Formats of Therapeutic Antibodies

Ranibizumab (Lucentis®) against VEGF for neovascular age-related macular degeneration has been expressed as Fab (comprising variable and constant domains of light and heavy chains of antibody) fragment in *E. coli* (Ferrara et al. 2006). The short half-life of Fabs can be increased by polyethylene glycol (Choy et al. 2002). Sugar chain-modified antibodies (Ishida et al. 2012) and low molecular weight antibodies (Ferrara et al. 2006) are also being explored as next-generation products. Catalytic antibodies that not only recognize the target antigen but also degrade it have been proposed as therapeutic agents. The catalytic antibody specifically hydrolyzes the target antigen at the site recognized by it. Interestingly, catalytic antibody reducing the β-amyloid accumulation in the brain of mouse has been developed (Planque et al. 2015). Soluble cytokine receptors have been fused with the antibody constant region and such biological are designated as "traps". Trap involving TNF receptor 2 fused with Fc (etanercept, Enbrel®) has been approved to treat rheumatoid arthritis. Other ligand "traps" approved are for IL-1 (rilonacept, Arcalyst®) and VEGF (aflibercept, Eylea®) for retinopathy.

22.6 Potential of Therapeutic Human MAbs

Sixty-four MAbs have been approved by the US FDA up to 2018 for clinical use in humans. It is projected that by 2022, expected sales of the therapeutic antibodies may be around US$ 172.8 billion, which may constitute 20% of the global pharmaceutical market (Tsumoto et al. 2019). Fully human MAbs currently comprise more than 55% of the market and constitute two-thirds of new MAbs approved by the US FDA in 2017. In future, emerging economies such as China, Brazil, Russia, Turkey, Mexico, South Korea, India, and Saudi Arabia will increase the market share of therapeutic MAbs. To address competition from biosimilars, the use of MAbs for newer clinical applications is being proposed. For example, Humira®, the top selling

Mab, was originally approved by the US FDA in 2002 for rheumatoid arthritis. This MAb has been approved in 2005 for the treatment of psoriatic arthritis, in 2007 for Crohn's disease, in 2008 for plaque psoriasis and idiopathic juvenile hidradenitis suppurativa, in 2016 for uveitis, and in 2017 for fingernail psoriasis. The use of therapeutic MAb initially dominated in the area of autoimmune diseases and cancers. However, new therapeutic antibodies are being developed for a variety of clinical conditions including viral and bacterial infections, obesity, diabetes, celiac disease, Alzheimer's disease, skin diseases, osteoporosis, etc.

Development of new technologies such as phage-display libraries, single-B cell PCR followed by cloning and expression of antibody in mammalian cells, and high-throughput screening systems leading to selection of appropriate clones and culture in miniatured bioreactor systems have led to cut down the response time to generate therapeutic antibody. Therapeutic human antibodies are now one of the viable options in addition to drugs and vaccines for emerging diseases. For example, during recent COVID-19 pandemic, bio-neutralizing MAbs against COVID-19 have been made using the above platforms as possible therapeutics, before vaccine (Yu et al. 2020).

22.7 Concluding Comments

Due to exquisite target specificity, MAbs are being increasingly used as therapeutics and have become one of the important portfolios of the pharmaceutical/biotech industries. Development of mouse-human chimeric antibody and humanized antibody has reduced the risk of development of human anti-mouse antibody and thus has made a significant contribution in their clinical applications. In recent times, due to the substantial reduction in the time required for the development of fully human therapeutic antibodies using single-cell PCR and cloning, high-throughput screening systems, and improved method of their production, these are being increasingly used in various clinical manifestations. Development of antibody-drug/radioisotope conjugate for targeted delivery, bispecific antibody capable of recognizing two different targets, have further broaden the scope of MAbs in clinical use. In addition to cancers and autoimmune disorders, therapeutic MAbs are being developed for diverse clinical disorders such as obesity, diabetes, celiac disease, Alzheimer's disease, viral infections, etc. which are likely to further increase their market share in future.

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Unleashing Potential of Bone Mimicking Nanodimensional Hydroxyapatites and Their Biomedical Applications

23

Seema Kapoor, Uma Batra, and Suchita Kohli

Abstract

Ionic substitution in nanodimensional hydroxyapatite (HA) offers numerous advantages including control of physicochemical properties, strong host interactions, biocompatibility, bioactivity, osteoconductivity, etc. In this chapter, the facile route of synthesis of novel hydroxyapatites, strategies to substitute ions, heat treatment of nanopowders, structural stability and thermal stability, in vitro biomineralization, and coating applications have been discussed. Nanotechnology, ionic substitution, and heat treatment have shown improvement in physicochemical characteristics of novel hydroxyapatites, which offer potential in a wide range of biomedical applications.

Keywords

Hydroxyapatite · Nanodimensional · Ionic substitution · Heat treatment · Coatings

23.1 Introduction

Hydroxyapatite (HA) is considered as a mineralized bone-like phase, and its potential for bone regeneration orthopedic surgeries has been well recognized. Despite the undeniable biocompatibility of HA, the overall prospect of hydroxyapatite for

S. Kapoor (✉) · S. Kohli
Dr. S.S.B. University Institute of Chemical Engineering and Technology, Panjab University, Chandigarh, India
e-mail: seemakapoor@pu.ac.in; seemakap2014@gmail.com

U. Batra
Department of Materials and Metallurgical Engineering, PEC (Deemed to be University), Chandigarh, India

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biomedical applications such as gene therapies, biomedical implants, controlled drug delivery, cancer therapies, tissue engineering, etc. is yet underutilized because of several drawbacks of HA. HA needs to be tailored for a specific application, and each novel structure of HA has to be treated as a new biomaterial before its application. The biological response of HA depends on its (a) crystallite size, shape, and crystallinity, (b) biocompatibility, (c) bioactivity, (d) relatively simple synthesis protocols, (e) functionalization, and (f) capacity to load therapeutic agents (Supova 2015).

A better HA product can be obtained by transition to nanodimensional material because such powders are characterized by a homogeneous structure, small crystallite size, and enhanced performance (Chen et al. 2002). The modification of hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ through its composition via cationic, anionic, or their simultaneous substitution can significantly affect its properties due to the formation of nanodimensional particles in the basic structure, which is of great interest for medical applications as a component of artificial bones and implants. The ionic substitution of HA is also essential for promising rate of bone tissue regeneration and physicochemical parameters close to those of natural bone. Each substitute ion can affect the features of the lattice, thus impacting its crystal size, crystallinity degree, stability, and morphology, all promoting its bioactivity and solubility. The most reported substitute ions for hydroxyapatite for biomedical applications are Mg^{2+}, Mn^{2+}, Sr^{2+}, and Zn^{2+} for calcium ions and $CO_3{}^{2-}$ and $SiO_4{}^{4-}$ for phosphate ions and F^- for hydroxide ions (Norhidayu et al. 2008).

In this chapter, the influence of ionic substitution in HA is examined. The nanodimensional hydroxyapatite powders substituted with various ions $Ca_{10-x}A_x(PO_4)_{6-y}B_y(OH)_{2-z}C_z$ (where x, y, and z indicate substitution for Ca^{2+}, $PO_4{}^{3-}$, and OH^-) were precipitated by wet chemical method from solutions. Powder characteristics like particle size distribution, morphology, phase composition, specific surface area, etc. have been studied. It was observed that the partial substitution of calcium ions, phosphate ions, or hydroxide ions or any two of the three leads to a reduction in the particle size to nanoscale. Furthermore, the amount of substitution also affects the crystallite size, shape, and crystallinity, biomineralization, bioactivity, etc. of synthesized powders.

Several methods can be used for the synthesis of hydroxyapatite, but most of these do not result in good quality hydroxyapatite having high crystallinity, acceptable biocompatibility, nanoscale particles as compared with natural bone tissue and enhanced resorption rate, necessary for application in implants, bone reconstruction, and other applications. Existing synthesis methods lead to the formation of secondary phases like α-, β-, and γ-tricalcium phosphate $Ca_3(PO_4)_2$, affecting biological properties. The degree of crystallinity required in HA structure can be attained by heat treatment at temperatures between 400 °C and 1300 °C, but it leads to sintering of powders, thereby increasing the particle size from nanodimension to microscale. This deteriorates associated characteristics of nanodimensional level initially obtained during HA synthesis.

It is reported that high crystallinity of nanodimensional particles positively impacts the growth and development of bone cells. An assessment of particle size

distribution in the as-synthesized powders and distribution of grains in heat-treated powders confirmed that the latter acquires the structure of the as-synthesized powders, which has a significant effect on its physicochemical characteristics.

23.2 Stoichiometric Hydroxyapatite and Associated Concerns

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) is identical to bone-like apatite structure and is a vital inorganic ingredient of bone as it provides rigidity to bones and teeth. Pure HA is stoichiometric apatite phase having molar ratio Ca/P as 1.67, hexagonal structure with P63/m space group, and lattice parameters $a = b = 9.418$ Å and $c = 6.884$ Å. It is the most stable crystalline phase of apatites and has high biocompatibility with natural bone (Kweh 1999; Kheradmandfard and Fathi 2013).

It possesses great biological properties like absence of immunological reactions, non-toxicity, and lack of inflammatory response (Vallet-Regi 2000). Chemical bonding of HA with the host tissue presents greater benefit in clinical applications. When HA is implanted into a bone location, several physiochemical interactions occur with the biological environment, causing the buildup of interfacial layers, which helps in the adhesion of implant material to bone tissue (Jennifer et al. 2005), resulting in implant stabilization and its superior fixation with adjoining tissues.

HA can stimulate new bone ingrowth via osteoconduction without causing any localized toxicity and inflammation response. It also inhibits the growth of cancer cells (Sadat-Shojai et al. 2013). Thus, HA has been widely recognized for repairing damaged or diseased bone tissues (Ming-Fa et al. 2001; LeGeros 2008). It has been effectively used as aesthetic restorative, bone filler, filler of inorganic/polymer composites, and coating of orthopedic implants (Pramanik et al. 2009). It can also be used as a carrier in drug delivery systems and catalysis (Constantin et al. 2012).

The application of stoichiometric HA in the form of powder, thin films, and porous or dense blocks is in plenty at the microscale level (Prakasam et al. 2015). But poor bioresorbability is an undesirable characteristic of microscale HA, as it inhibits the rate of bone regeneration (Kivrak and Tas 1998). Micron size HA has strong crystal-to-crystal bond and a low surface area as compared to bone mineral crystals which are nanodimensional and have loose crystal-to-crystal bond and large surface area. Stoichiometric HA also has poor thermal stability and mechanical properties, restricting its use for medical applications (Chen and Miao 2004; Kim et al. 2005).

There are considerable differences between stoichiometric and biological apatites. Biological apatites are nonstoichiometric carbonated compounds and are substituted with trace amounts of numerous ions (Combes et al. 2016; Supova 2015). These ions have a considerable biological role, directly affecting host cell response and/or exerting a therapeutic role; hence, their amount and presence in the peri-implant environment are essential.

Current findings for ion-substituted hydroxyapatite (HA) could mark the path towards its substantial growth in biomedicine, along with a prominence on a novel generation of dentistry and orthopedic applications.

23.3 Ionic Substitution in Hydroxyapatite

Ionic substitution in hydroxyapatite (HA) has received much attention in the recent past, as substitution of different ions in HA can modify its physicochemical characteristics, alter specific biological responses, and therefore help in producing multifunctional HAs (O'Neill et al. 2018). Even a small extent of ionic substitution in HA can significantly change its properties such as morphology, particle size, solubility, porosity, and specific surface area and helps in increasing its ability for involvement in the natural bone remodeling process. Ionic substitution in HA presents a key role in the biochemistry of bone, dentin, and enamel (Zhang et al. 2014). Hence, it is very important to unleash the potential of ionic substituted HA for various biomedical applications like medical implants, tissue engineering, gene therapies, drug delivery, etc.

Several cationic and anionic substitutions are feasible in HA due to its high stability and flexible structure. The biocompatibility and bioactivity of synthetic HA can be enhanced by substitution of particular trace ions like cations (K^+, Na^+, Sr^{2+}, Mg^{2+}, Ba^{2+}, Zn^{2+}, Mn^{2+}, Pb^{2+}, Tb^{3+}, Y^{3+}, Eu^{3+}) and anions (Cl^-, F^-, CO_3^{2-}, HPO_4^{2-}, SiO_4^{4-}) within the lattice structure (LeGeros 1991; Norhidayu et al. 2008). The substitution of physiologically significant ions in HA can affect its chemical and physical properties like morphology, lattice parameters "*a*" and "*c*," crystallinity, solubility, thermal stability, and osteoconductivity (Capuccini et al. 2008; Bracci et al. 2009).

23.3.1 Types of Ionic Substitutions in HA

Several cationic and anionic substitutions can be done in the structure of hydroxyapatite (Jiang et al. 2019); however, the extent and type of such ionic substitutions can be altered.

23.3.1.1 Single-Ion Substitution in HA

One of the efforts in the development of substituted HA is single-ion substitution, either by a cation or an anion. Cationic substitutions can occur in HA for the calcium ions, and anionic substitutions can occur in HA for PO_4^{3-} ions or OH^- ions.

In the stoichiometric HA, the cationic sites can take up vacancies for a maximum of 2 sites out of 10 available sites (Rey 1998). Cations smaller than Ca^{2+} such as Zn^{2+}, Mg^{2+}, and Mn^{2+} or low concentrations of slightly larger cations with strong interactions can be accommodated in site Ca (I), while larger cations like K^+ and Sr^{2+} at high concentrations can be accommodated in site Ca (II) (Boanini et al. 2010).

Potassium (K) has an impact on the biomineralization process (Kannan et al. 2006), and it also exhibits versatile nature in the regulation of biochemical processes. It can be substituted into HA lattice without significant changes in structural parameters. Zinc (Zn) is recognized as an important bone mineral, which is competent in enhancing biomineralization, bone formation, and osteoblast proliferation. It also incites alkaline phosphatase activity (Ovesen et al. 2001; Hall et al. 1999;

Yamaguchi and Yamaguchi 1986). Its deficiency causes a decrease in bone density. It helps in normal bone growth due to its involvement in numerous metabolic mechanisms (Prasad 1995; Yamaguchi 1998). Its substitution in HA impedes crystal growth and thermal stability (Bigi et al. 1997; Kanzaki et al. 2000). Magnesium (Mg) is involved in bone growth and skeletal metabolism by avoiding osteopenia and increasing osteoblast cell activity and reducing bone fragility and thus plays a critical role in bone remodeling (Cox et al. 2014). Mg ion has an obvious prohibiting influence on the growth and nucleation of HA (Kumta et al. 2005; Wang and Nancollas 2008). Its substitution in HA exhibits enhanced solubility in comparison to stoichiometric HA (Landi et al. 2008). Strontium (Sr) is known to modify bone turnover in favor of bone formation by promoting osteoblast activity and proliferation (Rapuntean et al. 2018; Reginster et al. 2009) as well as by lowering bone resorption (Hurtel-Lemaire et al. 2009). At low concentrations of Sr, there occurs a decrease in coherent length of the crystal, whereas at high concentrations, its crystallite size and crystallinity increase (Bigi et al. 2007). Europium (Eu^{3+}) is a suitable ion with fluorescent property that can be easily substituted into HA lattice as a result of its similar ionic radius. It can be used as a biological fluorescent probe due to its excellent luminescent properties. It exhibits favorable optical properties for use in laser hosts. It is used as luminescent probe in the study of the crystallographic structure of activator centers. It is also used as a tool to analyze the local occupancy and symmetry of the cationic locations in the apatite structure (Ciobanu et al. 2014).

The most important anionic substitutions in HA involve CO_3^{2-} and SiO_4^{4-} for PO_4^{3-} or F^- for OH^- groups (LeGeros 1965). Biomimetic HA contains a significant amount of carbonate (CO_3^{2-}) ions. The carbonate content in the individuals differs according to age. B-type carbonate-apatite (carbonate replaces phosphate) is ample in young individuals, whereas old individuals have more A-type carbonate-apatite (carbonate replaces hydroxyl) (Rey et al. 1991). B-type substitution improves the solubility of apatite at bodily pH without altering surface polar property, which helps in the affinity of osteoblastic cells. Carbonate substitution in HA generally results in a poorly crystalline structure with improved solubility (Wang and Nancollas 2008; Featherstone et al. 1983; Murugan and Ramakrishna 2006). Silicon (Si) is a vital trace element present in bones, which increases the rate of bone regeneration and biomineralization by stimulating the extracellular matrix secretion of chondrocytes. The presence of Si in HA impedes grain growth, thus promoting solubility and bioactivity of HA (Porter et al. 2003). Si is also substituted in HA to promote early bone healing. Fluorine is an indispensable trace element in bone tissue which can enhance the crystallization of calcium phosphate and further hasten the mineralization during the progression of bone formation (Shah et al. 2014; Kleerekoper 1996). It is present in teeth and bones of humans as a vital element against dissolution. The bond between implant and the bone is improved by its substitution (Sundfeldt et al. 2002a, b; Qu and Wei 2006). It also strengthens the bone structure (Bhadang et al. 2010) and boosts the thermal stability of HA (Barinov et al. 2003). Therefore, hydroxyapatite substituted with fluoride ions (FHA) is a good substitute material for bone repair (LeGeros et al. 1988).

23.3.1.2 Dual-Ion Substitution in HA

Dual-ion substitution is being regarded as a promising approach for enhancing biological and physicochemical properties of HA. Primarily, both of the two substituted ions are cations (Lowry et al. 2018; Robinson et al. 2017), whereas in some cases the anion/anion (Landi et al. 2010; Fahami et al. 2016) and anion/cation (Kumar et al. 2012; Kolmas et al. 2011) combinations have also been explored. When two or more ions are present, they can have a synergistic or antagonistic effect on properties of HA. It has been found that when Mg^{2+} and CO_3^{2-} are co-substituted in HA, there is a synergistic effect on the dissolution properties and crystallinity, whereas when CO_3^{2-} and F^- are co-substituted in HA, there is an antagonistic effect and F^- is more dominant.

Zn^{2+} and Mg^{2+} co-substitution in HA enhances bone formation (Kaygili and Keser 2015). Zn^{2+} ions are found to be more favorably substituted into Ca site in HA as compared to Mg. With an increase in the content of Mg^{2+} and Zn^{2+}, crystallinity, cell parameters, and unit cell volume of HA decrease drastically and also show an improved biocompatibility. In vitro studies demonstrated a notable improvement in cell proliferation, attachment, and adhesion in comparison to stoichiometric HA. Lowry et al. (2017) and Ullah et al. (2020) successfully substituted Zn and Sr ions in HA by various methods. Zn^{2+} partially substituted for Ca^{2+}, whereas Sr^{2+} got completely substituted for Ca^{2+}.

In vitro studies in human osteoblast-like MG63 cells for HA co-substituted with Sr and Mg (SrMgHA) displayed an enhanced cell attachment, proliferation, and differentiation (Geng et al. 2016). Landi et al. (2013) studied the extended release of Mg during the bone regeneration process and also studied the anti-osteoporotic and anticaries properties of Sr ion in SrMgHA. Yoruc and Aydinoglu (2017) synthesized Na- and Mg-substituted HA by a precipitation technique. The in vitro study revealed an improved bioactive behavior, as the growth of osteoblast cells was encouraging.

Dual substitution of anions presents another way to improve the cytocompatibility of HA. Investigations have been carried out with various combinations of anions including Cl^-, F^-, and CO_3^{2-}. Fahami et al. (2016) found that the co-substitution of F^- and Cl^- in HA could counteract probable side effect of Cl^-, e.g., dementia in elderly patients. They also found that the addition of F^- could improve the differentiation and proliferation of bone cells.

The cation-anion co-substitution in HA can influence its morphology, lattice parameters, crystallinity, and crystallite size. Douglas et al. (2017) observed enhanced dissolution and reduced crystallinity in the case of CO_3^{2-} and Mg^{2+} co-substituted HA. When carbonates are co-substituted with Sr^{2+} or Zn^{2+}, bone remodeling is enhanced. The combination of Zn and carbonate in HA hinders the crystal growth. The dual substitution of CO_3^{2-} and Sr^{2+} in HA helps in combining bioactivity of carbonate and therapeutic function of Sr^{2+} to enhance the new bone formation rate and osteointegration (Landi et al. 2008; Kumar et al. 2012).

With an increase in CO_3^{2-} and Na^+ content, the crystallite size of CO_3^{2-} and Na^+ co-substituted HA decreases (Zyman and Tkachenko 2013). The Sr and Si co-substitution in HA helps in the substitution levels of both the elements.

23.3.1.3 Multiple-Ion Substitution in HA

The simultaneous incorporation of more than two vivid ions within the HA lattice has not been extensively examined, due to the intricacy of the hydroxyapatite structure (Sprio et al. 2008). Efforts are on to synthesize multi-substituted HA to imitate chemical composition of biological apatite for enhancing chemical, physical, structural, and biological properties of HA. HA with simultaneous incorporation of CO_3^{2-}, F^-, Cl^-, Na^+, Mg^{2+}, and K^+ was synthesized by wet precipitation method (Kannan et al. 2011) to enhance CO_3^{2-} incorporation within HA lattice. The Mg-, Zn-, and Co-substituted HA presented a better cell viability, superior bioactivity, and antibacterial activity in comparison to pure HA (Rajendran et al. 2018). In vitro and antimicrobial activity of Mg- and Ni-substituted silicate hydroxyapatite displayed a quicker dissolution rate in SBF (Alshemary et al. 2015). Gopi et al. (2012) synthesized Sr/Mg/Zn HA and observed that simultaneous substitution of Sr, Mg, and Zn in HA not only provided growth of apatite but also hastened growth onto itself.

HA with simultaneous substitution of Mg, Zn, and SiO_4^{4-} ions was found to improve the growth proliferation and adhesion. In vitro studies showed the collagen synthesis of human osteoblasts (Corina et al. 2020; O'Neill et al. 2018).

23.4 Synthesis of Novel Hydroxyapatites

In the present work, various novel hydroxyapatites have been developed. These HA products have particle sizes in the nanodimensional range in which single, dual, or multiple ions have been substituted in HA according to the requirement for a specific biomedical application. The sol-gel technique used for their synthesis is a facile method, which can be easily scaled up for commercial production to yield physically as well as chemically uniform product.

The protocol for the synthesis of novel nanodimensional hydroxyapatites by sol-gel technique is illustrated in Fig. 23.1. The respective moles of precursors for various ionic substituted HAs are given in Table 23.1.

23.4.1 Stoichiometric Nanodimensional Hydroxyapatite

Stoichiometric nanodimensional hydroxyapatite (HA) was synthesized utilizing sol-gel method. The precursors for calcium and phosphorus were used as calcium nitrate tetrahydrate (CNT, $Ca(NO_3)_2.4H_2O$, Merck, AR grade) and potassium dihydrogen phosphate (KDP, KH_2PO_4, Merck, AR grade), respectively. Solution A containing 1.0 M CNT and Solution B containing 0.6 M KDP were made in double-distilled water (DDW), and molar ratio Ca/P was kept at 1.67. Solution A was added dropwise to Solution B at a stirring rate of 1000 rpm for 1 h at 25 ± 2 °C. The pH was adjusted to 10 ± 0.1 throughout by adding 25% ammonium hydroxide solution (NH_4OH, Merck, AR grade). Aging of the gel was done at 25 ± 2 °C for 24 h. Gelatinous precipitates formed were centrifuged, and thorough washing of

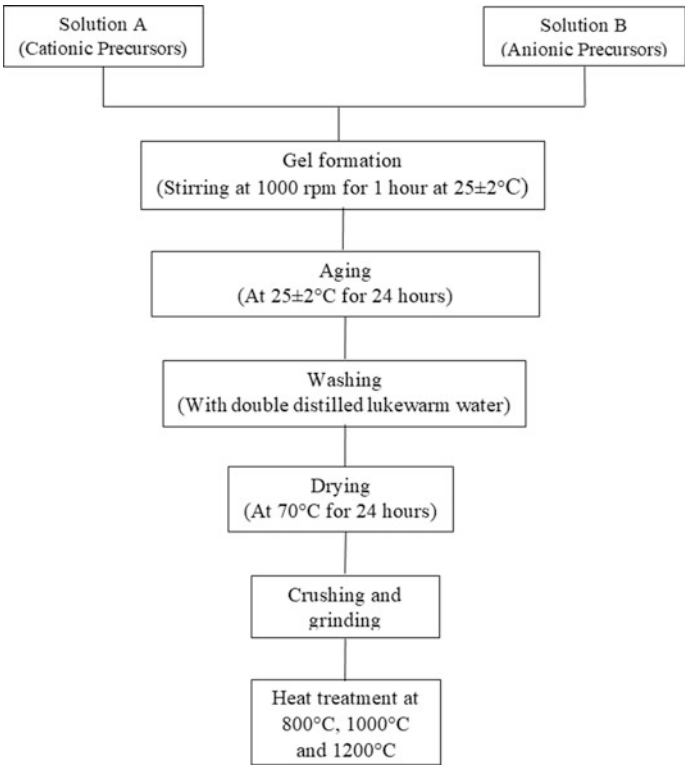

Fig. 23.1 Protocol for the synthesis of nanodimensional hydroxyapatites by sol-gel method

these precipitates was done with double-distilled lukewarm water. This step was followed by drying the precipitates for 24 h at 70 °C. Mortar and pestle was used to crush and grind the dried mass into fine powders.

23.4.2 Single-Ion-Substituted Nanodimensional Hydroxyapatites

Zinc nitrate tetrahydrate (ZNT, $Zn(NO_3)_2.4H_2O$, Merck, AR grade) and magnesium nitrate tetrahydrate (MNT, $Mg(NO_3)_2.4H_2O$, Merck, AR grade) were used as zinc and magnesium precursors for synthesizing zinc-substituted nanodimensional hydroxyapatite (ZnHA, $Zn_{0.2}Ca_{9.8}(PO_4)_6OH_2$) and magnesium-substituted nanodimensional hydroxyapatite (MgHA, $Mg_{0.2}Ca_{0.98}(PO_4)_6OH_2$), respectively. 1.0 M Solution A was made by adding appropriate amounts of CNT and ZNT or MNT in the case of ZnHA and MgHA, respectively, and 0.6 M Solution B was made by adding appropriate amount of KDP.

Europium nitrate pentahydrate (ENP, $Eu(NO)_3.5H_2O$, Merck, AR grade) was used as europium precursor for the synthesis of europium-substituted nanodimensional hydroxyapatite (EuHA, $Eu_{0.2}Ca_{9.8}(PO_4)_6(OH)_2$). Appropriate

Table 23.1 Precursors and their respective moles used for the synthesis of nanodimensional hydroxyapatites

Sample	HA	ZnHA	MgHA	FHA	SiHA	KSiHA	ZnFHA	SrFHA	MgSrHA	MgSrFHA	EuHA
Precursors used	CNT, KDP	CNT, ZNT, KDP	CNT, MNT, KDP	CNT, KDP, AF	CNT, KDP, TEOS	CNT, KN, KDP, TEOS	CNT, ZNT, KDP, AF	CNT, SNT, KDP, AF	CNT, MNT, SNT, KDP	CNT, MNT, SNT, KDP, AF	CNT, ENP, KDP
Respective moles	1.0, 0.6	0.98, 0.02, 0.6	0.98, 0.02, 0.6	1.0, 0.6, 0.1	1.0, 0.58, 0.02	0.98, 0.02, 0.58, 0.02	0.98, 0.02, 0.6, 0.1	0.88, 0.12, 0.6, 0.1	0.86, 0.02, 0.12, 0.6	0.86, 0.02, 0.12, 0.6, 0.1	0.98, 0.02, 0.6

amounts of CNT and ENP were added to make 1.0 M Solution A and appropriate amount of KDP to make 0.6 M Solution B.

Ammonium fluoride (AF, Merck, AR grade) and tetraethoxysilane (TEOS, Merck, AR grade) were used as fluorine and silicon precursors for the synthesis of fluorine-substituted nanodimensional hydroxyapatite (FHA, $Ca_{10}(PO_4)_6OHF$) and silicon-substituted nanodimensional hydroxyapatite (SiHA, $Ca_{10}(PO_4)_{5.8}(SiO_4)_{0.2}(OH)_2$), respectively. In the case of FHA, 1.0 M Solution A was made by adding appropriate amount of CNT and Solution B was made by adding 0.1 M AF and 0.6 M KDP, maintaining P/F ratio at 6.0. In the case of SiHA, 1.0 M Solution A was made by adding appropriate amount of CNT and 0.6 M Solution B was made by adding appropriate amounts of KDP and TEOS.

All other steps in sol-gel synthesis of nanodimensional single-ion-substituted hydroxyapatites were adopted as such.

23.4.3 Dual-Ion-Substituted Nanodimensional Hydroxyapatites

To synthesize potassium and silicon nanodimensional hydroxyapatite (KSiHA, $K_{0.2}Ca_{9.8}(PO_4)_{5.8}(SiO_4)_{0.2}(OH)_2$), appropriate amounts of CNT and potassium nitrate (KN) were added to make 1.0 M Solution A and 0.6 M Solution B was made by adding appropriate amounts of KDP and TEOS. To synthesize zinc and fluorine co-substituted nanodimensional hydroxyapatite (ZnFHA, $Zn_{0.2}Ca_{9.8}(PO_4)_6OHF$) and strontium and fluorine co-substituted nanodimensional hydroxyapatite (SrFHA, $Sr_{1.2}Ca_{8.8}(PO_4)_6OHF$), 1.0 M Solution A was made by adding appropriate amounts of CNT and ZNT or SNT and Solution B was made by adding 0.6 M KDP and 0.1 M AF while maintaining P/F molar ratio at 6.0.

Magnesium nitrate tetrahydrate (MNT) and strontium nitrate (SNT, $Sr(NO_3)_2$, Merck, AR grade) were taken as magnesium and strontium precursors for the synthesis of magnesium- and strontium-substituted nanodimensional hydroxyapatite (MgSrHA, $Mg_{0.2}Sr_{1.2}Ca_{8.6}(PO_4)_6OH$). 1.0 M Solution A was made by adding appropriate amounts of CNT, MNT, and SNT and 0.6 M Solution B was made by adding appropriate amount of KDP. Rest same procedure of sol-gel synthesis as adopted for HA was followed for all dual-ion-substituted nanopowders.

23.4.4 Multi-Ion-Substituted Nanodimensional Hydroxyapatite

For synthesizing magnesium, strontium, and fluorine multi-substituted nanodimensional hydroxyapatite ($Mg_{0.2}Sr_{1.2}Ca_{8.6}(PO_4)_6OHF$), appropriate amounts of CNT, MNT, and SNT were added for making 1.0 M Solution A and Solution B was made by adding 0.6 M KDP and 0.1 M AF while maintaining P/F molar ratio at 6.0. Rest same procedure of sol-gel synthesis as adopted for HA was followed for multi-ion-substituted nanopowder.

23.4.5 Heat Treatment of Novel Hydroxyapatites

The hydroxyapatite powders have potential applications as biomedical products in the form of scaffolds and coatings on metallic implants; therefore, their high temperature behavior needs to be understood. For this, heat treatment of as-synthesized novel hydroxyapatites was done at 800 °C, 1000 °C, and 1200 °C for 1 h at a heating rate of 10 °C/min in a silicon carbide furnace under controlled atmosphere.

23.5 Ionic Substituted Nanodimensional Hydroxyapatites

23.5.1 Elemental Analysis of Novel Hydroxyapatites

The elemental analysis of as-synthesized novel hydroxyapatite powders was carried out in order to confirm the substitution efficiency using wavelength dispersive X-ray fluorescence spectroscopy (WD-XRF, Bruker, Germany). Approximately 8 g of powder was used to make pellets of 1.5 mm thickness and 34 mm diameter. The test was conducted for 17 min. Photoluminescence spectroscopy (PL) of Eu-substituted HA powder was conducted using a Shimadzu UV-2401PC spectrophotometer. The excitation was done at 325 nm wavelength of He-Cd laser with an integrating sphere attachment using reference compound as $BaSO_4$. Diffuse reflectance UV-visible absorption spectra (DRUVS) were recorded. Micro-Raman and photoluminescence studies were also conducted via Raman microscope by Renishaw using a 514 nm wavelength of Ar laser.

The substitution of ions in hydroxyapatite was confirmed in all ionic substituted powders though the concentration of substituted element was lesser than the amount added during synthesis (Table 23.2). The substitution of ions in hydroxyapatite affects its stoichiometry. The Ca/P of as-synthesized nanodimensional stoichiometric HA powder was 1.67. With ionic substitution, there is a deviation from a Ca/P of 1.67. Most of the as-synthesized nanopowders had Ca/P molar ratio less than 1.67 except for SiHA and KSiHA (Table 23.2).

PL of Eu-substituted nanodimensional HA powder showed the luminescence at ~590 nm and ~612.6 nm with the transition of 5D0 → 7F1 and 5D0 → 7F2 of Eu^{3+}, respectively. At higher wavelength region, the weak peaks also appeared. These weak peaks might have occurred from direct excitation of Eu^{3+} from ground state to higher levels in 4f6 configuration. The two prominent characteristic peaks from 5D0 → 7F1 (590 nm) and 5D0 → 7F2 (612 nm) were predominant in emission spectra. These results infer that substitutions have occurred successfully in Ca sites for cationic elements like K, Zn, Mg, Sr, and Eu; OH sites for anionic elements like F; and PO_4 sites for anionic elements like Si.

Table 23.2 Mole% of substituent in hydroxyapatites and Ca/P ratio of nanopowders

Sample	HA	ZnHA	MgHA	SiHA	KSiHA	ZnFHA	SrFHA	MgSrHA	MgSrFHA
Mole% of substituent (x, y) added during synthesis	–	2	2	2	2, 2	2	12	2, 12	2, 12
Mole% of substituent in synthesized powder	–	1.4	1.98	1.314	1.417, 1.213	1.47	11	1.91, 10.8	1.7, 11.7
Ca/P	1.67	1.65	1.62	1.78	1.82	1.63	1.625	1.62	1.625

23.5.2 Structure of Novel Hydroxyapatites

The powder morphology was observed by using transmission electron microscope (TEM, Hitachi, 7500) at an accelerating voltage of 80–100 kV with a resolution of 0.2 nm. The powder samples were subjected to ultrasonication in ethanol and a drop of this suspension was dropped on a 300 mesh carbon-coated copper grid. As-synthesized stoichiometric and ionic substituted HA powder particles were nanodimensional (<40 nm). These powders exhibited either flakelike or rodlike morphology as shown in TEM micrographs (Fig. 23.2). The length parameter of the powder particles decreased on ionic substitution of HA, though the extent of decrease varied depending on the type of substitution and crystal size of substituting element (Table 23.3).

TEM micrographs of novel HA nanopowders after heat treatment showed two modifications. One modification is the increase in particle size as compared with the corresponding as-synthesized HA nanopowders, and the other is its morphology (Fig. 23.3). The particle morphology changed from flakelike or rodlike to a regular hexagonal shape on heat treatment from 800 °C to 1200 °C, and also there was a notable increase in the size of particles.

XRD analysis of as-synthesized and heat-treated nanodimensional powders was carried out to determine their structure using Philips X'Pert 1710 X-ray diffractometer using CuKα radiation, λ = 1.54 Å, step size 0.017°, time per step 20.03 s, between the range of 20–80 degree, and speed of scan 0.005°/s. The phases, crystallinity, lattice parameters, and mean crystallite size were determined from XRD spectra. The relative proportions of various phases were determined on the basis of peak intensity variation by means of external standard method. The XRD spectra were compared with JCPDS files: JCPDS Card No. 9-432 for

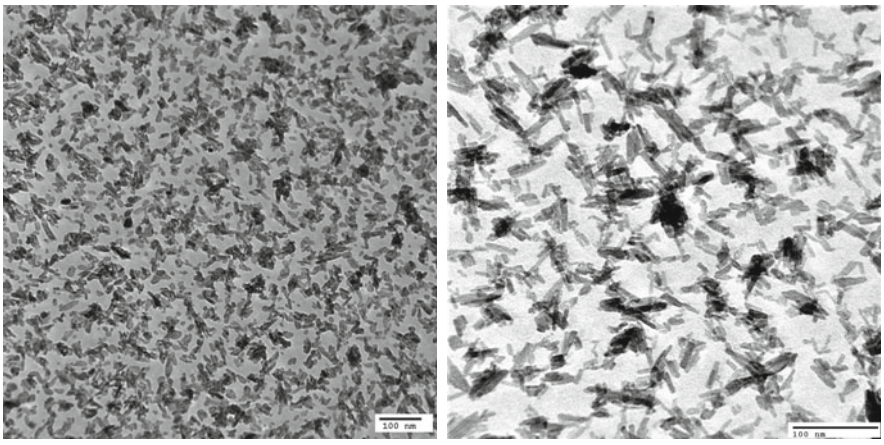

Fig. 23.2 TEM micrographs of two morphologies of as-synthesized novel hydroxyapatites

Table 23.3 Morphology and mean particle size of as-synthesized nanodimensional powders from TEM micrographs

Sample	HA	ZnHA	MgHA	EuHA	SiHA	FHA	KSiHA	ZnFHA	SrFHA	MgSrHA	MgSrFHA
Morphology	Flakelike	Flakelike	Flakelike	Rodlike	Flakelike	Rodlike	Flakelike	Rodlike	Flakelike	Flakelike	Flakelike
Particle size, nm	$L = 28–40$	$L = 26–36$	$L = 15–19$	$L = 39$ $D = 8.49$	$L = 12–22$	$L = 22–36$ $D = 3.8–4.6$	$L = 20–31$	$L = 15–38$ $D = 3–6$	18–22	30–34	25–29

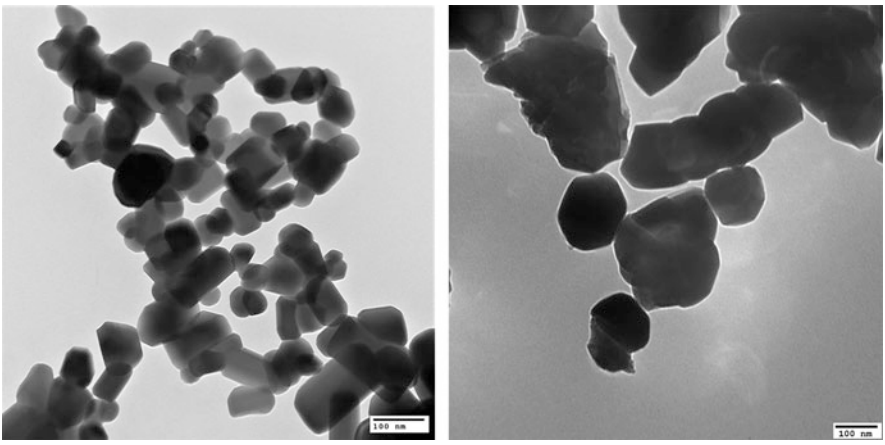

Fig. 23.3 TEM micrographs of heat-treated novel hydroxyapatites

hydroxyapatite; JCPDS Card No. 15-0876 for fluorapatite; JCPDS Card No. 09-0348 for α-tricalcium phosphate (α-TCP); JCPDS Card No. 09-0169 for β-tricalcium phosphate (β-TCP). The structural stability of heat-treated powders was also assessed from the phase transformations at higher temperatures. As reported by Dorozhkin (2003), Cullity and Stock (2001), and Kannan et al. (2007), the thermal decomposition of CDHA ($Ca_{10-x}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x}$) takes place above 1000 °C, resulting in biphasic mixture consisting of hydroxyapatite phase (HAp) and β-tricalcium phosphate phase, the equation for which is given below:

$$Ca_{10-z}(HPO_4)_x(PO_4)_{6-x}(OH)_{2-x} \rightarrow (1-x)\,Ca_{10}(PO_4)_6(OH)_2 + 3xCa_3(PO_4)_2 + xH_2O \quad (23.1)$$

where Ca/P = (10 − *x*)/6 and *x* is the calcium deficiency.

The mole fractions X_{HA}, $X_{\beta\text{-TCP}}$, and $X_{\alpha\text{-TCP}}$ of pure HA, β-TCP, and α-TCP phases present in various powders were determined. The external standard method was used to calculate the weight % of hydroxyapatite phase (W_{HAp}) and β-TCP phase ($W_{\beta\text{-TCP}}$) from XRD patterns. The weight % were then converted into mole fractions and used for calculating *x* and Ca/P values. The crystallinity degree (X_c) of nanopowders was calculated using the equation given below:

$$X_c = 1 - V_{112/300}/I_{300} \quad (23.2)$$

where $V_{112/300}$ is the intensity of hollow between (1 1 2) and (3 0 0) peaks and I_{300} is the intensity of (3 0 0) peak of HA. Verification for crystallinity was done according to the equation given below (Landi et al. 2000):

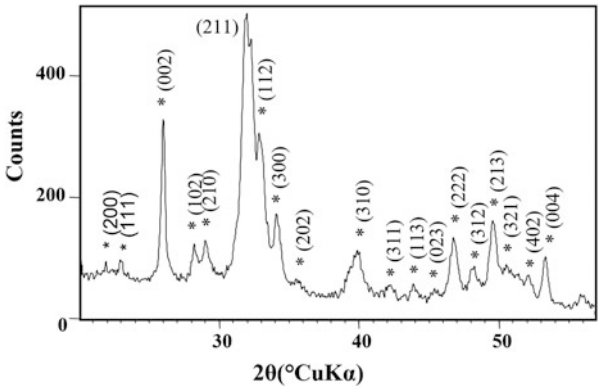

Fig. 23.4 XRD pattern of as-synthesized HA nanopowder

$$\beta_{002} 3\sqrt{X_c} = K \tag{23.3}$$

where β_{002} is the full width at half maximum (FWHM) of (0 0 2) peak in degree 2θ and constant K is equal to 0.24. In XRD patterns of as-synthesized nanopowders where any of (1 1 2) and (3 0 0) peaks were missing, FWHM values for (0 0 2) and (3 1 0) peaks were used to compare the crystallinity. The crystallite size of nanopowders was calculated using Scherrer's equation (Joseph and Tanner 2005; Clausen and Fabricius 2000):

$$X_S = \frac{0.9\lambda}{\text{FWHM} \cos\theta} \tag{23.4}$$

where X_S is the crystallite size in nm, FWHM is the broadening of diffraction line at half of its maximum intensity in radians, λ is the wavelength of X-ray beam, and 2θ is Bragg's diffraction angle (°). Instrument broadening was measured using silicon standard so as to correct the value of FWHM. Three high-intensity and well-separated peaks of XRD spectra were selected for evaluating the mean crystallite size of as-synthesized nanodimensional powders. For calculating the mean crystallite size of all heat-treated powders, three diffraction peaks (0 0 2), (2 1 1), and (3 0 0) of XRD spectra were chosen. The mean crystallite size of β-TCP phase was computed utilizing line broadening of (0 2 10) peak at around 31.0° (2θ) for heat-treated nanopowders (Ayed et al. 2001). The diffraction peaks at 25.8° (2θ) corresponding to (0 0 2) and 32.9° (2θ) corresponding to (3 0 0) were examined for calculating domain sizes along crystallographic axis "*a*" and "*c*" of nanodimensional powders. Cell parameters were calculated using the equation given below (Webster et al. 2004):

$$\frac{1}{d^2} = 4/3\left[\frac{h^2 + hk + k^2}{a^2}\right] + \frac{l^2}{c^2} \tag{23.5}$$

where d is the distance between adjacent planes in a set of Miller indices (h k l).

XRD patterns of HA and cationic substituted HA nanopowders showed only HA reflections. The reference XRD pattern is shown in Fig. 23.4. The as-synthesized

nanopowders were found to be hexagonal unit cell and matched well with JCPDS Card No. 09-432 for hydroxyapatite. The mean crystallite size, crystallinity, and lattice parameters of all as-synthesized nanopowders calculated from XRD diffraction peaks are given in Table 23.4. The changes in lattice parameters "*a*" and "*c*" of as-synthesized ionic substituted HA nanopowders showed the effect of ionic substitution in HA. Broader diffraction peaks of as-synthesized HA nanopowder indicated its amorphous nature. Its lattice parameters, i.e., "*a*" = 9.411 Å and "*c*" = 6.878 Å, matched well with hydroxyapatite. Both ZnHA and MgHA nanopowders showed a decrease in crystallite size. However, ZnHA nanopowder showed an increase in lattice parameters and MgHA nanopowder showed decrease in lattice parameters as compared to HA. The changes in lattice parameters with zinc substitution suggested possible Zn substitution at Ca sites in the apatite lattice. In MgHA nanopowder, a decrease in the value of lattice parameters was due to smaller ionic radii of Mg (0.66 Å) with respect to Ca (0.99 Å). EuHA nanopowder showed a decreased value of lattice parameter "*a*" and an increased value of lattice parameter "*c*" as compared to HA.

The XRD pattern of fluorine-substituted HA nanopowder was compared with JCPDS Card No. 15-0876 of fluorapatite. The XRD spectra of FHA nanopowder exhibited an identical behavior as HA.

FHA showed stronger XRD intensities and broader peaks than HA which indicated its lower crystallinity. The two peaks (211) and (112) combined because of fluorination. SiHA nanopowder showed increased crystallinity and decreased lattice parameters of HA on silicon substitution. KSiHA nanopowder showed "a"-axis contraction as compared to SiHA. But minor expansion of the *c*-axis was observed for KSiHA with respect to SiHA. The contraction in the *a*-axis was due to the substitution of silicon ion. Although the ionic radius of potassium ion (1.33 Å) is greater than the ionic radius of calcium ion, the further decrease in lattice parameter "*a*" of KSiHA nanopowder was due to the substitution of a bivalent cation (Ca^{2+}) by a monovalent cation (K^{+}), resulting in the decrease of channel diameter of "*a*" parameter (Kannan et al. 2007). Substitution of Zn and F in HA lattice showed increased crystallite size and lattice parameters. In MgSrHA nanopowder, XRD peaks shifted towards lower angle as compared to MgHA. The co-substitution of magnesium and strontium (MgSrHA) in HA showed lower crystallinity and increased lattice parameters. Multi-substituted MgSrFHA nanopowder showed variation in crystallite size and lattice parameters. MgSrFHA showed higher crystallite size than SrFHA nanopowder. Lattice parameter 'a' was smaller whereas lattice parameter 'c' was higher in MgSrFHA than SrFHA.

On heat treatment of as-synthesized nanopowders, change in mean crystallite size, crystallinity, lattice parameters, and phase transformations were observed (Fig. 23.5). The change in their respective values is summarized in Table 23.5. Crystallite size and crystallinity of nanopowders increased with an increase in heat treatment temperature. At higher heat treatment temperatures, the increase in crystallite size is due to the coalescence of small grains through grain boundary diffusion (Choodamani et al. 2014). The increase in crystallinity after heat treatment indicates

Table 23.4 Mean crystallite size, crystallinity, and lattice parameters of as-synthesized hydroxyapatite nanopowders from XRD patterns

Nanopowders		HA	ZnHA	MgHA	EuHA	FHA	SiHA	KSiHA	ZnFHA	SrFHA	MgSrHA	MgSrFHA
Mean crystallite size, nm		34	29	17	12.3	*a*-axis 11, *c*-axis 22	23.67	25.53	32	19.5	33	27
Crystallinity		Amor*	0.11	Amor*	0.195	0.11	0.038	0.12	Amor*	Amor*	Amor*	Amor*
Lattice parameter (Å)	**aÅ**	9.411	9.451	9.390	9.349	9.410	9.398	9.355	9.551	9.411	9.624	9.330
	cÅ	6.878	6.895	6.860	6.987	6.894	6.842	6.846	6.899	6.894	6.983	6.977

*Amor** amorphous

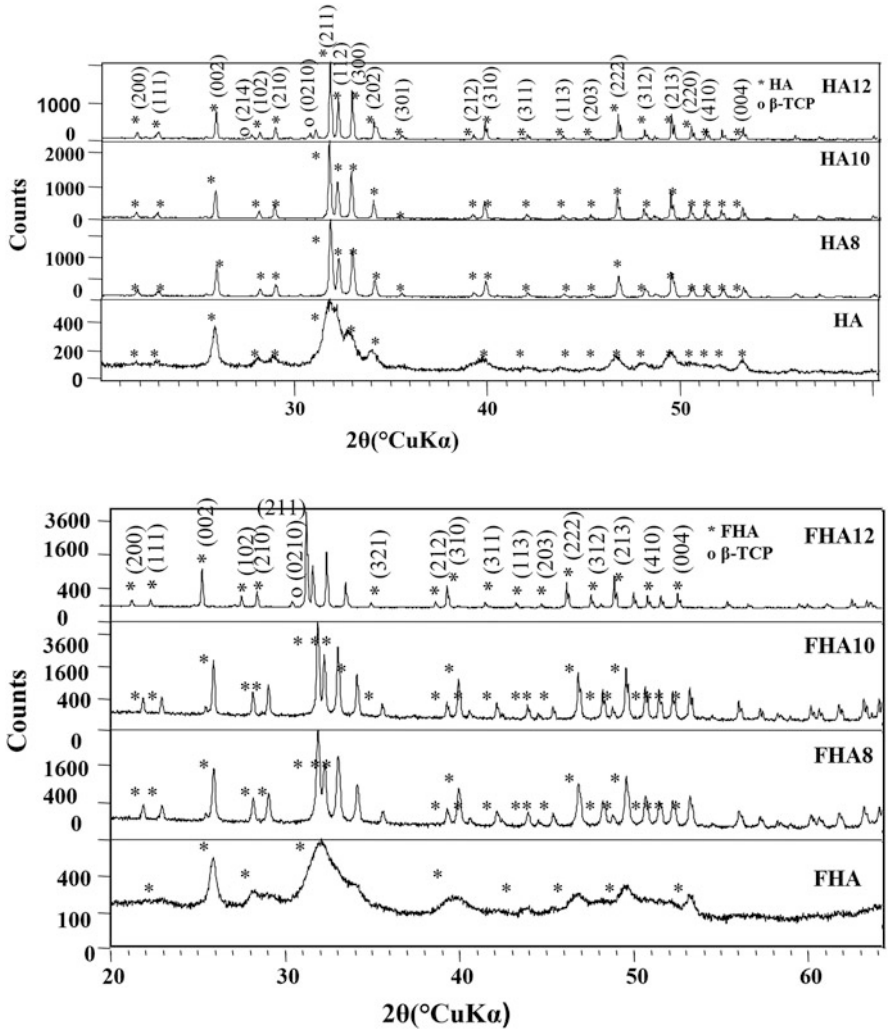

Fig. 23.5 XRD patterns of heat-treated nanopowders (HA8 and FHA8, HA10 and FHA10, and HA12 and FHA12: powders heat-treated at 800 °C, 1000 °C, and 1200 °C)

that the number of crystallites formed during heat treatment is large than that for as-synthesized hydroxyapatite powders (Pang and Bao 2003).

The mean crystallite size of heat-treated HA, ZnHA, MgHA, FHA, and SrFHA nanopowders at 1200 °C was lower than 1000 °C except for EuHA, SiHA, KSiHA, ZnFHA, and MgSrFHA, which may be due to the decomposition of HA to β-TCP. FHA nanopowder revealed the presence of only hydroxyapatite phase at 800 °C and 1000 °C, whereas β-TCP phase was observed as minor phase when heat treatment

Table 23.5 Mean crystallite size, crystallinity, and lattice parameters of heat-treated nanopowders from XRD patterns

	Mean crystallite size, nm			Crystallinity			Lattice parameters *a* and *c*, Å		
Nanopowder	800 °C	1000 °C	1200 °C	800 °C	1000 °C	1200 °C	800 °C	1000 °C	1200 °C
HA	65.00	71.00	68.00	0.88	0.91	0.94	9.415, 6.879	9.413, 6.877	9.411, 6.876
ZnHA	41.00	74.00	62.00	0.74	0.91	0.92	9.394, 6.895	9.405, 6.869	9.409, 6.880
MgHA	38.00	127.00	92.00	0.76	0.93	0.95	9.398, 6.757	9.399, 6.686	9.397, 6.747
EuHA	37.67	58.52	87.78	0.478	0.538	0.54	9.324, 6.820	9.478, 6.884	9.450, 6.817
FHA	58.00	87.00	67.00	0.9	0.91	0.95	9.387, 6.874	9.389, 6.877	9.388, 6.883
SiHA	41.41	71.71	113.70	0.81	0.93	0.96	9.393, 6.868	9.388, 6.841	9.409, 6.886
KSiHA	42.29	88.00	129.40	0.89	0.96	0.96	9.393, 6.868	9.384, 6.841	9.409, 6.881
ZnFHA	52.70	70.60	87.90	0.906	0.94	0.94	9.398, 6.885	9.399, 6.886	9.397, 6.885
SrFHA	105.44	116.75	57.94	0.88	0.89	0.91	9.410, 6.894	9.411, 6.894	9.366, 6.893
MgSrHA	65.38	76.26	81.01	0.755	0.819	0.822	9.625, 6.892	9.625, 6.869	9.625, 6.883
MgSrFHA	54.00	63.70	67.00	0.29	0.68	0.71	9.358, 6.981	9.296, 6.847	9.367, 6.822

Table 23.6 Weight % of HA and TCP phases in heat-treated nanopowders from XRD patterns

	Weight % HA			Weight % β-TCP and α-TCP		
Nanopowder	800 °C	1000 °C	1200 °C	800 °C	1000 °C	1200 °C
HA	100.00	100.00	77.00	0.00	0.00	23.00
ZnHA	84.60	67.50	50.82	15.40	32.50	49.18
MgHA	51.00	42.00	35.00	49.00	58.00	65.00
EuHA	83.50	67.24	59.45	17.50	32.76	40.55
FHA	100.00	100.00	87.00	0.00	0.00	13.00
SiHA	73.83	72.44	62.70	5.37, 20.44	11.09, 16.47	20.38, 16.91
KSiHA	84.99	73.79	69.99	6.52, 8.49	12.81, 13.41	21.37, 8.64
ZnFHA	100.00	92.23	63.48	0.00	7.76	36.52
SrFHA	73.00	72.00	71.00	27.00	28.00	29.00
MgSrHA	77.00	75.00	69.00	23.00	25.00	31.00
MgSrFHA	60.00	47.00	44.00	40.00	53.00	56.00

was done at 1200 °C. All other ionic substituted nanopowders showed a biphasic mixture of hydroxyapatite and TCP at 1000 °C and 1200 °C (Table 23.6).

SiHA and KSiHA nanopowders heat-treated at 800 °C showed both HA and α-TCP as major phases with β-TCP as the secondary phase. Although α-TCP and HA are primary phases in potassium and silicon co-substituted HA (KSiHA) nanopowder, the fraction of α-TCP decreased in KSiHA nanopowder in comparison to SiHA nanopowder. The fraction of β-TCP phase increased in the case of KSiHA nanopowder with respect to SiHA nanopowder. With an increase in heat treatment temperature, the calculated weight % of TCP phase increased. The order of structural stability is as follows:

$$\text{FHA} > \text{HA} > \text{ZnFHA} > \text{KSiHA} > \text{ZnHA} > \text{EuHA} > \text{MgSrHA} > \text{SiHA} > \text{SrFHA} > \text{MgSrFHA} > \text{MgHA}$$

Fourier transform infrared spectra were recorded in the region 400–4000 cm^{-1} using KBr pellets (1%, wt/wt) with a spectral resolution of 2 cm^{-1} to identify the various functional groups present in nanodimensional powders of HA such as phosphates, carbonates, hydroxyl, and nitrates. Figure 23.6 shows the characteristic peaks exhibited by the FTIR spectra of as-synthesized nanopowder. The FTIR spectrum of as-synthesized HA nanopowder showed all the specific peaks of pure HA, i.e., phosphate vibrations, ν_1 PO_4^{3-} (962 cm^{-1}), ν_2 PO_4^{3-} (473 cm^{-1}), ν_3 PO_4^{3-} (broad band 1031–1093 cm^{-1}), and ν_4 PO_4^{3-} (569 cm^{-1} and 602 cm^{-1}), and hydroxyl group of hydrogen bonded to OH^- at 3569 cm^{-1} and 631 cm^{-1}. The peak at 874 cm^{-1} could be attributed to P-O-H vibration in HPO_4^{2-}. Broad envelop between 3446 cm^{-1} and 3242 cm^{-1} and absorption bands between 2076-2001 cm^{-1} and at 1636 cm^{-1} could be attributed to O-H and H-O-H vibrations of absorbed water molecules in HA crystal structure, respectively.

The FTIR spectra of ZnHA nanopowder were similar to HA but with decreased strength of OH^- vibrational mode at 3569 cm^{-1} and 630 cm^{-1} and PO_4^{3-}

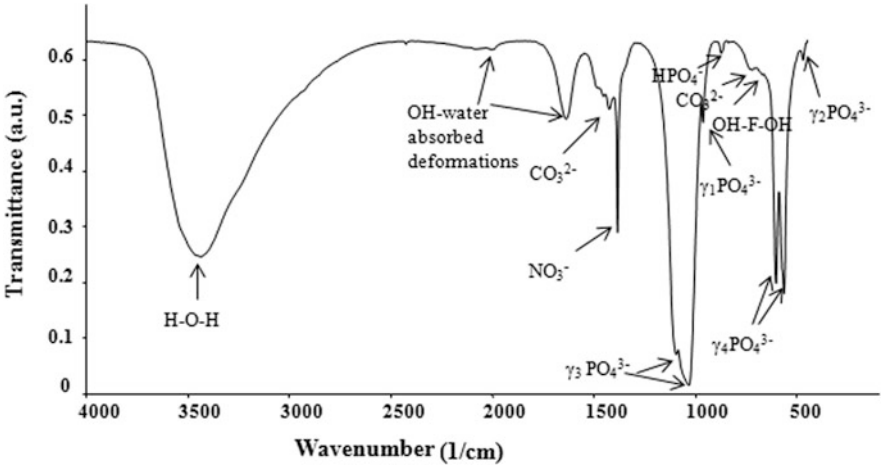

Fig. 23.6 FTIR spectra of as-synthesized fluorine-substituted hydroxyapatite nanopowder

vibrational mode at 569 cm^{-1} and 602 cm^{-1} and 1039 cm^{-1}. Similarly, MgHA showed decreased intensities of OH^- vibrations at 3568 cm^{-1} and 633 cm^{-1} and significant broadening at 700–1700 cm^{-1} for PO_4^{3-}. In EuHA nanopowder, all the characteristic peaks of hydroxyapatite were observed but with decreased transmittance. In FHA nanopowder, OH^- vibrations at 3569 cm^{-1} and 631 cm^{-1} were absent. The characteristic band of OH...F...OH appeared at 721 cm^{-1}. In SiHA and KSiHA nanopowders, bands corresponding to orthosilicate group appeared at 504 cm^{-1} and 892 cm^{-1}, respectively. In MgSrHA nanopowder, phosphate bands of ν_1 PO_4^{3-} and ν_4 PO_4^{3-} shifted towards lower frequency. The weak absorption peak at 875 cm^{-1} attributed to P-O-H vibration of HPO_4^{2-} was also observed. However, the band of OH...F...OH appeared at 716–727 cm^{-1} in ZnFHA, SrFHA, and MgSrFHA nanopowders.

The FTIR spectra of all heat-treated nanopowders showed almost similar patterns with decreased intensities. On heat treatment of ZnFHA nanopowders, the loss in intensity of the hydroxyl groups was observed around 3570 cm^{-1} (Fig. 23.7).

The vibration at 874 cm^{-1} related to P-O-H in HPO_4^{2-} disappeared on heat treatment, whereas peaks of phosphate became strong. In heat-treated ZnFHA nanopowders, bands ascribed to configuration FFOHFF were seen at 740 cm^{-1}. In SiHA and KSiHA heat-treated nanopowders, orthosilicate group (ν_1 symmetric stretch, ~752 cm^{-1}; ν_2 bending, ~504 cm^{-1}; ν_3 asymmetric stretch, ~892 cm^{-1}) was observed.

23.5.3 Thermal Stability of Novel Hydroxyapatites

The thermal behavior of as-synthesized HA and FHA nanopowders was investigated by thermogravimetric (TGA)/differential scanning calorimetry (DSC)/differential thermogravimetric (DTG) techniques. The approximate weight of samples taken

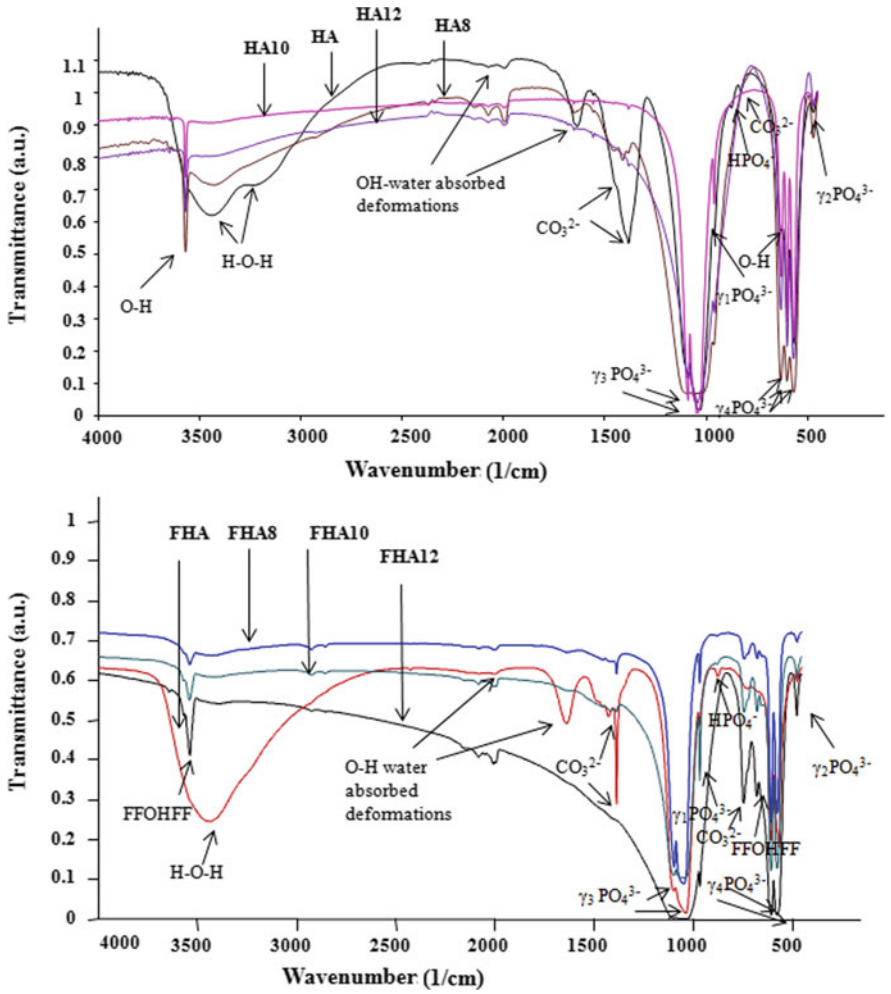

Fig. 23.7 FTIR patterns of heat-treated nanopowders (HA8 and FHA8, HA10 and FHA10, and HA12 and FHA12: powders heat-treated at 800 °C, 1000 °C, and 1200 °C)

for heat treatment was 10 mg. Samples were heated from room temperature to 1000 °C at a heating rate of 10 °C/min. On heating, as-synthesized HA nanopowders exhibited weight loss to different extents (Table 23.7). In general, the weight loss occurred in three steps: the first weight loss is due to dehydration, elimination of nitrogenated substances, and conversion of HPO_4^{2-} into P_2O_5; the second weight loss is owing to reaction of OH^- and P_2O_7 at temperatures between 400 °C and 700 °C, and the third weight loss above 700 °C is suggested due to phase transformations. HA nanopowder showed a total weight loss of 13.98%. There was a small weight loss of about 2.31% above 700 °C, which indicated its high thermal stability. The thermal stability of HA was affected by ionic substitution. Substitution of Zn or Mg in HA showed a decreased thermal stability due to the

Table 23.7 % Weight loss of as-synthesized hydroxyapatite nanopowders

Nanopowder		HA	ZnHA	MgHA	EuHA	FHA	SiHA	KSiHA	ZnFHA	SrFHA	MgSrHA	MgSrFHA
Weight loss, %	Below 400 °C	9.08	5.90	24.8	1.83	10.3	10.9	7.16	12.75	3.54	33.70	5.90
	400°C to 700 °C	2.58	2.84	8.2	3.20	3.31	1.27	2.35	6.41	3.26	1.47	2.84
	Above 700 °C	2.31	1.65	12.00	0.89	2.51	0.8	1.21	5.93	0.73	1.80	1.65
Total weight loss, %		13.97	10.39	35.0	5.92	16.1	12.97	10.72	25.09	7.53	36.97	10.79

formation of β-TCP at 800 °C, whereas no such peak occurred in HA even up to 1000 °C. In contrast, the substitution like Eu and F resulted in an increased thermal stability. Similarly, Si substitution and K and Si co-substitution resulted in an enhanced thermal stability in comparison with stoichiometric HA (Table 23.7). Zn and F co-substitution in HA resulted in a decreased thermal stability with a total weight loss of 25.09%. Co-substitution of strontium and fluorine in HA enhanced its thermal stability. Only 7.56% total weight loss occurred in SrFHA nanopowders. MgSrHA nanopowder exhibited only 1.8% weight loss after 700 °C indicating its higher thermal stability than MgHA. Among all the as-synthesized nanopowders, MgSrFHA nanopowder exhibited highest thermal stability with a total weight loss of 5.28%.

23.5.4 Surface Area of Novel Hydroxyapatites

The novel hydroxyapatites have potential application as implants, and therefore their mechanical properties are important, and these depend on the porosity of powders. The BET surface area of nanopowders was evaluated by N_2 adsorption using Quantachrome Instruments NOVA 2200e Surface Area Analyzer employing Brunauer-Emmett-Teller (BET) method (Joseph and Tanner 2005). The BET equation in linearized form is expressed as:

$$\frac{p}{v(p_0 - p)} = \frac{1}{v_m z} + \frac{z-1}{v_m z}\frac{p}{p_0} \tag{23.6}$$

where z is a constant related to energy of adsorption, p/p_0 is the relative vapor pressure of adsorbate, v is the volume of gas adsorbed, and v_m is the volume of gas adsorbed in a monolayer. The minimum resolution for p/p_0 was 2×10^{-5}. A linear regression of the left side of the BET equation and p/p_0 yielded a slope and intercept from which z and v_m were obtained. Table 23.8 presents the BET surface area of nanopowders as calculated from v_m (Currey 2005). Interestingly, ionic substitution lowered the surface area of HA nanopowders, indicating that substituted hydroxyapatites are denser than stoichiometric HA. The order of the BET surface area of nanopowders is as follows:

HA > ZnHA > FHA > SrFHA > MgHA > KSiHA > SiHA > MgSrFHA > MgSrHA

23.5.5 Bioactivity of Novel Hydroxyapatites

In vitro bioactivity estimation of nanopowders was performed in a simulated body fluid (SBF). The various reagents used in the preparation of SBF are given in Table 23.9. SBF has ionic composition matching human blood plasma (Table 23.10).

Table 23.8 BET surface area of as-synthesized nanopowders

Nanopowder	HA	ZnHA	MgHA	FHA	SiHA	KSiHA	SrFHA	MgSrHA	MgSrFHA
BET surface area (m^2/g)	205	189	106	112	80	93	108	31	47

Table 23.9 Chemical reagents used for the preparation of SBF (Kokubo 1996)

Reagent	NaCl	$(CH_2OH)_3CNH_2$	$NaHCO_3$	$MgCl_2.6H_2O$	$CaCl_2$	KCl	Na_2SO_4	$K_2HPO_4.3H_2O$	1 kmol/m^3 HCl
Amount (g)	7.996	6.057	0.350	0.305	0.278	0.224	0.071	0.228	40 cc + small amount to adjust pH

Table 23.10 Ionic composition of human blood plasma and SBF (Fatehi et al. 2009)

Ion	Na^+	K^+	Ca^{2+}	Mg^{2+}	HCO_3^-	HPO_4^-	Cl^-	SO_4^{2-}
SBF (mmol/L)	142.0	5.0	2.5	1.5	4.2	1.0	147.8	0.5
Human blood plasma (mmol/L)	142.0	5.0	2.5	1.5	27.0	1.0	103.0	0.5

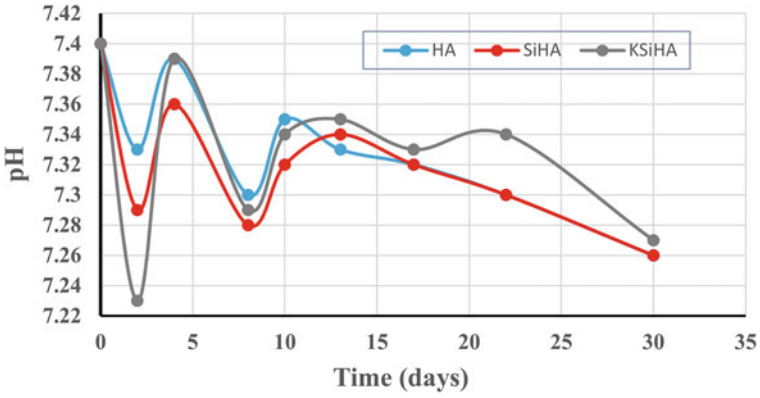

Fig. 23.8 pH change in SBF with time of immersion of as-synthesized nanopowders

To study the in vitro behavior, nanopowders were immersed individually in SBF in polystyrene bottles and were stored in a biological incubator for 30 days at 37 °C. Digital pH meter was used for recording changes in pH of SBF solution at regular intervals. Nanopowders were dried at 70 °C for 4 h in a hot air oven after 30 days. Dried nanopowders were analyzed for apatite formation on their surfaces using FTIR and TEM.

In vitro analysis of nanopowders on immersion in SBF for 30 days almost showed a similar trend of alternate decrease and increase of pH in SBF confirming the bioactive behavior of the nanopowders (Fig. 23.8). However, the variation in pH of SBF with time decreased with an increase in heat treatment temperature, indicating reduced bioactivity of heat-treated nanopowders than as-synthesized nanopowders. The FTIR spectra displayed a significant decrease in the relative transmittance of nanopowders after immersion in SBF, indicating the deposition of an apatite layer on the surface of nanopowders (Fig. 23.9). TEM micrographs also revealed that there was a growth of an apatite layer on the surface of nanopowders (Fig. 23.10), exhibiting bioactive nature of nanopowders upon immersion in SBF.

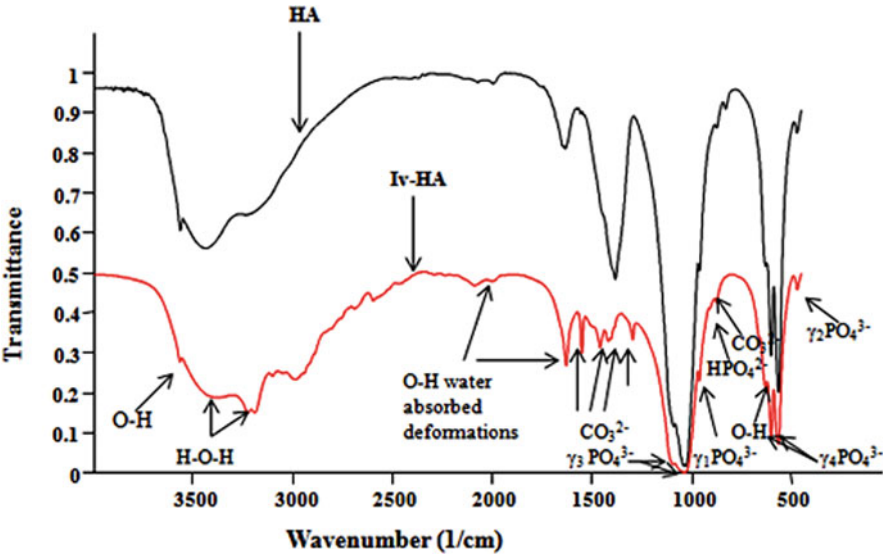

Fig. 23.9 FTIR spectra of hydroxyapatite nanopowder before (HA) and after immersion in SBF (IvHA)

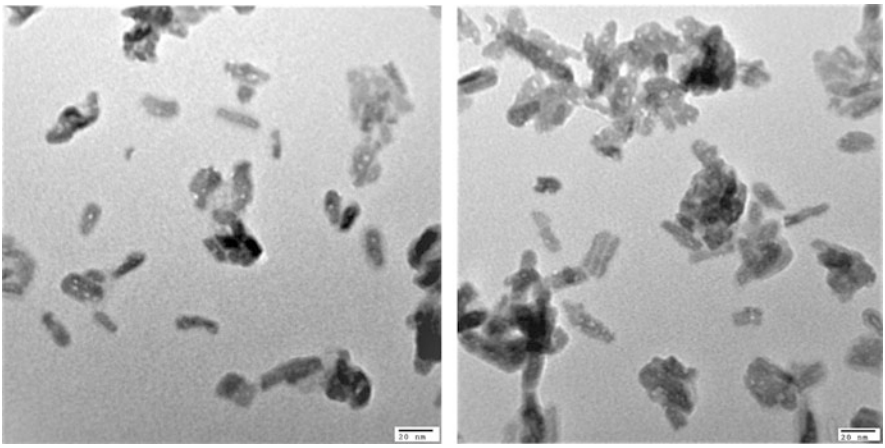

Fig. 23.10 TEM images of nanopowders after immersion in SBF

23.6 Applications of Novel Hydroxyapatite

The applications of novel HA nanopowders are demonstrated from the experimental results of the present work on coating of Zn and F co-substituted HA nanopowder on bio-grade 316L stainless steel. Metals such as stainless steel (SS), Cr-Co alloys, and

Table 23.11 Corrosion parameters from potentiodynamic polarization tests

Sample	OCP (V)	*ba* (V/decade)	*bc* (V/decade)	Ecorr (V)	jcorr (μA/cm^2)	Corrosion rate (mm/year)
Uncoated-316L SS	−0.324	0.335	0.8324	−0.492	20.13	0.23422
HA-316L SS	−0.275	0.123	0.415	−0.470	8.38	0.09732
ZnFHA-316L SS	−0.112	0.060	0.178	−0.136	0.17	0.00202

Table 23.12 Pitting corrosion characterization

Sample	Breakdown potential E_b (V)	Pit protection potential E_p (V)	$\Delta E = E_p - E_b$ (V)
Uncoated-316L SS	−0.143	−0.203	0.148
HA-316L SS	−0.131	−0.141	0.111
ZnFHA-316L SS	0.251	0.158	0.093

Ti alloys are commonly employed for making bone plates (Fadli et al. 2019). However, the use of metals becomes limited due to their poor biocompatibility with their hosts as well as limited organ function and influence on the bioactivity in the body. Metals also cannot regenerate new bone. In addition, when interacting with the host tissue, the corrosion products of the metals cause severe infection and implant failure. Biomaterials or bioceramics including hydroxyapatite coating on the metal surface possess great potential to overcome these drawbacks. Hydroxyapatite plays a double task of inhibiting the release of metal ions, making the metallic implant more corrosion resistant and also promoting bioactivity at the metal surface (Sridhar et al. 1997).

The results of uncoated and coated 316L stainless steel implant with HA and ZnFHA are presented in Tables 23.11 and 23.12. Table 23.11 compares the corrosion parameters from potentiodynamic polarization tests, and Table 23.12 compares the pitting corrosion characteristics for uncoated and coated 316L stainless steel. ZnFHA coated 316 stainless steel implant displayed lower corrosion rate, pitting resistance than uncoated and HA coated 316L stainless steel. The corrosion resistance of ZnFHA coated 316L stainless steel was found to be 115 times better than 316L stainless steel implant. The coating and passive layer both play a role in corrosion resistance.

Figure 23.11 shows the SEM micrograph of ZnFHA coated 316L stainless steel. Figure 23.12 displays the apatite formation on ZnFHA coating on immersion in SBF, indicating the bioactive behavior of coating. Therefore, ZnFHA coated 316L stainless steel can be a good candidate for bio-implant applications especially in bone repairs in dental and orthopedics and osteoporosis treatment.

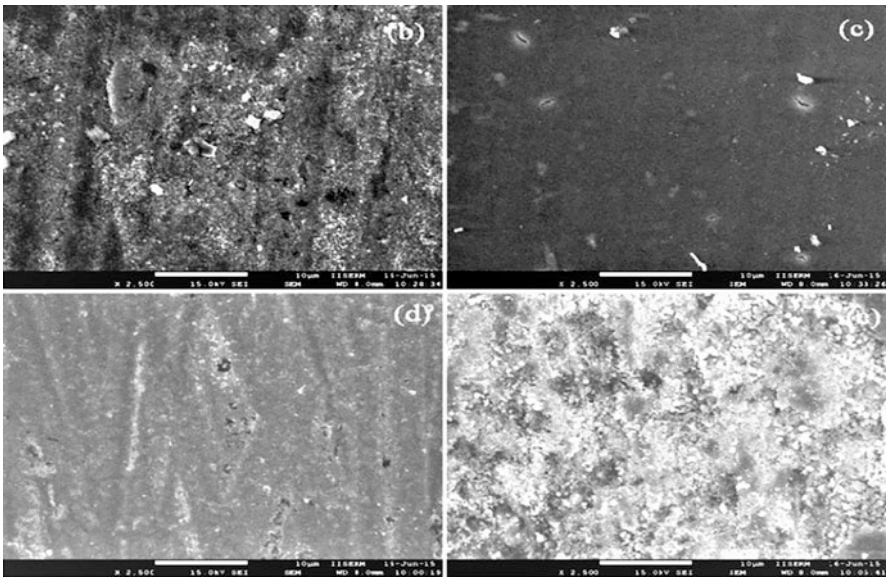

Fig. 23.11 ZnFHA coated 316L SS

23.7 Conclusions and Future Perspectives

This chapter presents findings on stoichiometric HA and HA substituted with ions like potassium, zinc, magnesium, strontium, europium, fluorine, and silicon as single, dual or multi-substituted and synthesized by sol-gel process, followed by suitable heat treatment. Ionic substitution and heat treatment have a significant influence on the crystallite size, crystallinity, lattice parameters "*a*" and "*c*," as well as morphology. In addition, the phase constitution indicating conversion of hydroxyapatite phase to β-TCP and α-TCP phases is affected by both the substitution and the heat treatment. The ionic substitution of HA with elements like F, K, and Zn enhances the structural stability, while Eu, Si, Sr, and Mg substitution results in lowering the structural stability. Furthermore, the substitution of HA with elements like Eu, F, and Si improves the thermal stability of HA, but ionic substitution with Zn and Mg is responsible in lowering the thermal stability. The degradation of ionic substituted powders in SBF during in vitro immersion study clearly indicates the bioactive behavior of the as-synthesized as well as heat-treated nanopowders. The mineralization of nanopowders, during immersion in SBF, resulted in the formation of apatite like phase on the surface of nanopowders. This indicates that the nanopowders are likely to help in promoting bone growth under in vivo conditions as well.

Literature reports that secondary phases form in hydroxyapatite on ionic substitution, due to which there is a significant change in Ca/P molar ratio. Moreover, a

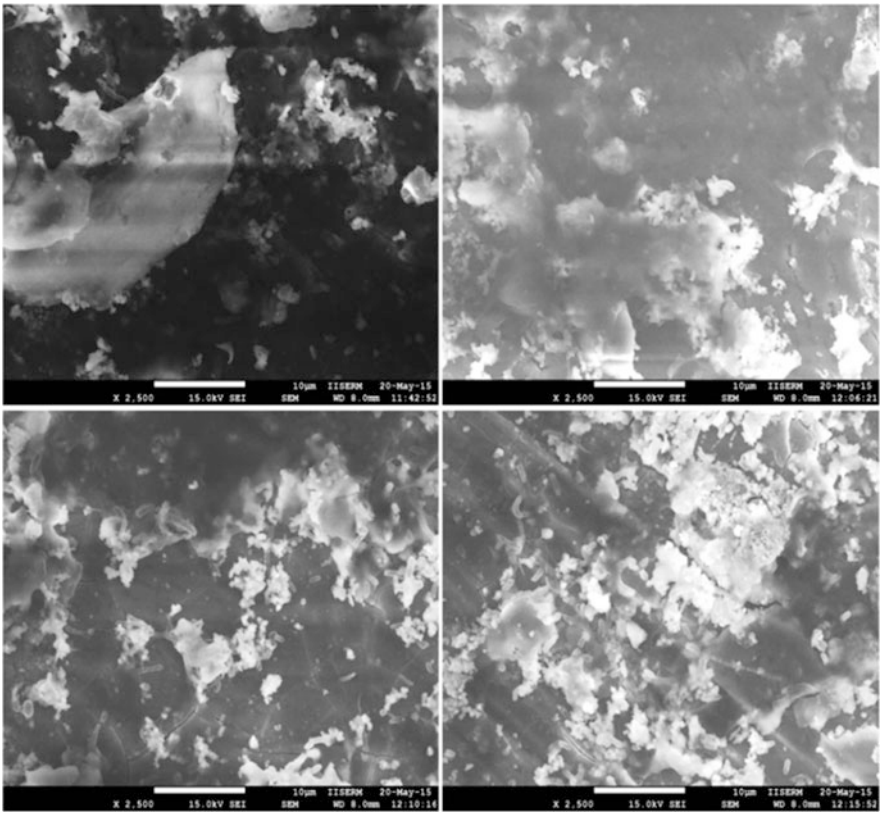

Fig. 23.12 ZnFHA coated 316L SS after immersion in SBF

small change in the Ca/P ratio of HA especially caused by substitution of ions can have a remarkable effect on its properties. In addition, there can be a noticeable effect on the crystallinity of nanopowder by ionic substitution in HA and its heat treatment. These changes are helpful when such nanopowders are used as drug release agents, bone grafts, and bone void fillers to deal with various diseases related to bone infections and injuries. Furthermore, the coatings on implants with ionic substituted HAs also show a great future potential in the protection of biodegradable metallic implants. Interestingly, many researchers have proposed that the ionic substituted HA mimic the natural bone, but their results are far from reality and mimicking of real composition of bone is still not achieved. The natural bone constitutes various trace ions, but the fact is that only one or two ions have been incorporated simultaneously in ionic substituted HAs.

Nevertheless, human bone has high magnesium amount, and it has more biological relevance, but a comprehensive study on its substitution is not available. Although human bone contains a very small amount of Sr, an extensive study on its substitution is reported in the literature. Besides this, various ions like Na, K, etc. are

present in the biological apatite, but these are not substituted in HA intentionally. In addition, the research on ionic substitution seems to be motivated by the possible therapeutic role of ions rather than materials characterization.

It is recommended that an extensive work should be carried out on ionic substituted HAs to fully exploit their potential. Some future scope in this field includes the following aspects:

- Comparison of ionic substituted HAs with stoichiometric HA.
- Effect of different ions on the structure and properties of HA should be compared.
- Composition close to natural apatite should be synthesized.
- Experiments on in vivo examination need to be enhanced.

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Recent Progress in Applications of Magnetic Nanoparticles in Medicine: A Review

24

Renu, Jaspreet Kaur, Sonal Singhal, and Anupama Kaushik

Abstract

Nanotechnology embodies an exceptional combination of conventional and contemporary material sciences to investigate physical matter at the nanometric scale. The unmatched potential of these nanoscale materials which have equivalent size to the biological molecules has come into being since the 1950s. This technology of nanomaterials has transformed the field of medicine through a plethora of applications including biological detection, diagnostic imaging, tissue engineering, drug/gene delivery, etc. Amongst all inorganic and organic nanoscale particles, magnetic nanoparticles have unique characteristics like nontoxicity, biocompatibility, and high level of accumulation in targeted tissues, injectability, and attraction to high magnetic flux density that make them the most common choice for medical applications. This review addresses the current status, advantages and limitations, toxicity, and future scope of magnetic nanoparticles in the area of medicine.

Keywords

Magnetic nanoparticles · Nanometric scale · Medicine

Renu · J. Kaur
Energy Research Centre, Panjab University, Chandigarh, India

S. Singhal
Department of Chemistry, Panjab University, Chandigarh, India

A. Kaushik (✉)
Energy Research Centre, Panjab University, Chandigarh, India

Dr. SSB University Institutes of Chemical Engineering and Technology, Panjab University, Chandigarh, India
e-mail: anupamachemarchive@gmail.com

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24.1 Introduction

Nanoparticles are small structures with a size range of 1–100 nm in at least one dimension, while nanotechnology comprises the engineering of these nanoscale structures at an atomic or molecular level. Nanoparticles can be synthesized or produced from both organic and inorganic materials or can be natural or synthetic. Various organic nanoparticles include polymeric nanoparticles, liposomes, dendrimers, and micelles (Romero and Moya 2012), while inorganic nanoparticles comprise quantum dots, carbon nanoparticles, magnetic iron oxide nanoparticles, etc. (Giner-Casares et al. 2016). These nanoparticles, due to tuning of particle morphology from micro- to nanosize, result in different characteristics compared to their micron-sized counterparts which helps in their versatile applications. The reason for unique and enhanced properties of these nanomaterials is their large surface-to-volume ratio, high surface forces, higher percentage of atoms and molecules on the surface, and quantum confinement effect.

Recently, a large focus has been towards the synthesis of different magnetic nanoparticles (MNPs) with their extensive applications in a large number of fields including biomedicine, biomedical, environmental remediation, and catalysis. As one of unique nanomaterials, these nanoparticles possess not only the general characteristics of nanomaterials but additional advantage of magnetic properties. These nanoparticles exhibit their best performance at a typical size range from 10 to 20 nm. The low-dimensional structures are characterized by superior magnetic moment and emergence of superparamagnetism (Khanna et al. 2018). Superparamagnetism results because of the thermal fluctuations which are sufficiently strong to naturally demagnetize a previously saturated assembly; hence, these nanoparticles display zero coercivity with the absence of hysteresis. Thus, the external magnetic field applied can magnetize the nanoparticles with greater magnetic vulnerability. On removal of the magnetic field, these nanoparticles show no magnetism. Due to superparamagnetic structures, they are able to respond immediately to magnetic fields applied. Moreover, these nanoparticles display large specific surface area, large surface-to-volume ratio, facile separation under magnetic field, and high mass transference, perfect characteristics for application in the field of biomedicine (Niemirowicz et al. 2012). These nanoparticles consist of various magnetic elements including iron, nickel, manganese, chromium, and cobalt and their compounds. One of the distinctive advantages of these nanoparticles is that they can be selectively attached to any functional molecule which allows their transportation under external magnetic field.

Magnetic nanoparticles, owing to their unique properties, are exceptional nanostructures which find applications in all the application areas of medical science including therapeutics, diagnostics, and imaging. There are diverse therapeutic applications of MNPs ranging from delivery of drugs, antimicrobial agents, vaccines, genes, and site-specific targeting to circumvent adverse effects of therapeutics (Parveen et al. 2012). Magnetic nanoparticles can be made biocompatible with surface modification, and hence can be used as vectors, facilitating directional transportation of drugs or genes under the influence of magnetic field to achieve

targeted therapy (Patitsa et al. 2016). It is well established that MNP-based therapy minimizes the side effects of therapy as compared to conventional drug delivery systems like chemotherapy, radiation, or immunotherapy because of their targeted approach. Furthermore, these nanoparticles due to their response towards magnetic field and efficient contrast agents are excellent candidates for magnetic resonance imaging (MRI) (Hola et al. 2015). These nanoparticles can be made to heat up in alternating magnetic field, which leads to their application as hyperthermia agents, conveying thermal energy to tumors, as chemotherapy and radiotherapy enhancement agents (Laurent et al. 2011). Magnetic nanoparticles are excellent candidates for sensors as they can be remotely and noninvasively employed for imaging probes and smart actuators. MNPs can also be integrated into the transducer materials and can be distributed in the samples and removed by magnetic field for active detection on the surface of biosensors.

The MNPs can be aptly envisioned as the future of medical science as they have the potential to become valuable tools for therapeutics, diagnostics, and imaging in the near future. Overall, the research in MNPs will not provide further development of the medical field but exciting applications of MNPs in related areas. This review gives an overview of various applications of MNPs in medicine including drug delivery, magnetic resonance and magnetic particle imaging, sensing, biomarker detection, antimicrobial agents, and regenerative medicine (Moradiya et al. 2019; Tay et al. 2018; Richard et al. 2017; Chen et al. 2017). The overall applications of MNPs are given in Fig. 24.1.

24.2 Magnetic Nanoparticles for Drug Delivery

Drug delivery serves as an alternative therapeutic technique towards the treatment of different human ailments such as cardiovascular disease, cancer, and microbial-attacked places (Hola et al. 2015). The concept of drug delivery encompasses biocompatible approaches and systems for the transportation of therapeutic agents to the specific site of action in the body. Unlike conventional chemotherapeutic agents, drug delivery offers the attractive protocol of targeting drug only to the intended area, thereby reducing the deleterious side effects of the drug to the surrounding healthy cells or tissues. Moreover, drug delivery overcomes the major problem of overdosing/underdosing cycle by releasing the drug in a controlled manner (Mou et al. 2015). For the accomplishment of these goals, the development of suitable vehicles for drug delivery is of utmost importance that can minimize the toxic side effects as well as assists in the enhancement of the therapeutic effect. In this context, MNPs can be harnessed as potent drug delivery vehicles due to their low cytotoxicity, magnetic attraction, target identification, proper drug uptake and release, biodegradability, biocompatibility, and reactive surface that can be easily modified with biocompatible coatings (Kariminia et al. 2016). The drug delivery by MNPs involves three basic steps: first, the immobilization of a drug in MNPs, followed by the introduction of the drug/carrier complex into the system/subject, and finally, the use of high gradient magnetic fields to direct and concentrate the

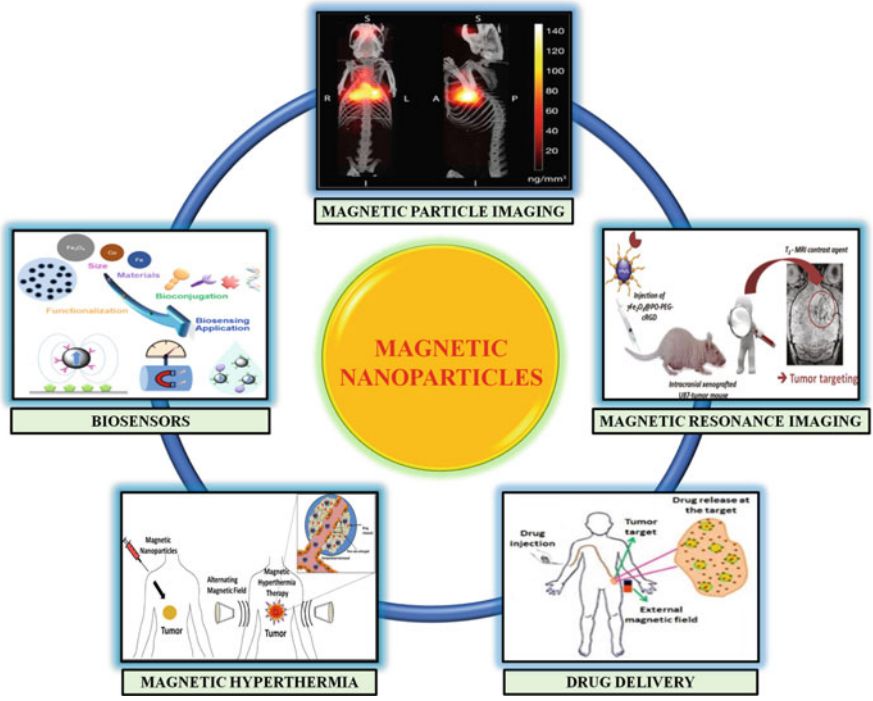

Fig. 24.1 Applications of magnetic nanoparticles in the area of medicine

complex at the affected area. Once the complex is reached at the target in vivo, the therapeutic agent is then released from the magnetic carrier due to specific triggers, such as pH, temperature, osmolality, or enzyme activity (Estelrich et al. 2015; Lungu et al. 2016).

Though, MNPs excellently meet the requirements for drug delivery but, the employment of MNPs for in vivo analysis, the MNPs ought to be stable and soluble in the aqueous environment which can be addressed by the surface functionalization of MNPs by inorganic or organic coating moieties. Following the surface functionalization approach for tailoring the properties of MNPs, Naqvi et al. (2020) fabricated dopamine-coated Fe_3O_4 NPs (Fe_3O_4/SiO_2@DA) for the drug delivery of doxorubicin. The coating of dopamine on the surface of Fe_3O_4 NPs imparts the stability and biocompatibility as well as exposes the hydrophilic sites for the adsorption of drug molecule. Fe_3O_4/SiO_2@DA offers higher loading of the drug and shorter releasing time with drug release percentage of up to 90% of drug (Naqvi et al. 2020). Similarly, Dhavale et al. (2021) synthesized chitosan-coated Fe_3O_4 NPs (MNP-CS), and the utility of the nanocarrier was assessed in the drug delivery of anticancer drug telmisartan (TEL) (Dhavale et al. 2021). Herein, chitosan entails dual performance: as a capping agent and as a bridge for efficient conjugation between the amino groups of MNP-CS and carboxylic group of TEL. The designed MNP-CS exhibited loading of TEL drug capacity around 50. Drug-loaded MNP-CS

nanoformulation demonstrated dose-dependent cytotoxicity towards PC-3 human prostate cancer cells. Similarly, Kariminia et al. also utilized chitosan-coated iron oxide nanocomposites for the pH-sensitive drug delivery of an antibiotic, ciprofloxacin. Herein, in slightly acidic medium, ciprofloxacin was loaded onto the nanocomposite via hydrogen bonding interactions. The in vitro analysis revealed that about 99% of the ciprofloxacin drug was loaded by drug delivery system and for the release of drug, externally applied ultrasound radiations behaved as trigger. These findings indicate that nanocomposites hold the potential to act as drug carriers for the controlled antibiotic delivery in human body (Kariminia et al. 2016).

With a prospective to attain a site-specific drug delivery of doxorubicin in neuroblastoma SH-SY5Y cells, Lerra et al. (2019) synthesized core-shell magnetic nanohybrids wherein graphene oxide (GO) and magnetic iron oxide (MNP) behave as core elements and curcumin-human serum albumin conjugate (C@HSA) as a capping agent. Due to the synergistic effects of GO, MNP, and C@HSA conjugates, the novel nanoplatform holds the ability of improved efficiency in viability assay, controlled release of cytotoxic agent, and enhanced therapeutic effect on cancer cells. Drug releasing experiments revealed the pH-responsive behavior of nanohybrid where higher release amount was observed in acidic medium as compared to neutral medium. The pH-responsive property of the nanohybrid propels the controlled release of anticancer drug into the exact tumor site by change in the surrounding pH environment (Lerra et al. 2019). Similarly, Benyettou et al. employed mesoporous carbon template for the preparation of nanocrystalline iron oxide NPs; loaded by anticancer therapeutic doxorubicin and further drug-loaded NPs was coated by thermoresponsive polymer Pluronic F108 and administrated into Hela cells. Due to the external triggering conditions like pH and temperature, the fabricated drug delivery system was able to release the drug via two modes: slow drug release and burst drug release. Gradual release of drug from the particles occurred in aqueous solution at low pH. For the burst drug release, high-frequency field was applied to induce heating in iron oxide NPs and onset of temperature to 41°C, Pluronic F108 polymer undergoes swelling, and wettability of polymer also changes. This heat-induced change assisted via magnetic hyperthermia ensures the drug delivery of doxorubicin (Benyettou et al. 2016). Yang et al. effectively designed slightly folate (FA)-grafted chitosan-coated magnetic nanoparticles with the addition of tripolyphosphate (TPP) crosslinker which not only avoid side effects of the drug but also ease the controlled release and location of drug at targeted site. The modified MNPs were practically successful for drug release through in vivo experiments using athymic BALB/c mice with human glioblastoma U87 cells in a hypodermal tumor model. It discovered that magnetic guidance of FA-grafted CS-DIX-TPP MNPs extensively decreased the tumor when they were injected through the tail vein (Fig. 24.2; Yang et al. 2017a).

Nowadays, curcumin has been extensively employed in the drug delivery of MNPs for the treatment of breast and ovarian cancer. In this context, Nosrati et al. (2018a) developed nanoscale carrier for curcumin (CUR) based on bovine serum albumin-coated MNPs (F@BSA NPs) via desolvation and chemical coprecipitation process. The cytotoxic effect of F@BSA@CUR NPs on MCF-7 breast cancer cells

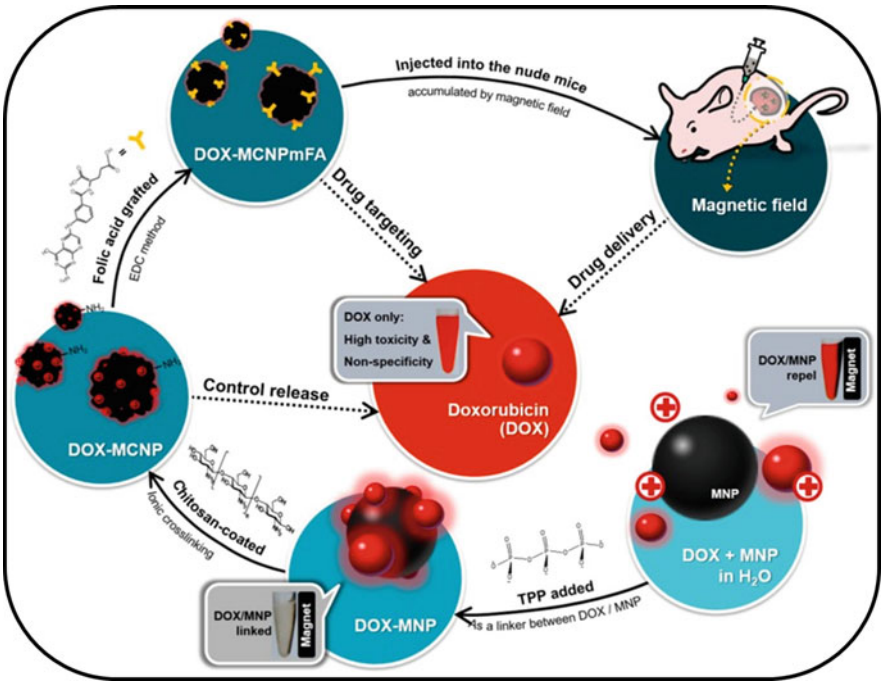

Fig. 24.2 Conceptual scheme of the approach for multitasking drug magnetic carriers. (Reproduced with permission from Yang et al. (2017a) (open access))

was investigated, and it was unveiled that F@BSA@CUR NPs possess much higher cytotoxicity than free CUR, against MCF-7 breast cancer cells owing to sustained drug release in a 96 h incubation time (Nosrati et al. 2018a). Furthermore, Kalita et al. employed magnetic Fe_3O_4@zirconium phosphate core-shell NPs as efficient vehicles for the delivery of curcumin to treat breast cancer (Kalita et al. 2016). A similar study was conducted by Mancarella et al. which involves the designing of functionalized Fe_3O_4 MNPs via coating with two different polymers: dextran and poly(L-lysine). Further, the obtained nanohybrid was subjected to loading of curcumin for the treatment of ovarian cancer (Mancarella et al. 2015).

In drug delivery stream, the transfer of therapeutic agent to the brain is the most challenging approach. But the development of such an approach is the need of the hour due to the growing prevalence of brain cancers and low efficiency of the available treatments. Nosrati et al. managed to develop MRI-monitored paclitaxel (PTX) delivery vehicle across the blood-brain barrier. In the current method, L-aspartic acid-coated IONPs were fabricated, and further these were conjugated by PTX, PEG (polyethylene glycol), and GSH (glutathione) for enhancing the brain delivery of PTX. Furthermore, the biocompatibility, cytotoxicity, and hemocompatibility of the developed drug system were monitored by MRI (Nosrati et al. 2018a).

24.3 Magnetic Nanoparticles for Magnetic Particle Imaging

Magnetic particle imaging (MPI) is an emerging tomographic imaging technique that provides the fast acquisition of 3-D volumes with high temporal resolution for in vivo imaging. The principle of MPI is based on the nonlinear magnetic susceptibility of MNPs (Paysen et al. 2019). In the presence of an oscillating magnetic field, there is a shift in the magnetization of MNPs, which leads to the generation of response signal from MNPs. MPI performance for clinical diagnostics strongly depends on the type of the magnetic material assessed. Amongst a variety of available MNPs, iron oxide NPs (IONPs) have the potential to be used as MPI tracers attributed to its superparamagnetic nature, tendency for magnetic saturation, and nonlinear magnetization curve. Additionally, IONPs are metabolizable and nonradioactive, induce linearly qualitative images, and assist in long-term tracking of targeted cells. As MPI directly locates and produces images of the IONPs in the targeted area, thereby, the concentration of IONPs governs the intensity of the MPI signal obtained (Meola et al. 2019). Moreover, MPI signal is only originated from MNPs without signal contributions from anatomical structures as human body tissues are diamagnetic in nature and thus cannot produce any signal that might be deemed as background noise (Tomitaka et al. 2019). MPI has an edge over other diagnostic techniques as it does not employ any source of radiation for imaging purposes. To summarize, MPI offers a potential biomedical imaging technique with same protection as magnetic resonance imaging, speed as of X-ray computed tomography, and sensitivity that of positron-emission tomography (Khandhar et al. 2017).

These attributes offer a plethora of clinical applications such as cardiovascular imaging, cancer diagnosis, brain injury detection, lung perfusion imaging, and in vivo tracking of magnetically labelled stem cells. Moreover, the in vivo preclinical diagnosis by MPI is reckoned clinically safe due to the biocompatible nature of IONPs and nonemployment of any ionizing source for imaging purposes.

Additionally, MNP, has the potential to label the cells and these MNP-labelled cell can be visualized by MPI, generating three-dimensional view of distributed MNP-labelled cells in the body. On the basis of this, Song et al. tailored Fe_3O_4 NPs encapsulated by fluorescent semiconducting polymers to create Janus Fe_3O_4NPs@semiconducting polymers which were assessed for the in vivo labelling and tracking of cancer cells. In comparison with fluorescence imaging and MRI, Janus Fe_3O_4NPs@semiconducting polymers by MPI offered superior sensitivity, deep tissue penetration, and excellent linearity between the tracer amount and the signal intensity (Song et al. 2018). Similarly, Jung et al. labelled exosomes released by both hypoxic tumor cells and normal tumor cells with superparamagnetic particles. The labelled exosomes were then traced by MPI (Jung et al. 2018). Furthermore, Zheng et al. utilized standard superparamagnetic iron oxide (SPIO) particles for the labelling of mesenchymal stem cells (MSCs) in order to trap them in the target tissue. This experiment was performed on the mice in which labelled stem cells were injected into the mice through the tail vein and then these distributed stem cells were tracked through MPI (Zheng et al. 2016).

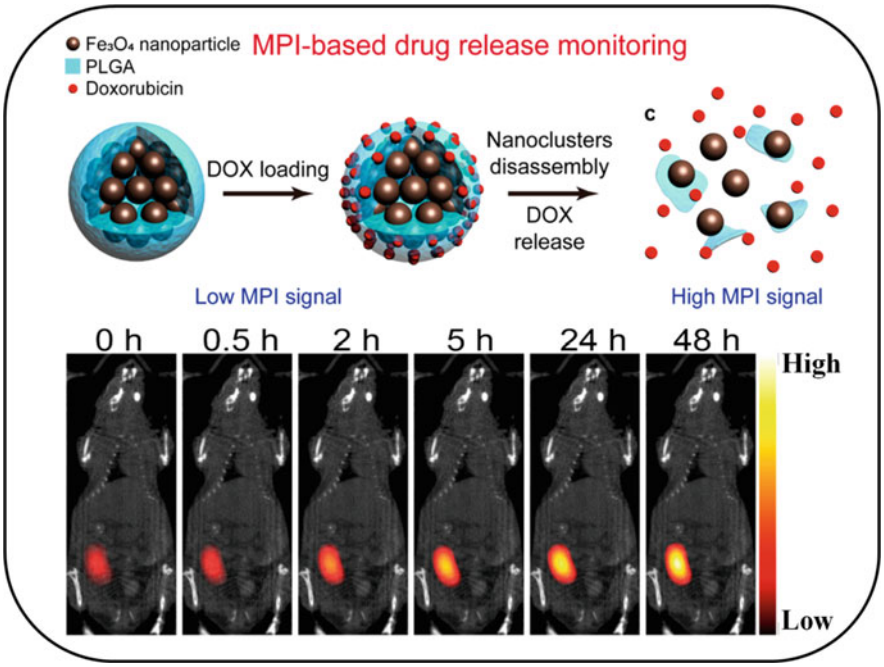

Fig. 24.3 Schematic representation for drug release using Fe_3O_4@PLGA and its MPI-based monitoring in tumor-bearing mice. MPI signal intensity within the tumor gradually increased with time after injection of drug-loaded Fe_3O_4@PLGA into mice. (Reproduced with permission from Zhu et al. (2019))

MPI can also be implemented for in vivo drug release monitoring to guide drug dosing on the desired location. This approach will enable the physicians to track drug doses along with real-time adjustments of doses in order to keep them within permissible limit. Basically, the integration of MPI with drug delivery will aid in visualization and quantitative spatial distribution of drug in the human system. The similar strategy was adopted by Zhu et al. for the monitoring of doxorubicin drug release in a murine breast cancer model. For this purpose, doxorubicin, a chemotherapy drug, was loaded on core-shell superparamagnetic Fe_3O_4 nanocluster@ poly (lactide-*co*-glycolide acid) (Fe_3O_4@PLGA) nanocomposite which can be degraded under mild acidic conditions, thereby releasing the doxorubicin. After the release of the drug, Fe_3O_4 core disassembles allowing the quantification of the drug via MPI. Employment of MNPs aids in significant enhancement of MPI signals as presented in Fig. 24.3 (Zhu et al. 2019).

MPI has been extensively explored in the blood pool imaging as MPI detects only the superparamagnetic moiety in the blood and the surrounding tissues which do not possess MNPs do not contribute any signal in MPI. Thus, MPI generates images that assist in distinguishing blood and the surrounding tissue. Khandhar et al. developed a MPI tracer LS-008 based on the coating of polyethylene glycol (PEG) onto the

surface of SPIO NPs. The PEG coating on SPIO provides an additional advantage to extend the blood half-life time of SPIO NPs. The tracer exhibited superior colloidal stability and persistent intravascular MPI signal for the generation of blood pool tracers for MPI (Khandhar et al. 2017). Moreover, Orendorff et al. presented the first three-dimensional imaging of the initial stage of traumatic brain injury and corresponding hematoma in the closed skull via utilization of SPIO in MPI modality. This study demonstrated the potential of MPI modality reinforced with MNPs in noninvasive diagnosis of internal bleeding for patients suffering from trauma in the emergency setting and thereby, assist in differentiating between mild and moderate injuries. Thus, MPI-based device can be harnessed for the determination of location, severity, and depth of the bleeding from the closed skull (Orendorff et al. 2017).

Additionally, Arami et al. presented a strategy for targeting the cancerous cells using IONPs coated by biocompatible PMAO-PEG copolymer molecules, which were further conjugated to lactoferrin to enhance the tumor-targeting activity of IONPs. Afterwards, external magnetic field was applied and MPI generated three-dimensional images from only nanoparticles that were embedded in tissues, based on their intrinsic magnetic responses. This first preclinical study of cancer-targeted NPs using a MPI system paves the way to explore new strategies for the diagnosis of cancer (Arami et al. 2017).

24.4 Magnetic Nanoparticles for Biosensing Applications

A biosensor is a potential device capable of converting biological event into an easily detectable signal. Biosensors comprise three parts: a biorecognition element (antibodies, nucleic acids, cell receptors, enzymes, etc.), transducer (physicochemical, optical, piezoelectric, and chemical), and signal processor. Firstly, biosensing of analyte entails the attachment of biorecognition element onto the surface of the signal transducer, accompanied by robust interaction of biorecognition element with the target analyte, and, finally, generation of an optical or electric signal by the transducer. Moreover, the biomolecule immobilization is the defining parameter for controlling the performance of biosensor.

In this regard, MNPs provide a suitable platform for the immobilization of enzymes/biomolecules owing to their high surface area, biocompatibility, and easy amenability of surface functional groups. The efficacy of MNP towards in vivo studies can be epitomized basically by three strategies. The first strategy involves magnetic preconcentration of an analyte where one can preconcentrate the analyte via interaction between MNPs and analyte. In this manner, analyte bonded to MNPs can be attracted onto the surface of the sensor with the application of gradient magnetic field, and thus, analyte can be detected with minimal interferences from the sample matrix. Another method emphasizes the involvement of functionalized MNPs as tags for the visualization and selective detection of immunocomplexes with a target analyte. The third approach involves the integration of MNPs into the transducer material or the surface functionalization of the sensor for the amplification of the output signal (Farka et al. 2017).

Selectivity for biosensors is really an imperative parameter for clinical diagnostics as rapid and accurate reading of analyte is very crucial for the treatment of different ailments. MNPs come out as a potential sensing material for the selective and sensitive detection of different biological moieties. In order to detect glucose in human blood serum, an enzyme-free biosensor was developed by Yang et al. (2017b) based on the electrochemical response of the hierarchical porous Co_3O_4/graphene (Co_3O_4/GR) microspheres. The synergistic effect of Co_3O_4 and graphene leads to high performance for glucose detection in terms of high sensitivity, remarkable stability, and superior selectivity (Yang et al. 2017b). Similarly, Pakapongpan and Poo-arporn (2017) developed an electrochemical biosensor for the detection of glucose which was constructed by the self-assembly of glucose oxidase on reduced graphene oxide-Fe_3O_4 (RGO-Fe_3O_4) nanocomposite; further modified on magnetic screen-printed electrode (Pakapongpan and Poo-arporn 2017).

In addition to this, Chauhan et al. (2017) designed a novel electrochemical biosensor for the estimation of a neurotransmitter, acetylcholine. For this purpose, FTO electrode was modified via poly(3,4-ethylenedioxythiophene) which was further covalently linked with rGO and Fe_2O_3 NPs. The constructed sensor was appraised for desired sensing, and many remarkable features were observed including significant selectivity, fair analytical recovery, multiple reusability, fast response, wide linear range, and a low detection limit of 4.0 nM (Chauhan et al. 2017).

A novel fluorescence biosensor based on aptamer was designed by Wu et al. (2015) for the determination of chloramphenicol. In the sensing probe, the biosensor utilized upconversion nanoparticles as signal labels and aptamer-conjugated MNPs as recognition and concentration moiety (Wu et al. 2015). The fabricated biosensor displayed sensitive, selective, and rapid sensing of chloramphenicol. Mikani et al. constructed an electrochemical biosensor with magnetic nanocomposite Fe3O4@SiO2@NH2 as a biosensing platform to quantify urea in human blood. The biosensor exhibits superior sensing attributes such as wide linear range from 5 to 210 mg/dL and a LOD of 3 mg/dL for urea (Mikani et al. 2017).

Pang et al. presented a Fe_3O_4@Ag magnetic nanoparticle-functionalized SERS biosensor for the ultrasensitive detection of microRNA biomarker present in cancerous cells. They followed the preconcentration strategy via utilizing the superparamagnetic nature of Fe_3O_4, thereby assisting in concentrating and capturing the target microRNA. The presented biosensor showed detection limit as low as 0.3 fM which is quite desirable in clinical analysis (Pang et al. 2016).

Recently, Chauhan et al. proposed an electrochemical immunosensor based on Fe_3O_4/polyacrylonitrile fiber (Fe_3O_4-PANnFs) composite which was electrospun onto the surface of the indium tin oxide (ITO)-coated glass electrode. The hydrolyzed Fe_3O_4-PANnFs/ITO electrode was used as an immobilization matrix for covalently attaching the monoclonal antibody specific to vitamin D_3 by using EDC-NHS chemistry. The immunoelectrode showed a limit of detection of 0.12 ng/mL and was functional within a wide detection range of 10–100 ng/mL (Chauhan et al. 2018).

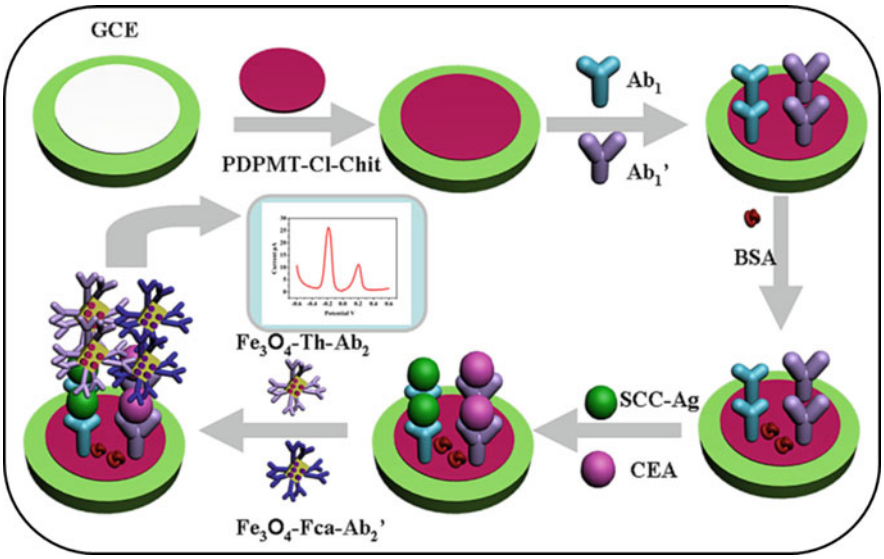

Fig. 24.4 Schematic representation of preparation of immunosensor. (Reproduced with permission from Zhang et al. (2014))

MNPs either individually or in integration with other signal mediators can also be exploited as magnetic tags for the amplification of an analytical signal; for example, Zhang et al. proposed the simultaneous detection of two tumor markers; squamous cell carcinoma-associated antigen (SCC-Ag) and carcinoembryonic antigen (CEA) were achieved by the electrochemical determination of two MNP-based probes on the electrode surface as shown in Fig. 24.4. For the accomplishment of this strategy, the primary antibody was immobilized on poly(1-methyl-3-(1-methyl-4-piperidinylmethylene)thiophene-2,5-diyl chloride)-modified GCE electrode, and the differently surface-functionalized Fe_3O_4 NPs (thionine and ferrocenecarboxylic acid) were associated with secondary antibody to fabricate labels. During the electrochemical analysis, two distinct peaks were obtained, thereby providing simultaneous determination of analytes (Zhang et al. 2014).

24.5 Magnetic Nanoparticles for Magnetic Resonance Imaging

With the increase in tumor morbidity rates, it has become imperative to detect these at an early stage so that a timely treatment can be provided. Magnetic resonance imaging (MRI) is a very important noninvasive imaging technique extensively used in diagnostics based on high soft tissue contrast with ultrahigh spatial resolution for the detection of proton relaxation in an externally applied magnetic field. The protons present in water and lipid molecules majorly contribute to the MRI signals where MNPs act as contrast agents to provide strength to the signals. For practical

MRI applications, high Ms. magnetic materials that possess superparamagnetism along with high stability in the biological environment are required. To enhance the specificity of contrast agents, the surface of the MNPs is modified with functional group that is specific to the receptor binding site. Wu et al. synthesized porous carbon-coated magnetite nanoparticles (PCCMNs) by one-pot solvothermal method. The PCCMNs were later on modified with hyaluronic acid to specifically target CD44 receptors present on the surface of various cancer cells. Moreover, these hyaluronic acid modified PCCMNs not only used for in vivo MR imaging and fluorescent cell imaging but can also act as a drug carrier, and thereby it can provide a versatile platform for cancer theranostics (Wu et al. 2019). Additionally, Khmara et al. choose natural polymer chitosan to modify Fe_3O_4 NPs which not only enhance the stability of the MNPs but also make the NPs more biocompatible. The authors demonstrate even after surface modification the MNPs retain their high Ms. and thus certainly provide strong potential as MRI contrast agent (Khmara et al. 2019). Similarly, Esmaeili et al. produced dendrimer-like structure on the surface of the Fe_3O_4 NPs using (3-aminopropyl)triethoxysilane and polyamidoamine with step-by-step addition of methyl acrylate and aminosilane in a cycling manner. The dendrimer-modified MNPs were shown to be nontoxic even at concentration as high as 100 μg/mL and excellent efficacy for MR imaging and MH applications (Esmaeili et al. 2019). Xiong et al. produced novel MNPs in which oleic acid-tailored Fe_3O_4 NPs were modified with polylactic acid-polyethylene glycol-D-glucosamine (Fe_3O_4@OA@PLA@PEG@DG), and these were found to exhibit great potential application as MRI contrast agents for tumor imaging (Xiong et al. 2017). Cha et al. proposed star polymers that were composed of β-cyclodextrin core and poly(2-(dimethylamino) ethyl methacrylate arms to be used for surface modification of Fe_3O_4 NPs. As compared to linear polymer, star polymers not only strengthen the MRI signals but also provide stability to the MNPs in the biological environment (Cha et al. 2017). Atabaev et al. studied the potential of polyethylene glycol-coated dysprosium-doped Fe_3O_4 MNPs for MRI applications. A moderate doping with dysprosium into the Fe_3O_4 lattice enhanced the magnetization and consequently improved the sensitivity of MRI signals (Atabaev 2018). Gholibegloo et al. developed a novel theranostic system composed of cyclodextrin nanosponge polymer anchored on the surface of Fe_3O_4 NPs (Fe_3O_4/CDNS NPs). Further, Fe_3O_4/CDNS NPs were modified with folic acid that acts as a targeting agent. Moreover, the system Fe_3O_4/CDNS-FA showed selective cytotoxicity and excellent MRI contrast efficiency along with smart drug release capability (Gholibegloo et al. 2019). Arsalani et al. extracted natural rubber latex (NRL) from *Hevea brasiliensis* and used it as a covering agent for Fe_3O_4 NPs. Magnetization was found to be increased upon increasing the NRL content in Fe_3O_4 NPs, and thus NRL can be considered as an effective natural biocompatible stabilizing agent to be used for improved MRI applications (Arsalani et al. 2019). Su et al. developed magnetic hybrid composed of Fe_3O_4 NPs and Schiff base containing dextran nanogels to be employed for biomedical applications. Magnetization capability increased dramatically after encapsulating the Fe_3O_4 NPs into the dextran nanogels. Results obtained from MRI studies suggest that the presence of multiple aldehyde groups and Schiff base

linkages on the hydrogel matrix allows simplistic design of a multifunctional nanoplatform as the MRI-guided drug delivery system (Su et al. 2019). Nosrati et al. synthesized two different amino acid (L-lysine and L-phenylalanine)-modified Fe_3O_4 NPs. The authors revealed that the modified MNPs worked well as contrast agents in the early diagnosis of tumor cells using MRI. Also, these modified MNPs provide a suitable and appropriate system for delivery of anticancer drug (curcumin) to breast cancer cells (Nosrati et al. 2018b).

24.6 Magnetic Nanoparticles for Hyperthermia Treatments

Hyperthermia is a conventional noninvasive method for the treatment of cancer with the aid of high temperature (Obaidat et al. 2019). When the cancerous cells exposed to high temperature (above 41 °C), fluidity and the permeability of the cell membrane increases whereas the rate of production of nucleic acid and protein decreases. The exposure to high temperature induces protein denaturation and ultimately destructs cancerous cell. However, extensive heat is required to be applied that may cause negative effects on healthy tissues. Thus, to overcome these side effects, plentiful heat must be transferred only to the targeted cells, i.e., tumor cells. Magnetic hyperthermia (MH) employs the MNPs which produce heat in the local region of the tumor cells through vibration or rotation stimulated by altering magnetic field (AMF). Thus, MH overcomes the drawbacks of local hyperthermia where heat is transferred directly to the cancerous cells thereby minimizes the side effects and allows for deeper penetration to cancer cells. For practical applications of MNPs in MH, nanoparticles must possess sufficiently large M_s that will produce enough heat in cancer cells upon exposure to AMF. Besides large M_s, another requisite is that the MNPs should be superparamagnetic so that in the absence of the external magnetic field, MNPs lost their magnetism and thus ensure their colloidal stability. Amongst the various available MNPs, superparamagnetic iron oxide nanoparticles such as magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3) are very popular candidates to be used for MH applications because of their good magnetic properties, ease of commercial availability, biocompatibility, and biodegradability. The magnetic properties of these MNPs can be tailored by (1) varying the method of synthesis, (2) changing the size and shape, and (3) modifying the surface functionalizing moieties. Kubovcikova et al. made use of poly-L-lysine to improve the stability and biocompatibility of Fe_3O_4 nanoparticles. The modified MNPs were subsequently attached with the specific antibodies for the detection of tumor cells mediated by the antibodies. The results have confirmed the potential of the synthesized nanoparticles for the combined detection of the antibody-derived tumor cells and their therapy in combination with MRI and hyperthermia (Kubovcikova et al. 2019). Ramos-Guivar et al. synthesized completely nontoxic low-cost maghemite (γ-Fe_2O_3) nanoparticles embedded in a nanohydroxyapatite matrix to study their potential for the MH application. MH experiments have shown that the heating response tends to increase by increasing concentration of the modified MNPs in water (Ramos-Guivar et al. 2020). Recently, Soleymani et al.

proposed the low-cost hyaluronic acid-coated Fe_3O_4 nanoparticles (Fe_3O_4@HA NPs) that could act as a multifunctional platform for diagnostic and therapeutic applications. Hyaluronic acid simultaneously acts as a biocompatible coating layer and as a targeting ligand CD44 receptor present on the surface of breast cancer cells. The authors have synthesized highly uniform-sized particles along with colloidal stability for more than 3 months at biological pH. Moreover, Fe_3O_4@HA NPs were found to exhibit almost negligible toxicity, high heating efficacy, and 50 times higher potential for MH applications as compared to commercially available Fe_3O_4 NPs (Soleymani et al. 2020). Fotukian et al. successfully prepared monodispersed $CuFe_2O_4$ NPs using triethylene glycol as a solvent, reductant, and stabilizer. The $CuFe_2O_4$ NPs were found to exhibit higher heat generation capability as compared to bare Fe_3O_4 NPs that could be attributed to the lower anisotropic energy of $CuFe_2O_4$ NPs leading to higher Ms. (Fotukian et al. 2020). Umut et al. proposed the coprecipitation method for the synthesis of uniformly distributed tetramethylammonium hydroxide (TMAH)-coated $NiFe_2O_4$ nanoparticles to be used as theranostic agents (Umut et al. 2019). Darwish et al. prepared magnetic nanoparticles coated with oleic acid (MNPs-OA) and studied the effect of viscosity of the carrier on hyperthermic properties. They found higher heating efficacy of the MNPs-OA in less viscous aqueous medium as compared to more viscous ethanol medium (Darwish 2017). Linh et al. reported well-dispersed, nontoxic, and biocompatible dextran-coated Fe_3O_4 NPs which were found to be appropriate for hyperthermia application. They clearly demonstrate that magnetic interactions between coated nanoparticles strongly influence induction heating efficiency. Reducing the Fe_3O_4 NPs concentration helps to decrease dipolar interaction field acting on colloidal particles resulting into the easier movement of the colloidal particles contributing to increasing heat capacity (Linh et al. 2018). Wang et al. reported a highly efficient novel material HPMC/Fe_3O_4 composed of hydroxypropyl methylcellulose (HPMC), polyvinyl alcohol, and Fe_3O_4 for the MH ablation of tumors. The proposed material is believed to promote bench-to-bed translation of MH technology besides bringing a new concept for biomaterial research field (Fig. 24.5) (Wang et al. 2017). Fopase et al. studied the effect of yttrium concentration on the MH properties of Fe_3O_4 nanoparticles. Doping of yttrium ion in the Fe_3O_4 nanoparticles helps to tune the structural and hence magnetic properties of later such that highest Ms. observed for a 0.5 molar ratio of yttrium. The synthesized nanoparticles were explored for in vitro MH studies to ensure its suitability for MH-based cancer treatment (Fopase et al. 2020).

24.7 Toxicity Associated With MNPs

MNPs are the potential elements in the clinical diagnosis and treatment of different ailments. However, it is equally crucial to examine that whether these MNPs are targeting the desired infected area or unnecessarily affecting the surrounding healthy cells and thereby prompting long-term health issues. Moreover, when MNPs are incorporated into the therapy and transplanted within the body, their behavior can be

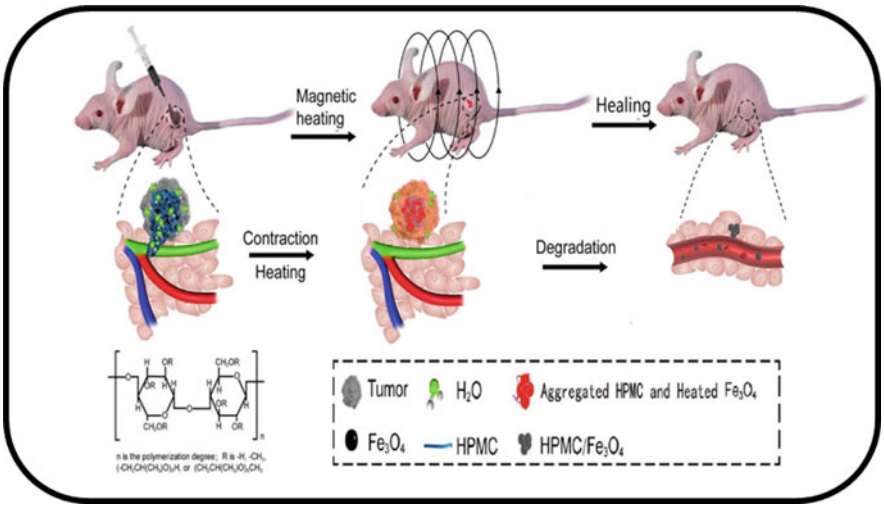

Fig. 24.5 Employment of HPMC/Fe_3O_4 for the magnetic hyperthermia ablation of tumors. (Reproduced with permission from Wang et al. (2017))

predicted not only from its properties but also by the mechanism by which MNPs operate that may interfere with the biological activities (Malhotra et al. 2020). Therefore, it is imperative to understand the toxic traits associated with MNPs. The toxicity of MNPs depends on the number of parameters including, chemical, physical, and structural properties of MNPs, amount of dose and intended usage of MNPs (Markides et al. 2012). Some studies have shown that appraisal of MNP in vivo studies can lead to some toxic effects such as oxidative stress, mutagenicity, genotoxicity, mitochondrial damage, and vascular embolism. This may arise due to the agglomeration of MNPs. Rare side effects include headache, back pain, hypotension, and urticaria (Zamay et al. 2020). Bourrinet et al. assessed the toxicity of MNPs via performing experiments on animal models and concluded that a concentration of $\leq$100 mg Fe/kg did not cause any toxic effects. Moreover, when MNPs were employed for diagnostic purposes in the range of 20–50 mg of Fe, it did not cause any toxic effects in humans (Meola et al. 2019; Bourrinet et al. 2006). However, suitable approaches could be developed to make MNPs biocompatible via surface passivation with natural polymers, amino acids, aptamers, etc. in order to minimize the toxicity to obtain a reasonable benefit-to-risk ratio.

24.8 Conclusion

The present study reviewed the importance of MNPs for the advancement of technology in the field of biomedical applications. MNPs are exponentially gaining popularity for clinical diagnosis and treatment using biosensing, targeted drug

delivery, magnetic hyperthermia, MPI, and MRI owing to their biocompatibility, biodegradability, high stability, and most importantly ease of maneuvering them using an external magnetic field. Moreover, the physiological and chemical properties of MNPs can be tailored according to the requirement by altering the method of synthesis and surface coatings. MNP-based drug delivery allows the MNPs to accumulate at specific locations such as tumor cells and enables demolition by releasing drug at the targeted cells without affecting the healthy cells. This MNP-based targeted therapy helps to mitigate the side effects and drug resistance as well as assist in the invasive medical interventions. MNP-based imaging is one of the most adaptable imaging techniques as it provides high-resolution images and allows diagnosing and grading diseases such as cancer at its earlier stage. In spite of several advantages in nanomedicine, MNPs are also associated with some limitations such as weak magnetization of biocompatible MNPs and requirement of the large-size MNPs to be applied for MH applications that causes difficulty in their excretion from the body. Thus, the future challenges will certainly involve the development of superparamagnetic nanoparticles of the optimized size that allows for stronger magnetic responsivity while maintaining their biocompatibility and enabling enhanced renal clearance.

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Chimeric Antigen Receptor T Cell Therapy: A Cutting-Edge Therapy for Multiple Myeloma

25

Eshu Singhal Sinha

Abstract

Despite advancements in treatment options for multiple myeloma, it still remains an incurable malignancy due to relapse and development of refractory disease. There is a need to develop therapeutic strategies that can combat the failure of currently available therapies. Immunotherapy using chimeric antigen receptor-engineered T cells is changing the outcome of multiple myeloma. These engineered anti-myeloma T cells express the chimeric receptors that target specifically myeloma cells through binding to B cell maturation antigen, CD19, CD138, signaling lymphocytic activation molecule 7 (SLAM7), or immunoglobulin light chains.

Keywords

Multiple myeloma · SLAM7 · Chimeric antigen receptors · B cell maturation antigen · Immunoglobulin light chains · CD138

25.1 Introduction

Multiple myeloma (MM) is a hematological malignancy caused by the cancerous transformation of B lymphocytes in which some plasma cells multiply abnormally within the bone marrow and interfere with the production of normal plasma cells. The malignant plasma cells also invade the solid bone structure and accumulate in the bone cavity to form multiple tumors. Hence, this disease is referred to as MM. Nearly 60% of the patients affected with MM develop myeloma bone disease

E. S. Sinha (✉)
Department of Biotechnology, Panjab University, Chandigarh, India
e-mail: eshu04@gmail.com

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due to uncoupling of the balance between bone formation and resorption, with an increase in the number and activity of osteoclasts and a decrease in the number of osteoblasts leading to enhanced osteoporosis and the development of lytic lesions (Coleman 1997; Walker et al. 2014). All these factors account for the key symptoms of MM including bone pain, pathological fractures, weight loss, spinal cord compression, hypercalcemia, kidney problems, frequent infections, and death (Coleman 1997; Croucher and Apperley 1998).

Treatment advancements for the past two decades have led to the development of many new drugs for MM including immunomodulatory agents (e.g., thalidomide and pomalidomide), proteasome inhibitors (e.g., bortezomib), histone deacetylase inhibitors, and monoclonal antibodies (e.g., daratumumab and elotuzumab). The incorporation of several agents alone or in combination into the MM treatment has resulted in improved survival and has dramatically changed the outcome of MM patients. However, despite these advanced therapies, MM still remains an incurable malignancy. Most of the MM patients eventually relapse and develop refractory disease. Since MM constitutes 1% of all malignancies and 10% of all hematological malignancies (Rajkumar et al. 2014; Vincent Rajkumar 2014), therefore, there is a need to develop alternative effective treatment strategies that can combat chemotherapeutic resistance in MM.

Immunotherapy has emerged as a promising new strategy to eliminate cancer cells. This strategy engages the host immune cells to produce its effects and relies on target antigen specificity. The immunosuppression of T cells in MM emphasizes the need for establishing treatments aimed at enhancing T cell anti-MM activity. Chimeric antigen receptor T cell therapy is a subtype of autologous adoptive cell immunotherapy in which T cells are taken from the patient, modified ex vivo, expanded, and then reinfused back to the patient. For this, the host T cells are engineered to express a new chimeric antigen receptor (CAR) (Fig. 25.1). Hence, this immunotherapy is referred to as chimeric antigen receptor T cell (CAR-T) therapy.

25.2 What Are Chimeric Antigen Receptors?

CARs are the engineered receptors that bind to a desired antigen and redirect the engineered effector cells to a specific target cell (myeloma cells in case of MM). Basically, CAR is a fusion construct with an extracellular antigen recognition domain, a spacer, a transmembrane domain, and a CD3ζ intracellular domain for signaling to stimulate T cell activation upon antigen binding (Fig. 25.2) (Jensen and Riddell 2015; Van Der Stegen et al. 2015). The extracellular antigen recognition domain of CAR is composed of variable region of the heavy (V_H) and light (V_L) chains of an immunoglobulin moiety. In contrast to the natural T cell receptors, CARs bind unprocessed antigens expressed by the target cells. This is noteworthy in view of the fact that similar to most cancers, MM is associated with downregulation of major histocompatibility complex and antigen presentation. Notably, an intracellular costimulatory signaling domain is also included in CAR T cells to mimic the

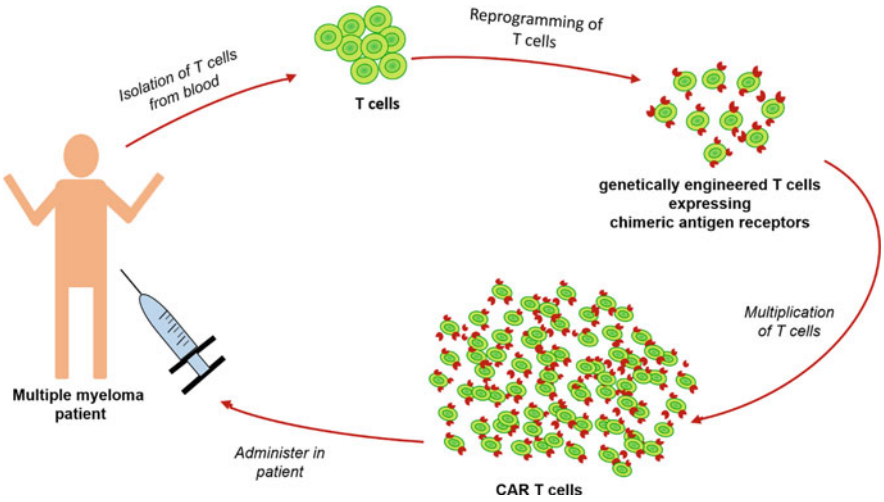

Fig. 25.1 Chimeric antigen receptor T cell (CAR-T) therapy. In CAR-T therapy, initially the T cells are collected from the patient's blood. Subsequently, these autologous T cells are genetically engineered to express CARs on the cell surface. The engineered CAR T cells are expanded in the laboratory to generate millions of CAR-transduced T cells, which are then infused in a process similar to blood transfusion in the MM patients

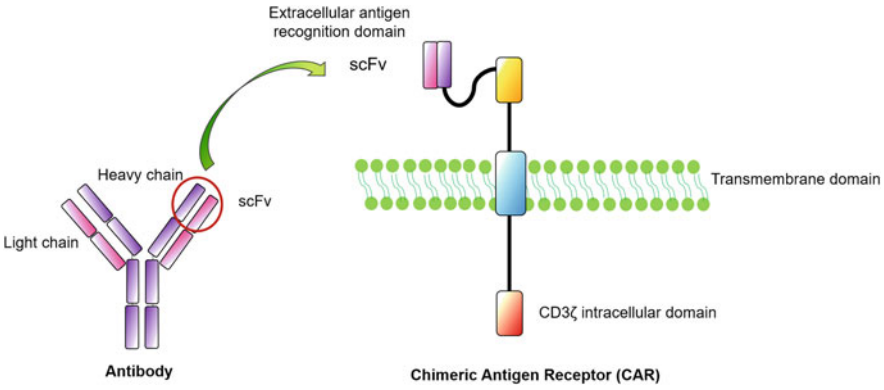

Fig. 25.2 Chimeric antigen receptor

second activator signal of a physiological T cell response (Van Der Stegen et al. 2015).

25.3 Procedure for CAR-T Therapy

CAR T cell therapy is a complex and multistep process in which initially the patients are evaluated through different tests to determine if CAR-T therapy is an appropriate treatment option. Subsequently, T cells are collected from the patient's blood. These autologous T cells are then genetically engineered to express CARs on their surface. The engineered T cells are expanded by growing those in the laboratory. When millions of CAR-transduced T cells are generated, the cells are infused in a process similar to blood transfusion (Fig. 25.1).

25.4 Targets for CAR-T Therapy

Choosing the right target is critical for the success of any therapy. Cancer being a heterogeneous disease contributes to the complexity in choosing the right target for CAR-T therapy. The antigens to be targeted must be expressed specifically on MM cells to avoid off-target side effects as are associated with most of the chemotherapeutics. Several tumor antigens are studied and are in clinical trials for CAR-T therapy for MM including B cell maturation antigen (BCMA), CD19, CD138, signaling lymphocytic activation molecule 7 (SLAM7), and immunoglobulin light chains.

25.4.1 B Cell Maturation Antigen

B cell maturation antigen (BCMA) is expressed in a subpopulation of B cells, normal plasma cells, and myeloma cells but not in other hematological cells like hematopoietic stem cells or normal tissues (Tai and Anderson 2015). BCMA is also absent on naive and most memory B cells (Xu and Lam 2001). An advantage of using BCMA as an antigen for CAR-T therapy is reduced off-target toxicity. However, a major disadvantage is that B cell tumors release/shed soluble BCMA into the surrounding tissues and blood which can negatively affect the recognition of $BCMA^{+}$ MM cells by BCMA-specific CAR T cells (Sanchez et al. 2016). Despite this, the demonstrated involvement of BCMA in MM development makes it the most popular target for MM CAR-T therapy. In MM, CAR T cells targeting BCMA have been investigated in a number of studies and have demonstrated important disease-remitting activity.

25.4.2 CD19

CD19 is a member of immunoglobulin superfamily and acts as a dominant signaling component on the surface of mature B cells. However, CD19 expression is lost in plasma cells. CD19 is rarely expressed on MM cells, but studies have revealed the expression of CD19 on MM stem cell subset. The MM stem cells are a distinct

population of tumor cells that are responsible for self-renewal and drug resistance (Johnsen et al. 2016). Besides MM, CD19 has been demonstrated to be present in many B cell malignancies including acute lymphocytic leukemia and chronic lymphocytic leukemia. This makes CD19 a potential target for CAR-T therapy in B cell malignancies including MM. Interestingly, CAR T cells targeting CD19 when used in conjunction with melphalan and autologous stem cell transplantation have shown therapeutic activity in relapsed/refractory MM (RRMM) (Garfall et al. 2015). These dual-target CAR T cells are in clinical trials for RRMM. Due to high response rates in patients, two CD19-engineered CAR T cell products, axicabtagene ciloleucel (Yescarta, Kite) and tisagenlecleucel (Kymriah, Novartis), have already been approved by the FDA for therapy of advanced B cell malignancies.

CD19- and BCMA-targeted CAR-T combination trial has shown significant results in RRMM. The CAR used in this study contains an anti-BCMA and anti-CD19 single chain antigen recognition fragment, cytoplasmic portion of the OX40 and CD28 costimulatory moiety, and a CD3ζ domain for signaling (Lingzhi et al. 2017).

25.4.3 SLAMF7

SLAMF7 (also known as CD319 or CS1) is an antigen abundantly expressed on the surface of activated B cells, monocytes, normal and neoplastic plasma cells, NK cells, some $CD8^+$ T cells, and dendritic cells. Since it is absent on non-hematologic organs and hematopoietic stem cells, SLAMF7 is under intense investigation as a target for CAR-T therapy in MM. Elotuzumab, a monoclonal antibody against SLAMF7, has gained FDA approval for the treatment of MM. SLAMF7-specific CARs have proven to be highly cytotoxic for MM tumor cells (MM cell lines and primary MM cells) when tested in vitro, ex vivo, and in MM xenograft mouse models (Gogishvili et al. 2017). Notably, SLAMF7-targeted CAR T cells are in clinical phase I trials.

25.4.4 CD138

CD138, also known as syndecan 1, plays a significant role in the development and proliferation of plasma cells. It is a surface molecule which is highly expressed on most malignant and normal plasma cells but is absent from T cells, B cells, and other hematopoietic cells. These facts make CD138 a specific and ideal target in MM treatment. Despite the attractiveness of CD138 as a target for MM, the shedding of CD138 from malignant cells and its expression in mature epithelial cells are two potential drawbacks. In a preclinical study with CD138-specific CAR T cells, no off-tumor toxicities were reported neither in vitro nor in animal model (Sun et al. 2019).

25.4.5 Light Chain

Malignant B cells are usually light chain-restricted cells. Light chain-specific CAR (κ.CAR) T cells were tested in a phase I clinical trial of autologous κ.CAR T cells where four of the seven RRMM patients remained stable for 2–17 months (Ramos et al. 2016).

25.5 Side Effects of CAR-T Therapy

Besides efficacy, early trials have demonstrated adverse clinical events associated with CAR-T therapy such as cytokine release syndrome and encephalopathy/neurotoxicity. Efforts are ongoing to reveal the underlying mechanisms of these toxicities and to manage them appropriately so as to prevent fatal complications. As compared to other hematological malignancies, these side effects are relatively milder and less frequent in MM.

25.6 Conclusion and Future Perspectives

CAR-T therapy is emerging as an attractive promising target for MM in the past few years. Numerous studies on CAR-T therapy with single antigen target or in combination have shown high overall response even in RRMM patients. Besides dual-target CAR T cell therapy for RRMM, CAR-T therapies targeting different antigens in combination with different drugs are under preclinical or clinical studies. BCMA-targeted CAR T cell product has demonstrated significant MM cytotoxicity and is expected to be approved by the FDA for clinical therapy of RRMM soon. CAR T cells targeting CD19, CD138, SLAMF7, and light chains also appear to be promising. Future research on combining CAR-T therapy with different treatment modalities including construction of antibody-drug conjugates and bispecific antibodies and reducing associated toxicities will certainly aid in utilizing CAR-T therapy in the management of MM. Emerging strategies of CAR-T with individualized treatment options may ultimately provide a cure for myeloma patients.

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Nanoparticle-Associated Lipopeptides: A New Class of Antimicrobials

26

Vivek Chauhan, Priya Gautam, and S. S. Kanwar

Abstract

Nanoparticles (NPs) are of size 1–100 nm and can be made from carbon, metal oxides or organic matter. All NPs have ability to show distinguished physical, biological and chemical properties at nanoscale. NPs exhibit properties like increased reactivity, increased stability in a chemical process, enhanced mechanical strength, dispersion, etc. These gather much attention because of their different and interesting properties, applications and advantages over bulk counterparts. NPs have different size, shape and structure. They can be hollow core, cylindrical, spherical, tubular, spiral, conical, flat or even irregular. They also differ in structure which can range from amorphous to crystalline with one or more crystals. NPs are generally eco-friendly and non-toxic, and hence they are amenable for biomedical applications. Lipopeptides (LPs) are amphiphilic molecules comprising of a lipid associated with the peptide head group. These are self-get together particles which can shape peptide-functionalized supra-atomic nanostructures. The self-get togetherness of LPs encourages the introduction of peptide functionalities at exceptionally high thickness on the outside of nanostructures, for example, fibrils, micelles and vesicles. LPs find use in different industries most important being medicine industry where they are used for preparation of antimicrobial drugs. LPs have shown prominent results in cancer treatment as well. NPs are vastly used in medical industry as a potent drug delivery system. LPs which have property of biosurfactants have capacity to be incorporated in the NPs. Surfactin, a type of LPs, can orchestrate both silver and gold NPs in which this surfactin lipopeptide utilized as a layout or settling operator assumes a key job in the adjustment of the NPs. Gold NPs' quality is guaranteed by their stability. LPs associated with NPs (LP-NP molecule) can act

V. Chauhan · P. Gautam · S. S. Kanwar (✉)
Department of Biotechnology, Himachal Pradesh University, Shimla, Himachal Pradesh, India
e-mail: kanwarss2000@yahoo.com

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as important medicinal molecules. A target-specific LP-NP molecule can result in efficient drug delivery, e.g. lipopeptide NPs are among the most potent NPs for the transfer of selective siRNA delivery in non-human primates and rodents. siRNA therapeutic is used for the treatment genetic disorders. Encoding of this siRNA on to the nanoparticle structure of lipopeptide ensures its target drug delivery up to the point of genetic disorder. Antimicrobial properties of the LPs can be explored in to make a target-specific drug in association of NPs. There is vast future of possibility for LP-NP molecules in the medicinal field. In this chapter we will try to address some ongoing research and a futuristic approach in which LP-NP association can result as a boon for medical industry.

Keywords

Nanoparticle(s) · Lipopeptide(s) · LP-NP association · Target specificity · Drug delivery

26.1 Introduction

Nanoparticles (NPs) are of size 1–100 nm and can be made from carbon, organic matter or oxides of different metals (Hasan 2015a, b). All the NPs have ability to show distinguished biological, chemical and physical properties at nanoscale. NPs exhibit properties like increased reactivity, and stability in a chemical process enhanced mechanical strength etc. (Smita et al. 2012). These have gathered a greater attention due to different and interesting properties, applications and inherent advantages over their bulk counterparts. NPs are of different shape and sizes like zero-dimensional, e.g. nanodots; one-dimensional, e.g. graphene; two-dimensional, e.g. carbon nanotubes; and three-dimensional, e.g. gold nanoparticles. They can be hollow core, cylindrical, spherical, conical, tubular, spiral, flat or even irregular in their shape (Machado et al. 2015). They also differ in structure which can range from amorphous to crystalline with one or more crystals (Ealia and Saravanakuma 2017; Hasan 2015a, b). NPs have biomedical applications, and thus the eco-friendly and non-toxic methods should be used for their synthesis. The preferable way to synthesize NPs is by using microorganisms, which is the most superior method as well as highly cost-effective (Zhang et al. 2011). Use of microorganisms to produce NPs has many advantages like energy efficiency and environmental friendliness, and also such produced NPs can be used to perform functions like drug carrier for target delivery, gene therapy, DNA analysis, biosensor and MRI (Li et al. 2011). Thus, it can be said that different microorganisms play a vast role in production of industrially important NPs. Taking this into consideration, the present article includes an overview of NPs, use of various microorganisms (especially bacteria) in production of different kind of NPs and their applications.

The design of antimicrobial lipopeptide (LP) drug carriers is of great interest in treatment of various microbial infections. LPs are low-molecular mass bioactive molecules which contain a lipid molecule connected to a peptide chain. These are

self-gathering particles which can shape peptide-functionalized supra-atomic nanostructures. The self-get together of LP molecules encourages the introduction of peptide functionalities at exceptionally high thickness on the outside of nanostructures, for example, vesicles, micelles and fibrils. Mostly present in bacterial species, they are able to self-assemble into different structures (Kirkham et al. 2016). LPs contain higher content of carbon atom ($\geq$14) in lipid tail, higher antifungal activity and antibacterial activity. LPs such as surfactin is utilized in synthesis of gold and silver NPs in which the LPs (surfactin) is used as a stabilizing agent or a template which plays a key role in stabilizing of the NPs (Reddy et al. 2009).

26.2 History and Development of Nanoparticles

Almost 4500 years ago, human civilization was already using ceramic matrixes which had natural asbestos NPs. Around 4000 years ago, Egyptians used NPs in a synthetic process for synthesis of ~5 nm diameter PbS-NPs for hair dye. Likewise, Egyptians only prepared and used the first synthetic pigment called 'Egyptian Blue' using a mixture of nanoparticle-sized quartz and glass approximately in third century BC. Egyptians and Mesopotamians in thirteenth and fourteenth century BC cited the beginning of metallic nanoparticle era by synthesizing metallic NPs via chemical method for making glass using metals (Schaming and Remita 2015). In 1200–1300 BC starting from late Bronze Age, Italy found red glass which was coloured by surface plasmon excitation of Cu-NPs. 400–100 BC period has been reported to contain the Celtic red enamels made from Cu-NPs and cuprous oxide. The most famous example of ancient metallic NPs usage is Roman glass work piece. Studies show that Lycurgus cups (Roman glass cups) reported during the fourth century was made up of Ag-Au alloy NPs in a ratio of 7:3 with an addition of approximately 10% copper. Reporting from 5000 BC, clay minerals with less thickness were used for preparation of natural NPs since antiquity.

NPs have been used without any prior knowledge up to this era without having any ability to understand the fundamental principle inheriting nanotechnological behaviour. The first scientific description to report nanoparticle preparation and initiating NPs history in scientific area was given by Michael Faraday in 1857. He reported the synthesis of colloidal Au-NP solution, revealing that optical characteristics of Au colloids were dissimilar to their bulk counterpart. SiO_2 nanoparticles are substitutes to carbon black for rubber enforcement started to be manufactured in 1940 (Rittner and Abraham 1998). The term nanotechnology was firstly introduced by Norio Taniguchi at the International Conference on Industrial Production held in Tokyo in year 1974 for description of the ultra-thin processing of material with nanometre accuracy and creation of nanosized mechanism. In 1980s, nanotechnology got major boost by development of cluster science and invention of scanning tunnelling microscope. This development helped in discovery of structural assignment of carbon nanotubes in 1991. First program of nanotechnology began in 1991 in the USA funded by National Scientific Fund. In 2001, National

Nano-technological Initiative (NNI) of the USA was approved. An antibacterial technology with trade name 'Silver Nanotm' was introduced by Samsung, Korea. By the end of year 2003, Mercedes-Benz brought nanoparticle-based clear coat for metallic as well as non-metallic paint finish. Addition of these NPs into the series production increased scratch resistance and enhanced the gloss.

In year 2005, Abraxane™, the human serum albumin NPs containing paclitaxel, was manufactured. Ferrofluids, commonly called liquid magnets, are the most stable suspension of small magnetic NPs bearing super paramagnetic properties; this liquid on onset of magnetic field will magnetize itself, which aligns the NPs in magnetic field direction (Alexiou et al. 2001). TiO_2 NPs have solar cells bearing dye-sensitization ability, exemplarily. In 2012, Logitech brought external iPad keyboard with this technique. Thus, nanotechnology emerged in 1960s, while during 1980s and 1990s, the development of nanotechnology started. Now it is, however, widely accepted that nanotechnology is now envisaged as a major factor for success in the twenty-first century and regarded as 'Next Industrial Revolution'.

26.3 Sources of Nanoparticles

26.3.1 Sources of Nanoparticles Are Mainly Classified in Three Prominent Categories

1. *Engineered NPs*: As the name suggests these are self-designed NPs engineered to have certain specific qualities and characteristics leading to desirable applications. Many anthropogenic activities as diesel or engine exhaust burning, cigarette smoke and building demolition can be used for synthesis of such NPs (Soto et al. 2005). NPs from health care are also included in this type. Thus, these are new genres of NPs which can completely show suspicious human health and environmental effects.
2. *Incidental NPs*: These are incidentally produced materials, which are actually the by-product of different industrial processes, like NP formation from vehicle welding fume, engine exhaust or some natural processes like photochemical reaction, forest fire, volcanic eruptions, ocean evaporation, etc. (Buzea et al. 2007a, b). These natural events result in production of high number of NPs. Likewise, some human activities like charcoal burning also lead to synthesis of NPs (Yano et al. 1990).
3. *Natural NPs*: These are vastly present and easily synthesized from bodies of plants, microorganisms, animals, insects and even human beings (Gong et al. 2015). Unlike engineered and incidental NPs, natural NPs are present in living organisms ranging from microorganisms like bacterial, algae and virus to complex organisms like plants, insects, birds, animals and humans. This knowledge of presence of NPs in microorganisms may be important because of their further use in biomedical applications (Buzea et al. 2007a, b; Jeevanandam et al. 2018).

26.4 Classification of Nanoparticles

There are four material-based categories in which NPs and nanosized materials may be organised (Table 26.1).

1. *Carbon-based NPs*: These types of NPs are mainly formed of carbon. Examples of their morphology are hollow tubes, ellipsoids, etc. These could be further classified into fullerenes, carbon black, carbon nanotubes (CNT), graphene, carbon nanofibres and activated carbon in nanosize (Kumar and Kumbhat 2016).
2. *Organic NPs*: These NPs unlike carbon-based NPs are made up of organic matter. Non-covalent interactions (being weak) are helpful for self-assembly and design of molecule in transformation of organic NPs into desired structure such as liposomes, dendrimers, micelles, ferritin, etc. (Tiwari et al. 2008). These NPs are mostly biodegradable and non-toxic, among which some form hollow core (such as micelles and liposomes) also known as nano-capsules, and they become sensitive to electromagnetic and thermal radiation. This characteristic makes such NPs as an ideal choice for drug delivery (Ealia and Saravanakuma 2017; Khan et al. 2017).
3. *Inorganic NPs*: Inorganic NPs are hydrophilic, non-toxic and biocompatible and highly stable when compared to organic materials. These are the metal and their oxide-based NPs. They have certain types into which they can be synthesized, out of which metals may include Au- or Ag-NPs (Salavati-Niasari et al. 2008); similarly metal oxides may include TiO_2, ZnO and even some semiconductors like silicon and ceramics (Tai et al. 2007).
4. *Composite NPs*: These are NPs of composite structures, including core shell structure, onion-like structure and gladiate composition. These NPs are multiphased, with one of their phases of nanoscale dimension. This characteristic helps them to combine different NPs and for a hybrid nanofibres structure or complicated structures as metallic organic frameworks. These composites can be

Table 26.1 Classification of nanoparticles

S. no.	Type of NPs	Sub-type	Example
1	Carbon	Fullerenes Graphene Carbon nanotubes Carbon nanofibres Carbon black	TiO_2
2	Organic	Dendrimers Liposomes Micelles	CdSe
3	Inorganic	Metal-based Metal oxide-based	Au and Ag ZnO and TiO_2
4	Composite	Simple hybrid Core or shell structured Multifunctional quantum dots	SiO_2

of different combinations like metal-based, organic-based or carbon-based NPs (Rane et al. 2018).

The drugs can be loaded on the NPs structure either by adsorption of drug on the surface or encapsulating them on NPs. Network of polymer can protect the drugs from degrading because of the enzymes secreted by the body. Drugs from the NPs can be released through various enzymatic degradation of the polymer, hydrolysis of the polymeric network, diffusion or by combination of different mechanisms.

26.5 Lipopeptide in NP Structure

Main failure of the anticancer drugs in the treatment purpose is due to distribution of drugs in the body at random sites and drawback of not being site-specific, which leads to very less effect of the complete dose of the drug given, resulting in the cause of excessive toxicity to the normal cells, too. Thus, in advanced searches, use or the research over NPs is showing utmost importance because of high drug loading capacity, better cancer targeting, improved bioavailability, prolonged circulation time and ease of manipulating drug release (Yu et al. 2010). By the morality of the nanosize, these particles are able to get collected at the specific site of cancerous cells due to the EPR effect. Blood vessels around the tumour cells are very poorly formed due to very fast growth of cancer cells which allows the passing of the NPs. However, if recognized by reticuloendothelial system, these NPs may get flushed out of the body; thus to minimize this risk, surfactin along with polyethylene glycol solution has been used. At the time of cancer treatment, several receptors are overexpressed which may be helpful in cancer targeting and thus offer higher amount of dose to the cancer cells (Morachis et al. 2012).

26.6 NP-Associated Lipopeptides

Perspective of NP-associated LPs not only comes from being cytotoxic agent but have several other roles when transformed in nanoparticle state. LPs like surfactin carrying biosurfactant properties such as amphiphilic structure and surface-active properties make them most suitable for transformation in nanoparticle state. Polymeric micelles, liposomes and noisome are the most acceptable form because of presence of their hydrophobic/hydrophilic core shell structure, and micro- and nano-type of emulsions are the options in which surfactin can be dispersed easily into nano-formulation. In several microemulsions, surfactin may act as an agent of anticancerous activities.

Scientists are using surfactin molecules because of their self-incorporation activities. They can be used as building blocks for several types of NPs such as micelles, liposomes, etc. Surfactin NPs are playing tremendous role because of the surface-active activity and the amphiphilic structure. Surfactin-loaded polyvinyl alcohol (PVA) nanofibres have been used as they bear the antiseptic properties and

can be used for wound dressing purposes, and also this can protect the prosthetic parts from secondary infection as well as from the biofilm formation. These nanofibres are formed by adding surfactin and PVA solutions and following gravity electrospinning (Ahire et al. 2017).

26.7 Mechanism for Synthesis of NP-Associated Lipopeptide (NP-LP Particles)

Lipopeptide class of biosurfactants have been reported in NP synthesis. Surfactants are commonly used as stabilizing agents in the synthesis of gold and silver NPs. In a common procedure (Fig. 26.1), NP synthesis involves reduction of the aqueous $AuCl_4$ using $NaBH_4$ in the presence of surfactin obtained from *B. subtilis*. Foam fractionation was done to recover surfactin from the culture supernatant which was further added to pale yellow colour chloroaurate solution that turned red-purple. This indicated the change in metal oxidation state and the formation of gold NPs. The NPs can be synthesized at a pH of varying range of 5–9 at 4 °C.

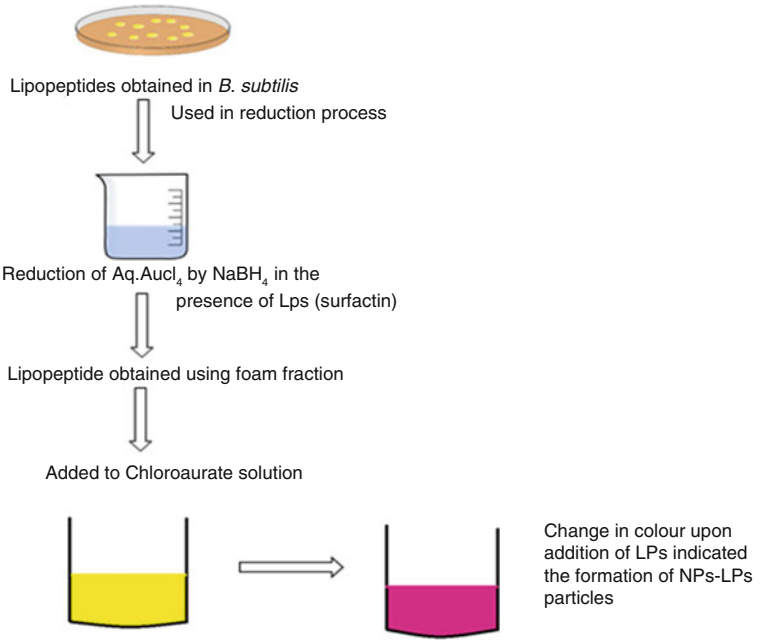

Fig. 26.1 General process for development of NP-LP particles

26.8 Important Functions of NP-LP Molecules

Many reports suggest that LPs produced from *Bacillus* spp. in nano-form are useful for controlling mould, fungal and bacterial pathogens (Ongena and Jacques 2007; Valodkar et al. 2011). Metal NPs have good antimicrobial activities, and NP-LP-based antimicrobial formulations could be an effective fungicidal as well as bactericidal agents (Rai et al. 2009). Surfactin-functionalized poly methyl methacrylate (PMMA) showed antimicrobial effect against *Escherichia coli* (300 μg/mL) of the NP-LP molecules. Also, the low haemolytic activity exhibited by these NPs reduces the amount of increased toxicity. A new surfactin-SMEDDS nano-formulation has been developed, although more confirmation was needed for their anticancerous activity (Hore 2019). The combination of surfactin along with nano-emulsion could act as a great drug delivery agent and which could be successfully used as in anticancer treatment (Table 26.2 and Fig. 26.2). Surfactin-based NPs also have uses in overcoming multidrug resistance in cancerous cells, for example, surfactin-loaded NPs loaded with doxorubicin. Doxorubicin has the purpose of stopping the growth of the cancerous cells. It is type of chemotherapy used for the treatment purpose of different type of cancer. β-Casein balanced out LP lyotropic liquid crystal NPs has been utilized, i.e. these are the NPs of lipopeptides and the mixture of doxorubicin which was an anthracycline, and therefore these lyotropic liquid NPs

Table 26.2 Describes some functions and applications of lipopeptides in different inorganic and organic nano-formulations

Nano-formulation	Type of nano-formulation	Surfactin function	Application of nano-formulation	References
Organic NPs	Polymeric NPs	Enhance sorption activities	Adsorbent and antibacterial	Hore (2019)
Surfactin-functionalized poly methyl methacrylate (PMMA) NPs				
Poly (methyl methacrylate) (core)-biosurfactant (shell) NPs	Polymeric NPs	Emulsifier, pH-responsive gate keeper, control release of drugs	The pH-responsive and controlled release nano-carrier	Ohadi et al. (2020)
Inorganic NPs	Metal NPs	Stabilizer	Antimicrobial	Ahmad et al. (2019)
Anionic surfactin-mediated silver NPs				
Anionic surfactin-mediated gold NPs	Metal NPs	Stabilizer	Anticancerous	Bali et al. (2019)
Cadmium sulphide NPs	Metal NPs	Stabilizer, capping agent	Antimicrobial	Santos et al. (2019)
Surfactin-stabilized biogenic silver nano-cubes	Metal NPs	Stabilizer	Antipseudomonal and antiendotoxin	Krishnan et al. (2017)

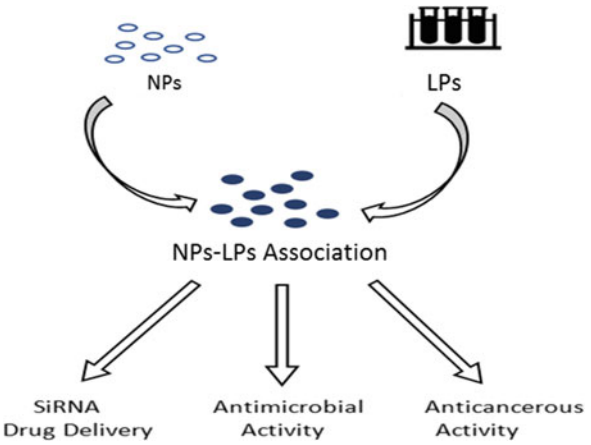

Fig. 26.2 General functions of NP-LP molecules

could be successfully used in the proper drug delivery and treatment of cancer (Kural and Gursoy 2011).

Lipopeptide NPs are among the strongest NPs for the exchange of specific siRNA delivery in non-human primates and rodents. siRNA therapeutics are used for the treatment of genetic disorders. Encoding of this siRNA on to the nanoparticle structure of lipopeptide ensures its target drug delivery up to the point of genetic disorder.

26.9 Limitations

The main limitation to the LP-NP molecules is about their stability which often leads to the prevention of the aggregation process causing loss in their antibacterial activity. The biological methods for NP-LP productions are still at a developing stage; also it is hard to control and achieve the desired shape, size and controlled dispersion in NP-LP production. Many different microorganisms have been successfully utilized for production of NP-LP particles (Ohadi et al. 2020), but impacts of such microbes, synthesis conditions and growth media which are probably responsible for their potent biological and physiochemical properties are still not understood properly.

26.10 Future Perspectives and Conclusion

With the advent of new inventions and research in field of nanotechnology, the concept of developing the nano-form of important biological molecules is possible today. Many scientists consider it as the future of medicinal science. NP-based drugs play significant roles in commercial development. LPs extracted from various microbial and plant sources have many medical applications. Antimicrobial and

anticancerous nature of LPs is being exploited by the scientists to develop different medicines. The incorporation of the nanomaterials like gold and silver with these LPs has shown wonders in antimicrobial and antitumor properties. NP-LP formulations though in the early stage of development have shown promising results by enhancing the antimicrobial properties in many LPs and their delivery and specificity. With future development and further research, NP-LP may become a pioneer cancer drug. Though development issues like cost and environmental factors, difficulty in achieving desires size and stability still prevail, but with dedicated research, these shortcomings can be minimized. As a concluding remark, it would not be wrong to say that NP-LP have boundless possibilities/applications and they may emerge as one of the most important class of medicinal molecules of twenty-first century.

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Declaration of Competing Interest Authors declare that they have no conflict of interest amongst themselves or with parental institute.

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Antimicrobial Applications of Engineered Metal-Based Nanomaterials

27

Moondeep Chauhan, Gurpreet Kaur, Bunty Sharma, and Ganga Ram Chaudhary

Abstract

Microbial resistance to the antibiotics is a serious problem at present and will become alarming in near future. It is therefore important to establish a new technology that has antibacterial potential without developing resistance against bacterial strains. Nanotechnology has come to a way forward to address these antimicrobial challenges. Metal-based engineered nanomaterials (ENMs) are the main core of nanotechnology. Development in the field of metal-based ENMs has shown the potential to use it as an antimicrobial agent. The current chapter will critically evaluate the potential of metal-based ENMs as antimicrobial agents. This will include their fabrication process, properties, interaction with bacteria, and mode of action and mechanistic view of bacterial killing.

Keywords

Microbial resistance · Metal-based nanomaterials · Bacterial killing

M. Chauhan
SAIF/CIL, Panjab University, Chandigarh, India

G. Kaur · B. Sharma
Department of Chemistry and Centre of Advanced Studies in Chemistry, Panjab University, Chandigarh, India

G. R. Chaudhary (✉)
Department of Chemistry and Centre of Advanced Studies in Chemistry, Panjab University, Chandigarh, India

SAIF/CIL, Panjab University, Chandigarh, India
e-mail: grc22@pu.ac.in

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27.1 Introduction

When we look back on the history of human diseases, infectious diseases have accounted for a very large proportion of diseases. It was, however, only in the latter half of the nineteenth century when microorganisms were discovered to be responsible for a number of infectious diseases that have been plaguing mankind since ancient times. Thereafter, the first antimicrobial agent salvarsan was formulated by Ehrlich in 1910 for the cure of syphilis. In 1935, a new class of synthetic drugs called sulphonamides was developed by Domagk and other researchers to cure bacterial infections successfully (Yousef et al. 2018). However, these compounds had limitations in terms of safety and efficacy. In 1928, Sir Alexander Fleming discovered penicillin, an excellent and outstanding antimicrobial agent in terms of safety and efficacy that lead to the golden period of antimicrobial therapy. It came into clinical use in the 1940s.

For the next two decades, new types of antimicrobial drugs were developed one after the other, leading to remarkable advances in the treatment of infectious diseases and fate of mankind (Saga and Yamaguchi 2009). This gave rise to an overwhelmingly optimistic view that infectious diseases will be eliminated in the near future, provided new antibiotics continue to be developed. However, the development of new types of antibiotics has slowed in the mid-1980s, and very few have been developed in the last quarter of a century (Shallcross et al. 2015; Silver 2011). At present, fewer new antimicrobial agents are being introduced in the market. In addition, infections with drug-resistant organisms remain an important problem in clinical practice that is difficult to solve. As a consequence of this, isolates are now appearing which are resistant to almost every antibiotic available, raising the spectre of untreatable infection even at the world's most advanced medical centres (Snitkin et al. 2012).

In fact, by the early 1940s, resistance to antimicrobials drugs has been observed and reported in various studies (Abd-El-Aziz et al. 2017). For instance, in 1941, Abraham and co-workers (1941) observed that cultures of *Staphylococci* sp. developed resistance by continuous subculture in the presence of penicillin, and in 1942, Rammelkamp and Maxon (1942) isolated four strains of penicillin-resistant *Staphylococci* sp. during treatment of local infections with penicillin. Within two decades of penicillin's introduction, most strains of *S. aureus* isolated in large hospitals were resistant to penicillin and to other antibiotics including streptomycin, tetracycline, and erythromycin.

Widespread scientific consensus has concluded that the human overuse and mismanagement of antimicrobial agents have contributed to the rapid development of antimicrobial resistance (AMR) in microorganisms. AMR arises when the microorganisms which cause infection (e.g. bacteria) survive exposure to a medicine that would normally kill them or stop their growth. This allows those strains that are capable of surviving exposure to a particular drug to grow and spread, due to the lack of competition from other strains. Antibiotics are the preferable treatment for the cure of chronic bacterial infection. These antibiotics have a potent outcome and also it is cost-effective, but with time the overuse and misuse of antibiotics create resistant towards broad spectrum of bacteria. This has led to the emergence of 'superbugs,'

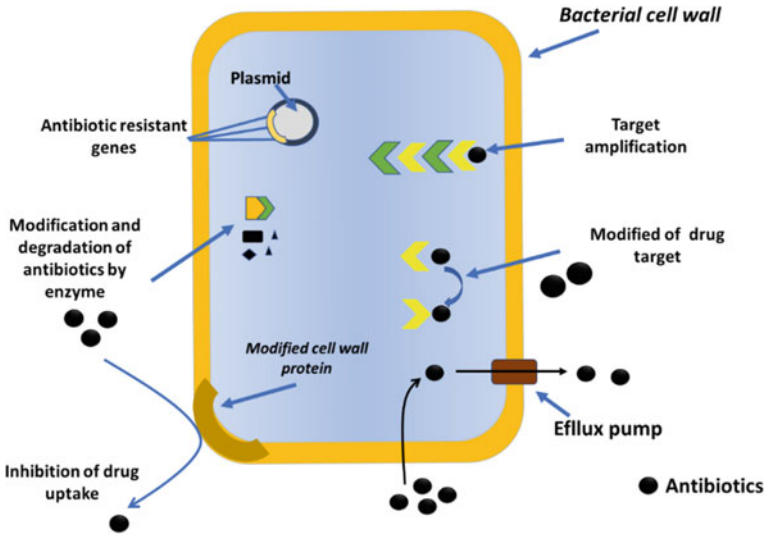

Fig. 27.1 Schematic representation of the bacterial resistance to the antibiotic

bacteria which are difficult or impossible to treat with existing medicines such as methicillin-resistant *S. aureus* (MRSA), *S. aureus* resistant to vancomycin, and extremely drug-resistant tuberculosis (Arguaete et al. 2013).

For bacteria, several mechanisms of resistance have evolved, including decreased membrane permeability (Delcour 2009), overexpression of specific efflux pumps (Piddock 2006), development of the mechanisms to degrade or alter the conventional antibiotic (Munita and Arias 2016), and biological differentiation of the antibiotic target site (Blair et al. 2015; Poole 2002; Jayaraman 2009). On broadly AMR against bacterial cell divided into two types: acquired (due to protein, enzyme, genetic and physical change in bacterial cell) and intrinsic (natural type of resistance in which bacteria retard the drug penetration through the cell wall and also modified the target) (Cope and Cope 2013) (Fig. 27.1).

Moreover, another study revealed that resistance in bacteria was created by a special gene in bacteria called NDM-1 which is called New Delhi metallo-β-lactamase-1. The bacteria which produce this NDM-1 have resistance to β-lactams, aminoglycosides, and fluoroquinolones (Pitout 2010). Bacteria possessing just one of these resistance mechanisms can be treated through an alternative class of antibiotic; however, it is becoming increasingly common for single strains of bacteria to simultaneously possess the genes for more than one of these resistance mechanisms.

Gravely, as antibiotics are becoming increasingly ineffective, the human population is expected to lose its most powerful weapon against infectious diseases, taking us back to the pre-antibiotics days, where minor wounds, injuries, and other sources of infection, including routine surgery, could potentially be life-threatening. Obviously, microorganisms have challenged modern science, and the global mortality rate is estimated to reach 10 million lives per year (i.e. one person in every 3 s) in

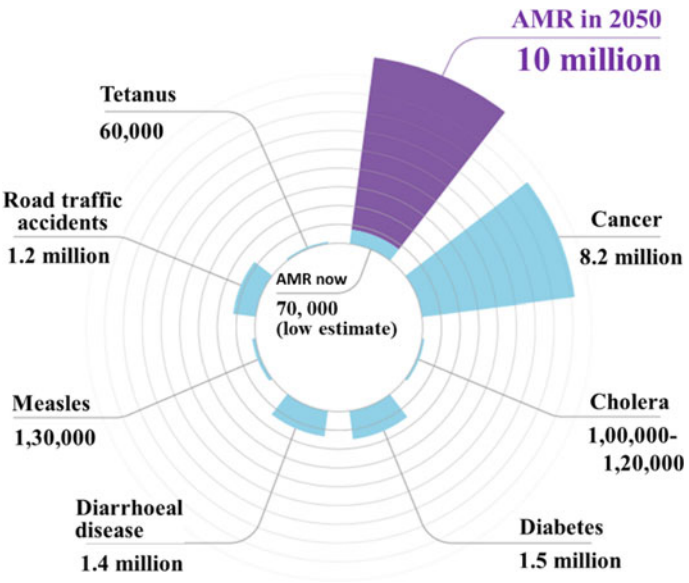

Fig. 27.2 Annual deaths attributable to AMR compared to other major causes of death (O'Neill 2018)

2050 (Fig. 27.2) that will add an economic burden of 100 trillion USD, unless serious measures are taken against microbial resistance.

Thus, there is a critical need for the development of new antimicrobial technologies as alternatives to, or to work in combination with, conventional antimicrobial treatment methods. The use of metal-based engineered nanomaterials (ENMs) possessing antimicrobial properties has already started addressing many of these criteria, with varying success. In particular, metal and metal oxide ENMs have been studied extensively as they possess a range of innate antimicrobial mechanisms, including the disruption of the cellular membrane, diffusion into and degradation of internal cellular components such as DNA, RNA, and enzymes and the release of ions with antimicrobial activity. Common materials include but are not limited to silver (Ag), gold (Au), copper (Cu), zinc (Zn), titanium (Ti), magnesium (Mg), calcium (Ca), nickel (Ni), iron (Fe), palladium (Pd), tellurium (Te), platinum (Pt), silicon (Si), and their corresponding oxides in some cases, with a range of shapes and sizes (typically in nanometre range) (Table 27.1).

27.2 Fabrication Techniques

The design of synthetic techniques has been commonly recognized as a key area for understanding and application of ENMs. The size and shape of ENMs have strong effect on their properties, thus, demands their morphology and dimensions to be controlled precisely during synthesis. Metal-based ENMs can be easily synthesized

Table 27.1 Assessment of metal-based ENMs for antimicrobial activity

ENMs	Size (nm)	Bacteria	Mode of action	Synthesis	References
Ag/ Chitosan	10 ± 5.4	*E. coli* *S. aureus* *C. albicans*	Release of silver ion and its soluble complexes and Cell membrane damage	Hydrothermal synthesis	Biao et al. (2017)
Ag/GO	5–10	*E. coli* *S. aureus*	Release of silver ion and Cell membrane damage	Microwave-assisted synthesis	Zhao et al. (2017)
Ag	10–70	*E. coli* *S. aureus, MRSA* *P. aeruginosa*	ROS generation	Pulsed laser ablation	Korshed et al. (2016)
Au	6–34, 20–40	*E. coli* *B. subtilis* *S. aureus* *K. pneumonia*	Disrupted their respiratory systems and induced ROS generation	Chemical reduction process	Shamaila et al. (2016)
Cu	2–60 (∼ 25)	*E. coli*	Release of Cu ions and generation of hydroxyl radical in the cytoplasm	–	Rispoli et al. (2010)
Cu	10–16	*E. coli*	ROS generation	Biological synthesis	Lv et al. (2018)
TiO_2	4–10	*Influenza virus strain (H3N2)*	Fragmentation of viral envelope	Chemical based synthesis	Mazurkova et al. (2010)
CaO	14–24 (∼ 18)	*P. aeruginosa* *S. epidermidis* *C. tropicalis*	ROS generation	Microwave irradiation synthesis	Roy et al. (2013)
Se	115 ± 38	*E. coli* *S. aureus*	Penetrate the outer membrane and modifying the role of enzymatic conveyors	Pulsed laser ablation in DI water	Guisbiers et al. (2016)
Se	90–150	*E. coli* *S. aureus*	ROS generation	Biogenic synthesis	Medina Cruz et al. (2018)

(continued)

Table 27.1 (continued)

ENMs	Size (nm)	Bacteria	Mode of action	Synthesis	References
Se	30–70	*E. coli* *S. aureus*	Deposition of NPs in the peptidoglycan layer and release of soluble Se species	Redox reactions	Tran et al. (2015)
CuO/W	39–53	*E. coli* *S. aureus*	ROS generation, Induction of oxidative stress–	Co-precipitation synthesis	Raba-Páez et al. (2020)
HfO_2	3.7 ± 0.6	*S. mutans*	Bacterial DNA fragmentation	Sol-gel synthesis	Ostadhossein et al. (2018)
α-Al_2O_3	~20	*S. aureus* *K. aerogenes* *E. coli* *P. desmolyticum*	NPs interaction with outer membrane and subsequent channel formation in the cytoplasmic membrane and ROS generation	Solution combustion synthesis	Prashanth et al. (2015)
CeO_2	6 ± 3.5, 15 ± 4.3, 22 ± 5.7, 40 ± 10	*E. coli* *S. oneidensis* *B. subtilis*	NPs-bacterium interactions and general stress response	Hydrothermal synthesis	Pelletier et al. (2010)
NiO	20–30	*P. aeruginosa* *B. subtilis*	Membrane disruption and cell lysis	Sol-gel synthesis	Rakshit et al. (2013)
Co_3O_4	10–25 (Co_3O_4-I) 100–150 (Co_3O_4-II)	*S. aureus* *E. coli*	Interaction of NPs through endocytosis across the plasma membrane into the cytoplasm	Pyrolytic synthesis	Ghosh et al. (2014)
Fe_3O_4	10–30	*E. coli* *P. mirabilis* *B. subtilis*	ROS generation, Penetration of NPs into membrane	Co-precipitation synthesis	Arokiyaraj et al. (2013)

and/or chemically modified for a desired application. A large number of synthetic techniques for the synthesis of ENMs with varied morphologies, sizes, and dimension have been explored by using different chemical and physical approaches.

Generally, to fabricate ENMs synthetic techniques can be broadly categorized into top-down and bottom-up approach. As suggested by its name, the top-down approach starts with a bulk material which is broken down into nanoscale dimension, using techniques such as ball milling or attrition. Although this is a simple technique for fabrication of ENMs, however, this technique results in a broad size distribution and nonuniform particle geometries and contains increased impurities.

On the contrary, bottom-up approaches utilize diverse techniques to build ENMs from raw chemicals and physical environments to a completed, finished product. This approach can be time-consuming, however, permits for precise control over the chemical output and produces consistent particle shapes, sizes, and geometries with little defects. Examples of this fabrication technique include solution-based method, sol-gel method, electrochemical method, colloidal methods, atomic layer deposition method, solid state thermal conversion method, etc. (Table 27.1). Several comprehensive reviews are available, providing a thorough discussion of the basic understanding and properties in metal-based ENMs synthesis.

27.3 Mode of Action of ENMs Against Bacteria

ENMs have unique chemical, electrical, magnetic, thermal, physical, and biological properties. Due to these interesting properties in ENMs, these were used as a potential antibacterial agent. Effect of ENMs varies from exposer time of ENMs against different bacterial culture with the environmental condition influences the antibacterial activity. The mechanism of antibacterial property varies for different ENMs. Yet, for all the ENMs, a mechanistic way is still not established for the antibacterial property by which it kills the bacterial cell (Xie et al. 2018; Cai et al. 2019; Li et al. 2020; Huang et al. 2020).

27.3.1 Reactive Oxygen Species (ROS) Generation

The term ROS denoted the reactive radicals which includes, singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide (O^{2-}), hydroxyl ions (OH^-), and free radicals ($OH^\bullet$), which are produced as a side product of some biological processes. ENMs generate ROS in presence of light, ultrasounds, or sometimes without light give oxidative stress to bacterial cell. These ROS action modes are applied for a short period which is also induced by catalase and superoxide. Normally, ROS plays an important role in the different physiological processes in animals and plants, but the excess level of ROS causes oxidation of different cellular components such as DNA, lipids, and proteins which is a menace to cell survival. Out of all these abovementioned ROS, $^1O_2$ is a highly toxic type of ROS which is responsible for the killing of bacteria. Normally, the $^1O_2$ is generated by putting a suitable

wavelength light on photosensitizer, and this phenomenon is known 'photodynamic therapy.' There were many ENMs like ZnO, TiO_2, MoO_2, MoO_3, WO_3, metal-organic framework (MOF), etc. that act as good metal-based photosensitizers which are used in photodynamic therapy (Sun et al. 2018). When ROS is generated in the presence of ultrasound, then the process is called son dynamic therapy (You et al. 2016).

The oxidative stress caused by ROS to the bacterial membrane causes the variation in the membrane permeability which leads to cell membrane damage. There are many examples in the literature related to the ENMs which generate ROS and produce oxidative stress to the cell and causes death.

27.3.2 Self-Antibacterial Property of ENMs

During the study of the antibacterial property of metal based ENMs, the reorganization of metal is very important because some metals have inherited antibacterial property in their bulk form (e.g. Cu, Ag, Zn, etc.), while some become antibacterial in its nanoparticles (NPs) form. Interestingly the self-antibacterial property of ENMs successfully applied against both Gram-positive (GP) and Gram-negative (GN) bacteria. Antibacterial properties are affected by the NPs surface to volume ratio. As the ENMs size decreases, then their more surface area is available for the dose for the surrounding environment, so different ENMs with an increase in surface to volume thus enhance the antibacterial effect. There are relevant variables like shape, chemistry, size, and surface charge which affect the antibacterial properties of ENMs. For example, the antibacterial property of Au, Ag, Cu based NPs have shown interesting antibacterial nature without creating any resistance so far (Yasuyuli et al. 2010).

A study by Yoon et al. report 90% killing of *E. coli* and *B. subtilis* by Ag NPs with the dose of 58.4 and 32.1 μg/mL NPs, respectively, while for Cu based NPs, it required 33.49 and 28.2 mg μg/mL, respectively (Yoon et al. 2007). This study has shown that Cu based NPs are more effective as compare to Ag based NPs. Due to its cheaper availability, greener synthesis, and the inherited antibacterial property of Cu against wide range of microorganism, it has been recognized as the first metallic antibacterial agent in 2008 by the American Environmental Protection Agency (Vincent et al. 2016). Other than bacteria, metallic Cu coating is also effective against yeast and virus killing. In this way, it was reported that Cu followed all the mechanisms of bacteria killing, i.e. ROS generation, plasmid and genomic DNA damage, lipid peroxidation occurrence, cell membrane rupturing and generating dissolve ions (Grass et al. 2011). Zinc oxide (ZnO) is also a well-known antibacterial NPs which shows antibacterial property against both GP and GN bacteria. Tiwari and co-workers fabricated the ZnO ENMs and applied it against *A. baumannii* which is a multidrug-resistant (MDR) pathogen. This also shown the ROS leads to bacterial cell damage and caused the killing of the cell (Tiwari et al. 2018; Alves et al. 2017).

27.3.3 Interruption of Protein Oxidation, Membrane Collapse, and Electron Transport

Protein, cell membrane, and electron transport are important component of bacterial cell structure, and their smooth functioning is important for cell survival. Protein oxidation occurred at cellular level, and these modifications lead to functional changes which disturb the cellular metabolism. The disturbance in electron transport chain effects the redox reactions which coupled to produce adenosine triphosphate (ATP). Positive zeta potential on the ENMs plays a crucial role in interaction with anionic bacterial cell wall which binds strongly to each other and enters the cell membrane. In this way, it may rupture the cell wall of bacteria and disturb the electron transport and also oxidize the protein of the cell which results in cell death (Allaker 2010). Contact of the ENMs to cell also creates different oxidative and non-oxidative stress to bacterial cell wall which also changes cell physiology and indorses DNA deprivation (Jayaram et al. 2017). Shi and co-workers found that by irradiating the light on silver NP, light is absorbed and transfers energy to protein which is oxidized and gets aggregate protein which leads to cell death (Shi et al. 2019).

Recently, Singh and co-workers used ZnO NPs on *D. radiodurans* which is an extremophilic bacteria and can survived in all toughest environment. ZnO NPs tempt DNA harm and protein oxidation by internalization of ENMs inside the *D. radiodurans* cell (Singh et al. 2020). Cadmium oxide was tested against *S. aureus*, *S. dysenteriae*, and *P. aeruginosa* which shows that on generating ROS protein leakage is observed from bacterial cell (Azam et al. 2020). Further, interruption of electron transport and leakage of DNA, protein, and carbohydrate occurred when ZnO ENMs generates the ROS and damaged the cell wall (Wang et al. 2014). Chen and co-workers fabricated CuO ENM by a biosynthesized method using soil borne pathogenic *R. solanacearum* bacteria which shows that CuO interacts with the bacterial cell wall and disturbs the ATP synthesis which is followed by cyto-membrane damage (Chen et al. 2019).

27.3.4 Release of Dissolved Ions

The ability of the dissolved metal ions to interact with bacterial cell is also considered as a major mechanism for bacterial cell killing. Wang and co-workers studied the antibacterial effect of NiO and ZnO and α-Fe_2O_3, γ-Fe_2O_3, and Fe_3O_4 ENMs on photo-bacterium phosphorus bacteria. The effect of these metal oxide particles was combined with the release of ions which attributed to the antagonistic, synergistic, and the additive effect of ENMs (Wang et al. 2014). Ag NPs are well studied for the release of such ions (Ag^+) which interact with the cell metabolic system by penetrating the bacterial cell wall. These Ag^+ ions then damage the DNA of the cell (Niskanen et al. 2010; Stensberg et al. 2011).

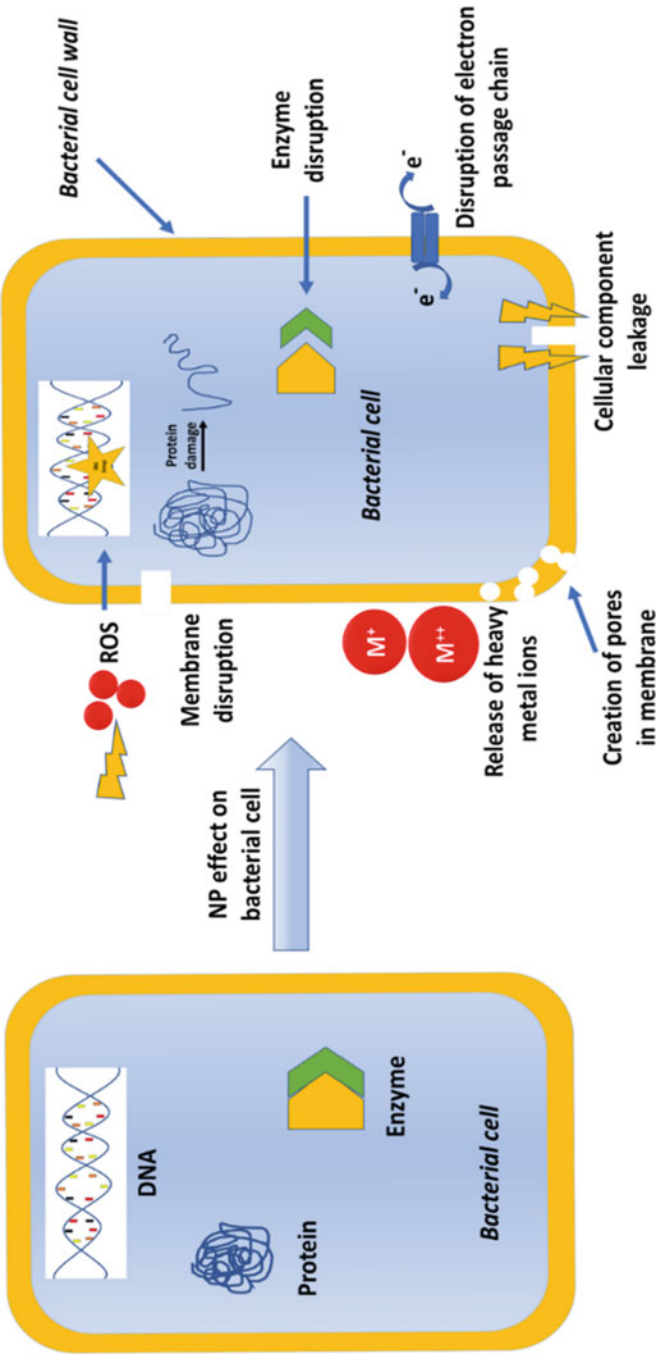

Fig. 27.3 Schematic representation of bacterial cell membrane damage by metal-based ENMs

Figure 27.3 gave the schematic representation of the possible mechanism of bacterial cell damage by the ENMs. Since, these ENMs have followed many mechanisms for its antibacterial property and due to this reason, there is a very rare chance of getting bacterial resistance towards these metal-based NPs in near future. For bacteria to develop resistance against ENMs, the microbial cells have to undertake several gene mutations that are not feasible instantaneously. In literature, the researchers have reported various greener methods of ENMs fabrication that enhance in antibacterial property against different types of bacteria.

27.4 Antimicrobial Effect of Some Common Metal-Based ENMs

Since the notable bacterial effects of reactive metal-based ENMs have been reported by Klabunde and colleagues (Stoimenov et al. 2002), there has been significant interest in other inorganic ENMs as antibacterial materials. Various metal-based ENMs and their corresponding oxides have been thoroughly studied for their distinct antimicrobial properties (see Table 27.1).

27.4.1 Silver (Ag)-Based ENMs

Ag and its compounds have been used for many millennia to prevent microbial infections, even before the realization that microbes were the agents of infection. In ancient times, the Greeks, Romans, Egyptians, and others used Ag to preserve water and food. Compared with other metals, Ag exhibits higher toxicity to microorganisms and lower toxicity to mammalian cells. Besides, it has been successfully used to treat multiple infectious diseases against a wide variety of microbes including bacteria, fungi, and viruses as well as non-infectious diseases, often with remarkable effectiveness. In many cases, with several microbial species growing resistant to antimicrobial drugs today, Ag-based ENMs are efficiently used to disinfect as well as coat medical equipment such as external fixation pins, heart valves, endotracheal tubes, cardiac, urinary catheters, etc. to reduce infections. Some permanent implants like mega-endo-prostheses which are implanted after the removal of bone tumours are coated with Ag.

Many studies have been conducted on the Ag-based ENMs to support its use in biological applications. The mechanism of the inhibitory effects of silver (Ag^+) ions on microorganisms is only partially known so far. Sondi and Salopek-Sondi (2004) first reported the antibacterial activity of elementary Ag NPs against *E. coli*. The results revealed that the increase in the permeability of the cell wall via incorporation of Ag NPs in the cellular membrane and formation of pits on its surface caused the cell death. Hassan and a co-worker (2018) fabricated the Ag NP-based scaffold which showed 100% antibacterial efficacy against *E. hirae* and *E. coli*. There were many green approaches to fabricate these ENMs which reduce toxic concerns and enhance the antibacterial property of the material (Roy et al. 2019).

To comprehend the antibacterial mechanism of Ag NPs, Li et al. (2010) studied the effect of Ag NPs on the permeability and the membrane structure of *E. coli* cells, as a model for GN bacterium. Results showed that Ag NPs seemingly enhanced the permeability of membrane and inactivated the activity of respiratory chain dehydrogenase enzyme, resulting in the leakage of the cellular materials and inhibition of respiration. In contrast, Kim et al. (2007) suggested that the antimicrobial activity of Ag NPs is related with the formation of free radicals and subsequent free radical-induced membrane damage.

27.4.2 Gold (Au)-Based ENMs

In the field of nanotechnology, Au-based ENMs have been widely analysed for various applications. Besides many applications, because of its various inherent properties like non-cytotoxicity, biocompatibility, inertness, high stability, polyvalent effects, ease of identification, photothermal activity, and easy functionalization, the antimicrobial property of Au-based ENMs have been largely exploited.

Studies have shown that ultra-small-sized Au nanoclusters (NCs) possess comparatively broad spectrum of antimicrobial activity than their larger counterparts. For instance, Zheng et al. (2017) found that the wide-spectrum antimicrobial properties can possibly be imparted to Au NPs (>2 nm) via precise control of Au size down to ultra-small NCs dimension (i.e. <2 nm). Au NCs was observed to kill both GP (*S. aureus*, *S. epidermidis* and *B. subtilis*) and GN bacteria, (*E. coli* and *P. aeruginosa*). This wide-spectrum antimicrobial activity of Au NCs has attributed their ultra-small size, which allowed them to better interact with bacteria. These interactions were reported to create a metabolic imbalance in cells, leading to about two- to threefold increase of intracellular ROS production that kills bacteria consequently. Overall, results suggested that the internalization of Au NCs, modulation of cell metabolism, and intracellular ROS generation were the determining factors for the high antimicrobial efficiency of Au NCs.

Unlike other metals, Au is generally considered as an inert and biocompatible material; therefore, several efforts have been performed to craft desired antimicrobial chemicals such as peptides, cationic ligands, and other antibiotics on the surface of Au NPs. Recently, Li et al. (2020) demonstrated that positively charged Au NCs stabilized with cationic ligand ((11-mercaptoundecyl)-*N*,*N*,*N* trimethylammonium bromide) hold a great potential to be used as an antimicrobial agent against MDR bacteria. Ultra-small size and positive charge on the surface were the reasons for the effective antimicrobial activity of Au NCs through a combined mechanism that includes destruction of cell membrane, DNA damage, and generation of ROS. Tiwari et al. (2011) investigated the antibacterial and antifungal activities of the Au NPs functionalized with 5-fluorouracil against *M. luteus*, *S. aureus*, *P. aeruginosa*, *E. coli*, *A. fumigates*, and *A. niger* microbes. The authors claimed that Au NPs were more effective against GN bacteria than GP bacteria due to their easy internalization into the GN bacterial cell membrane.

The generation of ROS is the cause of cellular death for most bactericidal antibiotics and ENMs. However, Cui et al. (2012) reported that bactericidal action of Au NPs is independent of oxidative damage-related mechanisms such as ROS generation. Au NPs have been shown to cause cell death specifically in two ways: firstly, to alter the membrane potential and suppress the activity of ATP synthase to decrease the amount of ATP, suggesting a general decrease in metabolism, and, secondly, to inhibit the ribosome subunit from tRNA binding, indicating a failure of the biological mechanism. In another study, Zhu et al. (2014) reported that the near infrared laser irradiation of the Au nanorod arrays resulted in fast photo-heating with noteworthy bactericidal properties which could be used for quick, efficient, and real-time killing of pathogenic bacteria and thus producing microbe-free water.

27.4.3 Zinc (Zn)-Based ENMs

Among several transitions metal oxide, ZnO is one of the most promising inorganic materials with a broad range of uses in the field of pharmaceutical, textile, cosmetic, catalysis, photoelectronic, environmental remediation, electronics, and so on. ZnO is registered as a safe substance by the US FDA, and owing to its distinctive electronic configuration, low production costs, and appropriate properties, ZnO is considered as one of the potential antibacterial materials (Joe et al. 2017; Abebe et al. 2020).

The key mechanism that has been identified for the antibacterial activity of ZnO ENMs involves the production of ROS, antimicrobial ion release (Zn^{2+}), electrostatic interaction, loss of cellular integrity, and internalization ENMs. Among different mechanisms, the most widely described mechanism in the literature for antimicrobial activity is the production of ROS, particularly, during the light absorption of characteristic wavelengths. ROS may include superoxide anions ($^{\bullet}O_2^{-}$), hydroxyl ($^{\bullet}OH$) and perhydroxyl radicals ($HOO^{\bullet}$), H_2O_2, and $^1O_2$ which can cause the destruction of cellular components such as DNA, proteins, and lipids. For the interaction of ENMs with the bacteria cell and production of ROS, the direct production of ROS inside the bacterial cell and indirect production of ROS outside the bacterial cell methods have been reported.

Semiconductor metal oxides such as TiO_2, α-Fe_2O_3, MgO, CaO, etc. have a specific band gap (such as 3.3 eV in case of ZnO NMs) that absorbs the characteristic wavelength of light for the generation of electron (e^{-}_{CB}) and hole (h^{+}_{VB}) pairs in the conduction and valence band, respectively. These electron-hole pairs have the probability of recombining in picoseconds to produce thermal energy without any chemical reactions or migrate/diffuse to the surface and initiate various reactions by reacting with other species such as O_2, H_2O, or other moieties adsorbed on the surface of the semiconductor. The ROS generated through different chain redox reactions are extremely reactive and believed to degrade the bacterial cell into CO_2, H_2O, and other nontoxic minerals; therefore, microorganisms in air and water can be destroyed when they come into contact with the surfaces of a photocatalyst.

To understand ROS generation mechanism and its relationship with the antibacterial potency of NPs, Li et al. (2012) studied the ROS generation kinetics of different metal oxide NPs (i.e. TiO_2, CeO_2, ZnO, CuO, SiO_2, Al_2O_3, and Fe_2O_3) and their bulk counterparts under the exposure of UV radiation (365 nm). The findings showed that different metal oxides had distinct photogenerated ROS kinetics, where TiO_2 and ZnO NPs were observed to generate all the three kinds of ROS ($^1O_2$, $^\bullet OH$, and $^\bullet O_2$), while the rest of metal oxides produced either one or two or did not produce any kind of ROS. NPs generated more ROS than their bulk counterparts presumably by having more UV radiation absorption sites due to the larger surface area. The average concentration of total ROS (the sum of the concentrations of three types of ROS) followed the order: $TiO_2 > ZnO > Al_2O_3 > SiO_2 > Fe_2O_3 > CeO_2 > CuO > ZnO$ (bulk) $> TiO_2$ (bulk). The ROS generation process was interpreted by comparing the electronic structures of metal oxides with the redox potentials of different ROS generation. Furthermore, a linear correlation was found between the average concentration of total ROS and the antibacterial activity of the NPs on *E. coli* cells as the model bacterium. However, in the dark, none of these metal oxides were reported to produce detectable ROS within the experimental period. Likewise, Lipovsky et al. (2009) related the toxic effect of ZnO to the elevated levels of ROS, namely, $^1O_2$ and $^\bullet OH$ radicals, when the aqueous suspension ZnO was irradiated with blue light (400–500 nm).

Padmavathy and co-workers studied the effect of size (ranging from micron to nm) on the antibacterial property of ZnO against *E. coli*. The results demonstrated that nanosized ZnO (10–50 nm) is more effective antimicrobial agent than bulk ZnO (2 μm). Comparatively high antibacterial effect of ZnO NPs was attributed to its abrasive surface texture due to rough edges and corners that contributed to the mechanical damage of the cell membrane. Moreover, with a decrease in size of ZnO particles, there is an increase in the generation of ROS, which killed bacteria more effectively (Padmavathy and Vijayaraghavan 2008). Jones and co-workers studied the antibacterial property of the purchased ENMs (ZnO, TiO_2, MgO, CeO_2, and CuO) against both GP (*B. subtilis*, *E. faecalis*, *S. pyogenes*, *S. epidermis*, and *S. aureus*) and GN bacteria (*E. coli*) and evaluated that among all, ZnO proved to be an excellent material with maximum antibacterial property (Jones et al. 2008).

Many studies have ascribed the antimicrobial activity of ZnO ENMs to the release of Zn^{2+} ions in a medium (Blinova et al. 2010; Wong et al. 2010; Heinlaan et al. 2008). When ZnO NMs are in solution, partial dissolution results in the release of Zn^{2+} ions, which have antimicrobial activity. Li et al. (2011) investigated the effect of dispersion medium on the toxicity of ZnO NMs and reported that the toxicity can be related with the concentration of the free hydrated Zn^{2+} ions or labile Zn complexes. In a typical study, five different aqueous medium, i.e. ultrapure water, NaCl (0.85%), phosphate-buffered saline (PBS), minimal Davis (MD), and Luria-Bertani (LB) were chosen to investigate the potential effect of water chemistry on the toxicity of ZnO NMs to *E. coli*. The results showed that the toxic effect of ZnO NMs in different media was in the order of ultrapure water > NaCl > MD > LB > PBS. The formation of precipitates like $Zn_3(PO_4)_2$ in case of PBS and complexes of Zn

with citrate and amino acids in case of MD and LB, respectively, were observed to decrease the concentration of Zn^{2+} ions, resulting in the lower toxicity in these media. These species drastically reduced the Zn^{2+} ion concentration, which resulted in lower toxicity in these media. Additionally, the isotonic and rich nutrient conditions improved the tolerance of *E. coli* to toxicants.

In another study, Jain et al. (2013) explored the physiological effects of the ZnO nanorods on the GP (*S. aureus* and *B. subtilis*) and GN (*E. coli* and *A. aerogenes*) bacterial cells. The findings showed that the antibacterial activity of ZnO nanorods were higher against GP bacteria than GN bacteria, suggesting that the structure of the cell wall plays a major role in the interaction with ENMs and displays a high sensitivity to the concentration of the particles.

The cause of antibacterial action in the dark is primarily attributed to the leaching of Zn ions from ZnO suspension into the cells, causing DNA damage and cell death. However, studies have shown the effective production of ROS even in the dark (Xu et al. 2013). For instance, Prasanna and Vijayaraghavan (2015) have reported that the production of ROS such as $^{\bullet}OH$, H_2O_2, $^{\bullet}O_2^-$, and $^{\bullet}HOO$ from the aqueous suspension of ZnO NPs in the dark can cause oxidative stress resulting in antibacterial activity. This work further confirmed that surface defects play a major role in the production of ROS both in the presence and absence of light.

27.4.4 Copper (Cu)-Based ENMs

Cu and its complexes are popular for their biocidal properties since ancient times. The earliest record of Cu being used for medicinal application can be found in Smith Papyrus, an ancient Egyptian medical text composed during 2600 to 2200 B.C.; it describes the use of Cu to sterilize chest wounds and drinking water (Borkow and Gabbay 2009). The doses required for the treatment of bacterial infections, however, are reasonably high enough to cause concomitant damage to healthy surrounding cells as well. Therefore, the direct use of Cu complexes for the treatment of bacterial infections is restricted in many cases. Recent studies have confirmed that Cu based ENMs have high antibacterial potency within a considerably low dose range. It implies that Cu based ENMs (metallic Cu, cupric oxide (CuO), and cuprous oxide (Cu_2O)) can be used as a potential antimicrobial agent. Kruk et al. (2015) reported the high antibacterial activity of monodispersed metallic Cu NPs (50 nm) against standard and clinical strains of GP bacteria (MRSA) and antifungal activity against *Candida* sp.

Oxides of Cu typically occur in two different forms, i.e. CuO and Cu_2O, which are both p-type semiconductors that have a band gap that ranges approximately between 1.21–1.55 and 2.2–2.5 eV, respectively. Comparatively, CuO is thermodynamically more stable and exhibit a broad spectrum of antibacterial activity. Some studies (Kumar et al. 2019; Gunawan et al. 2011; Hans et al. 2013) have observed high antimicrobial efficacy for Cu_2O than CuO, since it can generate cuprous ions (Cu^{1+}) which has been shown to be more toxic to bacteria than cupric (Cu^{2+}) ions. Meghana et al. (2015) reported in their study that antibacterial activity of CuO NPs is

closely dependent on the oxidation state and biocidal activity of Cu_2O against *E. coli* was found to be high than CuO. However, instead of Cu ion toxicity, ROS generation and binding of proteins were argued as major contributing factors for the antibacterial activity of CuO and Cu_2O NPs, respectively.

Applerot et al. (2012) studied the size-dependent (from the microscale size (800 nm) down to ultra-small nanoscale size (30 and 2 nm)) antibacterial activity of CuO particles. The antibacterial properties of CuO particles were found to be associated with their size, where smallest NPs were observed to have the highest biocidal activity. The effective antibacterial activity of CuO NPs was attributed to the increase of intracellular oxidative stress due to the generation of ROS by the NPs attached to the bacterial cells. Electron microscopy study further indicated that the ultra-small CuO NPs penetrated the bacterial cells. Similarly, Chauhan et al. (2019) investigated the effect of size and morphology on the antimicrobial activity of CuO NMs against the pathogenic bacteria (*S. aureus*) and reported that it varied as a function of size and surface area. Karim and co-worker fabricated CuO-based nanozymes which produce ROS in dark conditions, and upon irradiation of visible light, there was 20% enhancement in the ROS production. This enhanced ROS damages *E. coli* cell wall and leads to cell death (Karim et al. 2018).

27.4.5 Titanium (Ti)-Based ENMs

TiO_2 is one of the most extensively studied metal oxide semiconductors due to its great potential in the field of photocatalysis. Owing to various properties like biologically inertness, non-toxicity, substantial stability and production of ROS when illuminated with UV light makes TiO_2 the most suitable component for antibacterial applications. All of these radicals are known to be very reactive and easily disrupt organic compounds.

The novel concept of photochemical sterilization was first demonstrated by Matsunaga et al. (1985) using a powder of TiO_2 semiconductor loaded with platinum (TiOE/Pt). The authors reported the antimicrobial activity for TiOE/Pt powder under the exposure of metal halide lamp irradiation against bacteria (*E. coli* and *L. acidophilus)*, yeast (*S. cerevisiae*), and algae (*C. vulgaris*). Inhibition of respiratory function due to oxidation of coenzyme A has been identified as a cause of cell death. They further suggested that bactericidal effects of catalyst were not caused by toxic substances such as H_2O_2 and free radicals released during electrolysis and direct oxidation of the microbial cell was responsible for the loss of viability.

Following this initial study, research work on TiO_2 photocatalytic killing has been extensively conducted on a wide spectrum of organisms including viruses, protozoa, bacteria, fungi, algae, and cancer cells. Tsuang et al. (2008) investigated the photo-killing effects of TiO_2 NPs against five different bacteria (i.e. *E. coli*, *P. aeruginosa*, *S. aureus*, *E. hirae*, and *B. fragilis*) under UV light. At the end of the study, it was revealed that UV light alone did not affect the viability of bacteria, while TiO_2 NPs, especially under UV light exposure, showed significant effects on bacteria viability. Approximately, all the bacterial cells were killed within a 50 min

period indicating that TiO_2 NPs are very effective as antimicrobial agents. Liu et al. (2010) studied the effect of TiO_2, UV irradiation, and their combined exposure on the *E. coli* cells. After treatment with TiO_2 or UV light alone, cells sustained damage to the outer membrane due to lipopolysaccharide rupture to some degree, but the cells were still able to retain the initial rod morphology. However, the outer membrane of *E. coli* was severely compromised and totally removed in the presence of TiO_2 under the exposure of UV light.

Conventional TiO_2 ENMs are activated only under UV illumination, which is less than 5% of the solar spectrum compared to 45% of visible light (Ikram et al. 2020). In addition, overexposure to UV radiation has been known to cause serious genetic damage to human cells and tissues, which restricts the practical application of TiO_2 ENMs. To date, numerous studies have been carried out to design TiO_2 ENMs with an aim to draw its photoresponse into visible light region (Ismael et al. 2020). These include doping TiO_2 ENMs with metallic or non-metallic ion and coupling with narrow band-gap semiconductors. For instance, Yadav et al. (2014) reported the photocatalytic antibacterial activity of Ni-doped TiO_2 NPs under fluorescent visible light against GP (*S. aureus* and *B. subtilis*) and GN (*E. coli* and *S. abony*) bacteria. Ananpattarachai et al. (2016) studied the effects of cation (Ni) and anion (N) doping on the structure, visible light-absorbing capacity, and antimicrobial activity of the TiO_2 ENMs. N-doped TiO_2 ENMs were observed to show higher antibacterial activity than un-doped and Ni-doped TiO_2, which was attributed to the band-gap narrowing that leads to more visible light absorption and the superb antibacterial properties.

In another study, Hamal et al. (2010) reported the fabrication of Ag, S, and C co-doped TiO_2 composite NPs as an effective biocide/sporicide in dark and photocatalyst in visible light. It was reported that sporicidal efficacy of co-doped NPs increased with increasing Ag doping, while photocatalytic efficacy of co-doped NPs was higher at lower Ag concentration. According to this result, it is estimated that Ag/(C, S)-TiO_2 composite NPs can act as a potential biocide at higher loadings and a photocatalyst under visible light at lower loadings. Liu et al. (2017) reported the remarkable photocatalytic and antibacterial capability under visible light irradiation for TiO_2-Ag_2O heterostructure composite, which was mainly attributed to the synergistic effect between Ag_2O NPs and TiO_2 microsphere. Highly dispersed smaller Ag_2O NPs (5–30 nm) were suggested to enhance the visible light absorption and efficient separation of photo-induced charge carriers. To enhance the light absorption region of TiO_2 ENMs, Wang and co-worker (2010) fabricated the tri-doped TiO_2 NPs by doping with Er^{3+}, Yb^{3+}, and Fe^{3+} ions. By this method, they successfully broadened the light absorption region to the near infrared region which leads to more penetration effect and enhancement in antibacterial activity in this region.

In addition to extending the photoresponse to the visible or infrared region, doping has also been reported to improve the antibacterial properties of the parent

ENMs. Soo and co-workers (2020) have prepared Ag-doped TiO_2 nanofibers using calcination and electrospinning methods and evaluated the antibacterial property of $AgTiO_2$ against *S. aureus* and *S. albany* bacteria, which showed the enhancement in the antibacterial property of $AgTiO_2$ as compare to pure TiO_2 nanofibers.

27.4.6 Calcium (Ca)- and Magnesium (Mg)-Based ENMs

Among the widely explored metal oxides as antimicrobial agents, MgO and CaO are of particular interest because they are stable under harsh process conditions and generally regarded as safe materials to human beings. Moreover, they have antimicrobial activity without photoactivation, compared to TiO_2 which requires photoactivation. It has been verified that the antibacterial mechanism of CaO and MgO ENMs is driven by the generation of superoxide on the surface of these particles and also an increase in pH value by the hydration of CaO and MgO with water.

Nguyen et al. (2018) investigated the antimicrobial properties of MgO NPs against prevalent infectious bacteria (*E. coli*, *P. aeruginosa*, *S. epidermidis*, *S. aureus*, and MRSA) and yeasts (*C. albicans*, *C. albicans*, *C. glabrata*, and *C. glabrata*). The MgO NMs was reported to be more effective against GN bacteria than GP bacteria owing to differences in the structures of cell wall and membrane. The interactions of MgO NMs with cell wall and/or membrane were suggested to be the key mechanism for the lethal effects of MgO against planktonic bacteria. In another study, Yamamoto and colleagues (2010) fabricated the $CaCO_3$ (grain) and nanoscaled MgO (20 nm fine crystallite) based composite powder by thermal decomposition of dolomite for oral hygiene application. Gedda et al. (2015) fabricated CaO nanoplates with a length ranging 40–130 nm and breadth ranging 30–100 nm by using shrimp shells as the source. As-fabricated NMs were reported to possess effective antimicrobial activity against GP (*E. coli*) and GN (*S. aureus*) bacteria. The NPs were observed to accumulate around the cell membranes and inside the bacterial cells, thereby suggesting that NPs must have infiltrated the cells by destroying the cell membranes, which demonstrates the mode of bactericidal action of the CaO NPs.

According to the reports, MgO NPs damage the cell membrane and then cause the leakage of intracellular contents which in turn lead to death of the bacterial cells. Hewitt et al. (2001) reported that MgO initiated the some degree of cytoplasmic membrane de-polarisation changes in *E. coli* bacteria. Leung et al. (2014) described that strong antibacterial activity of the MgO NPs could be observed in the absence of any ROS production. They declared that the mechanism of antimicrobial activity might be due to the cell membrane damage. MgO NPs showed the bactericidal activity against both GP and GN bacteria. Sawai et al. (2000) investigated antibacterial activity of MgO against *E. coli* and *S. aureus*. They suggested that the presence of active oxygen, such as superoxide, on the surfaces of MgO NPs was one of the primary factors that affect their antibacterial activity.

27.4.7 Other Metal-Based ENMs

In addition to the conventional metal-based ENMs, there has been great interest in using other metals such as iron (Fe), palladium (Pd), platinum (Pt), tellurium (Te) and selenium (Se) based ENMs for antimicrobial applications.

Among these metallic NMs, Fe-based ENMs are widely explored for their biocidal properties with proven efficacy owing to their higher sensitivity to visible light for the formation of radicals, low cost of production, chemical stability (stable across a broad range of pH), ease of fabrication non-toxicity, abundance, and reasonable cost. Basnet et al. (2013) reported the fabrication of α-Fe_2O_3 nanocolumns and nanorods for visible light antimicrobial applications against *E. coli* bacteria. Under visible light exposure, the nanorod arrays were observed to be more efficient at inactivating *E. coli* than thin film samples. The increased biocidal effects of nanorods were attributed to their morphology, which promoted the longer contact times between bacteria and α-Fe_2O_3 surface and thus increased the probability that *E. coli* could be inactivated by direct photochemical oxidation of the intra cellular coenzyme A.

Lee and co-workers (2008) reported the high bactericidal activity of zero-valent Fe (Fe^0) NPs in aqueous solution against *E. coli*. A strong bactericidal effect of Fe^0 NPs was found under deaerated conditions, with a linear correlation between log inactivation and Fe^0 NPs dose (0.82 log inactivation/mg/L nano-Fe^0 h). The inactivation of *E. coli* under air saturation required much higher Fe^0 NPs doses due to the corrosion and surface oxidation of Fe^0 NPs by dissolved O_2.

Polyvinyl alcohol (PVA), often referred as green polymer, is among the most commonly used synthetic polymers for biomedical applications due to its solubility pattern and easy degradability and biocompatibility. Tran and colleagues (2010) studied the biocidal effect of PVA-stabilized Fe_2O_3 NPs on *S. aureus*. The results provided evidence that Fe_2O_3 NPs inhibited the growth of *S. aureus* and the antimicrobial inhibition behaviour was concentration-dependent. In addition, all cells were not adversely impacted in the presence of Fe_2O_3 NPs, especially osteoblasts (bone-forming cells), whose growth was observed to be enhanced. These studies have shown that Fe_2O_3 NPs can have a dual therapeutic role which could boost bone growth and inhibit bacterial infection as well. Finally, this research suggested that, with an appropriate external magnetic field, Fe_2O_3 magnetic NPs could be guided to destroy bacteria as required in the body.

Other than Au and Ag, precious metals such as platinum (Pt), palladium (Pd), rhenium (Re), etc. have also been studied for their antimicrobial activity. For instance, research has shown that platinum NPs have the capability to pass into the cell which makes it a potentially good candidate for antimicrobial therapy (Rice et al. 2019). Tahir et al. (2017) examined the antimicrobial activity of biosynthesized Pt NPs (2–7 nm) against GP (*B. subtilis*) and GN (*P. aeruginosa*) bacteria. The results showed the high antimicrobial activity of Pt NPs for both the bacteria.

Taking advantage of the exceptional catalytic properties, Pd based ENMs have been used in a number of important chemical reactions, manufacturing pharmaceuticals, degrading hazardous pollutants, and detection of different analytes. Different studies have been performed to examine the antimicrobial capacity of Pd NPs, which provides an indication of their usefulness as target antimicrobial compounds. Like, Adams et al. (2014) reported the first investigation of size-dependent antibacterial activity of Pd NPs against GN (*E. coli*) and GP (*S. aureus*) bacterial growth culture. Results showed that Pd NPs are highly antimicrobial, and fine-scale differences in size can alter their antimicrobial activity. Fang et al. (2018) reported the facet-dependent antibacterial properties for Pd NCs where {100}-faceted Pd cubes were observed to kill GN bacteria (*S. aureus*) more efficiently than {111}-faceted Pd octahedrons counterparts which displayed better results against GN bacteria (*E. coli*).

In recent years, tellurium (Te) and selenium (Se) (Guisbiers et al. 2016; Lara et al. 2018) based NPs have gained interest as potential antibacterial agents. Tellurite (TeO_3^{2-}) ions have antibiotic properties and have been used to inhibit the growth of a several microorganisms, including penicillin-insensitive bacteria. Lin et al. (2012) investigated the morphology-dependent antibacterial activity of Te NMs. In a typical experiment, Te NMs with four distinct morphologies (i.e. nanowires, nano-pencils, nano-rice, and nano-cubes) were fabricated and further tested for their antibacterial activity against *E. coli*. The antibacterial activity of Te NMs was obtained in the following order: nano-cubes > nano-rice > nano-pencils > nanowires. Moreover, antibacterial activity of Te NMs was reported to be higher than Ag NPs, while toxicity towards mammalian cells was lower than that Ag NPs, which according to authors clearly suggests that Te NMs have great potential for use as effective antibacterial agents.

In another study, Cruz et al. (2019) biosynthesized the rod- and cubic-shaped Te NPs by using the extracts of lemon, orange, and lime as reducing and capping agents via microwave-assisted reaction. Te NPs showed an important antibacterial activity against both GN (MDR *E. coli*) and GP bacteria (MRSA) in a range concentration from 5 to 50 μg/mL over a 24 h time period with a main mechanism of inhibition related to ROS production.

Se compounds have been extensively investigated because of their anticancer properties and low toxicity, and Se NPs have been shown to exhibit lower cytotoxicity relative to selenium compounds. Recently, Geoffrion et al. (2020) fabricated Se NPs by a novel green process called pulsed laser ablation in liquids technique. Se NPs showed a dose-dependent antibacterial effect towards both standard (*P. aeruginosa* and *S. epidermidis*) and antibiotic resistant, i.e. MDR *E. coli* and MRSA strains of bacteria at a range of concentrations between 0.05 and 25 ppm. Besides, Se NPs were reported to show a low cytotoxic effect on human dermal fibroblast cells up to a concentration level of 1 ppm as well as an anticancer effect on human glioblastoma and melanoma cells at the same range of concentration. Huang et al. (2019) fabricated the spherical shaped Se NPs with the size ranging from ~40 to 200 nm and further investigated the influence of size on the cytotoxicity and antibacterial activity. The antibacterial activity of the Se NPs was shown to be

strongly dependent on size where Se NPs with size of 81 nm showed the maximal growth inhibition and killing effect of methicillin-sensitive (MSSA) and methicillin-resistant *S. aureus* bacteria (MRSA). The Se NPs were reported to have multimodal mechanisms of action that includes the depletion of internal ATP, disruption of membrane potential and ROS production.

27.5 Summary

With ever-growing resistance against common disinfectants and antibiotics, microorganisms have challenged the modern science and medicine for the effective and sustainable treatment of infectious diseases. It is evident from the literature that application of metal-based ENMs can be considered as a suitable alternative to antimicrobial agents and appear to have high potential to solve the problem of the emergence of AMR. Several valuable studies have been documented in the field of antibacterial ENMs in the recent years.

However, the exact mode of action of these ENMs still remains elusive. Therefore, to address the main mechanism for antibacterial activity of metal-based ENMs will be worth to address in future research. There are still some unanswered questions on the penetration of metal-based ENMs into the bacterial cell wall. The ENMs have enormous therapeutic potential, but there are some toxicity issues that restrict their current usage and required to be addressed. Environmental disposal of NMs is also a matter of concern since they are reported to contribute to some environmental problems. A greener approach needs to replace the ongoing methods of synthesis of ENMs.

Therefore, in order to exploit ENMs for their antimicrobial potential, a perfect balance should be achieved highlighting the potentials of ENMs along with masking the limitations at the same time with utmost care. Finally, it can be concluded that in the near future, metal-based ENMs with minimal toxicity can plausibly be used as alternatives to conventional antimicrobial agents for eradicating the several pathogenic microorganisms.

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Part III

Traditional Drugs

Strengthening Immunity: Ayurveda and Medicinal Plants

28

D. R. Nag and Akshay Nag

Abstract

It is the desire of the human being to live a long healthy life for which he struggles throughout his living time. During the process he may fall ill and may get infected with the various diseases. How one gets infected or falls ill varies from man to man. It is directly related with the immune system of the individual: the stronger he is, the lesser chances to fall ill or get infected, and the weaker he is, the more prone to get infected. According to Hindu mythology and Ayurveda, the atmosphere in which we live is basically made of five fundamental elements called as *Panch Mahabhoot*. These five elements are *Prithivi* (earth), *Vayu* (air), *Akash* (ether), *Agni* (fire), and *Jal* (water). These are the bases of our life on this planet. Unbalanced harnessing of these bio-resources resulted in the various diseases and infections. These gross elements give rise to the concept of *Doshas, Dhatu*, and *Malas* in Ayurveda. Among these, *Doshas* play a vital role in determining the status of health of the person. Three forms of *Doshas* are *Pit, Kapha*, and *Vat* which reflect on the *Prakriti* of the individual. Imbalance of *Doshas* may lead to the disease or disorder. There is advisory to the persons of a specific *Dosha*s and *Prakrit*i to adopt specific *Dincharya-Ahar-Vihar* (daily lifestyle and food habits) and *Ritucharya* (seasonal lifestyle) for developing strong immune system to fight against the infection and remain healthy. The calendar year is divided in to six *Ritus*, 2 months of each under the *Ritucharya* concept. In each of the six *Ritus*,

D. R. Nag (✉)
Regional Cum Facilitation Center-NR-1, NMPB, Ministry of AYUSH, GOI, RIISM, Jogindernagar, HP, India

Medicinal Plants, AYUSH, RIISM, Government of HP, Jogindernagar, HP, India
e-mail: drnag04@gmail.com

A. Nag
Department of Biotechnology, Panjab University, Chandigarh, India

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there are guidelines laid down regarding what to take and do or perform and what not to take and perform. All the six *Ritus* are classified together in *Adan Kal* and *Visharg Kal*. *Adan Kal* is from December to mid of June when immune system of the body declines, whereas in *Visharg Kal* from mid of June to December, the immune system remains vigor. In addition to *Doshas*, *Dincharya*, and *Ritucharya*, practicing *Yoga* makes the mental health strong which brings the harmony between the mind and body. *Ritucharya lifestyle* and *practicing Yoga* together enhance the immune system of the body which is quite relevant in the present time to fight against COVID-19 pandemic. In addition *Aushadhi Dravya* (medicinal plants) described in Ayurveda are of immense value to act as remedial drugs to fight against the diseases and to develop strong immunity. The different herbal formulations mentioned in the *Charak Samhita*, *Sushruta Samhita*, and other classic texts of Ayurveda are derived from the herbal resources and are in use in the system. The varying agroclimatic conditions of the country from extreme tropical to the extreme temperate make it a one of the mega *hot spots* of the medicinal plants in the world. In the present write-up, there is attempt to short-list some such plants which are widely used as drugs or food supplements for enhancing the immunity of the body.

Keywords

Hindu mythology · Ayurveda · Medicinal plants · Herbal formulations · Immunity

It has been the desire of every human being to live a healthy, long and disease-free life. The new born child undergoes the various drastic physiological changes when it comes out from the mother's womb. His struggle starts immediately to adjust with the nature to the long journey of life in which he lives. During the course of time, he grows into an adult from childhood gradually. The consistent struggle on this earth continues till the death of the individual to live a painless, healthy, and disease-free life. But in fact, this does not happen, and the person usually may get infected by the various viruses and bacteria and may fall ill at the various stages of life. Many of these diseases are the result of lifestyles he adopts or by the curse of nature for its unethical exploitation.

28.1 Panch Mahabhoot

The nature has blessed us with the various elements on this earth which are necessary for living. These bio-resources are in the form of visible and non-visible; and living and nonliving forms. The visible elements which exist above the earth are plants, animals, and water, while the non-visible are air, sun rays, microorganisms, and ether. Hidden beneath the earth are the non-visible elements which are minerals, underground plants' parts, microbes, and animals. Broadly we can also classify these into organic: with living origin; and nonorganic with nonliving origin. The existence

of life on this earth is the balance sum up of these bio-resources. Any imbalance between these may harm the life.

Therefore, the judicious harnessing of these resources is necessary for the existence of life on this planet. The concept of the different bio-resources elements as mentioned above was well understood by our ancestors since time immemorial. It has been described in detail in *Vedas*, the oldest manuscript in Hindu religion about 200,000 years back and later on in Ayurveda classics. The widely acknowledged recorded theory of *Panch Mahabhoot* was mentioned in Ayurveda about 5000 years back. According to Ayurveda, everything in this universe is made up of five basic elements in different proportions. The five elements are *Prithivi* (earth), *Vayu* (air), *Akash* (ether), *Agni* (fire), and *Jal* (water) called as *Panch Mahabhoot*. Ayurveda believes that everything in this universe is made up of five basic elements. The human body is, therefore, basically also made up of these *Panch Mahabhoots* represented in different forms, for instance, Prithivi (earth) as bones and teeth, *Vayu* (air) as oxygen (*Pran Vayu*), *Akash*(ether) as space between matter and cavities, *Jal* (water) as blood and lymph, and *Agni* (fire) as metabolism. In the formation of universe, these basic elements get absorbed into each other forming the gross elements the *Panch Mahabhoot.* They are principle reflection of *a Prapancha,* the universe. These gross elements give rise to the *Doshas*, *Dhatus*, and Malas.

28.2 Concept of Tridoshas

The *Doshas* are the result of a dynamic interplay between the *Panch Mahabhootas*. These *Doshas* form the principle energy sources within the body which control all functions of the body ranging from the simple cellular processes to the most complex processes of biological functions. The term *Dosha* denotes the property which is "subject to change" or "quick to go out of balance." Doshas are the classic examples of energy and matter in dynamic accord. In a state of balance, they cannot be visibly detected, but when out of balance, they manifest a transgression from their normal functions. There are three forms of *Doshas*; the term denotes *Tridoshas* or three vital energies. These are the combination of pure elements becoming manifest in the physical world. These are *Vata* combination of ether and air, *Pitta* combination of water and fire, and *Kapha* combination of earth and water. These three *Doshas* are a reflection of *Prakriti* (primordial nature) of the human beings. Imbalance of *Doshas* in a person of specific *Prakriti* may lead to a disease or ailments.

28.3 Ritucharya

There is a detailed advisory laid down in Ayurveda for the persons of different acquired *Prakriti* on how to live a healthy, disease-free life by adopting the right way of daily lifestyle (*Dincharya)*, food habits, and living (*Ahar-Vihar)* by balancing *Tridoshas.* Concept of *Ritucharya* has been described in detail in Ayurveda (Charak

Sutar 6), where the guidelines have been laid down to adopt food habits and lifestyle in each of the six different *Ritus* of the year. In the western classification, there are four seasons in a calendar year, i.e., winter, spring, summer, and autumn, but in Hindu mythology the calendar of the year divided into six *Ritus*. Each *Ritu h*as 2 months (*dyumasa*). These six *Ritus* are:

Sishira	Later winter
Vasanta	Spring
Grishma	Summer
Varsha	Rainy season
Sharad	Autumn
Hemant	Early winter

The first three *Ritus* are together called as *Adan Kala* (*Uttarayana*, northern solstice tilt of the northern hemisphere of the earth away from the sun) and next three together called as *Visarga Kala* (*Daksinayana*, southern solstice tilt of the southern hemisphere of the earth away from the sun). *Adan* Kala is the period from mid-December to mid-June. During this period, the immune system of the body declines. *Visarga Kala* is the period from mid-June to mid-December. During this period, the strength and vigor remain enhanced. So food and regimen should be controlled to prevent diseases due to over nutrition. The diet and routine activities should be aimed at increasing strength and vigor. There is gradual decrease of *balam* (physical strength or immunity due to the effect of season) during *Uttarayana a*nd gradual increase of *balam* during *Daksinayana.* There occurs some special changes in environment and humans in every *Ritu*, and hence certain foods and exercises are specially told for each *Ritu*; this process is called as *Ritucharya* (source: Arunadatta, commentary on *Astang Haridya Sutra 3/1*).

There are certain seasonal changes in Dosha*s* which are mainly as:

1. *Chaya*—accumulation of deranged *Doshas* in its main site like *Vata Dosha* in the intestine.
2. Prakopa—accumulation of deranged Doshas in other main seats in the body like *Vata Dosha* in the low back region.
3. *Prasama—Doshas* coming back to normalcy after derangement.
4. As stated above, in every *Ritu,* the food habits and lifestyles have been indicated in detail in the Ayurvedic classic, the *Ashtanga Hridaya* for adaptation to live a healthy and disease-free life. It enhances the immunity of the individuals to fight against various infectious ailments. The period in between two *Ritus* when the season of one *Ritu* is almost over, and it is just the starting time of the next *Ritu*, is prone to many diseases due to change in the climatic conditions. This period is called *Sandhikala.*
5. At this juncture precaution is required to be taken in the food habits and lifestyle. In these six *Ritus* what is to be taken/performed, i.e., *Pathya* (dos), and what is not to be taken/performed, i.e., *Apathya* (don'ts), are advocated in *Ashtanga Hridaya.*

S. No.	Ritu	DOs	Don'ts
1.	Shishir Mid-December to mid-February	• Sweet, sour and salty food • Milk and its products • Honey, lukewarm water • Nutritious diet • Oil massage • Protection against cold • Physical exercise	• Bitter, astringent, and pungent food • Fasting • Use of cold water • Living in cold environment
2.	Vasant Mid-February to mid-April	• Drink lukewarm water • Use of wheat, barley • Appropriate use of fermented beverages • Physical exercise	• Sour and fried food • Heavy diet • Day sleeping
3.	Grishma Mid-April to mid-June	• Sweet, cold, and liquid diet • Plenty of water • Seasonal fruits like grapes • Coconut water • Morning walk	• Heavy exercise • Excess sexual indulgence • Hot, spicy, and salty food • Exposure to the sun
4.	Varsha Mid-June to mid-August	• Use of honey • Use of rice, wheat • Boiled water • Oil massage • Fermented beverages • Soup of pulses	• Cold water • Day sleeping • Heavy exercise • Surface water Excess sexual indulgence
5.	Sharad Mid-August to mid-October	• Sweet, bitter, and astringent food • Fruits like amla, grapes • Use of rice, wheat, green gram • Therapeutic purgation	• Fried food • Use of curd • Day sleeping • Exposure to strong sunshine
6.	Hemant Mid-October to mid-December	• Sweet, sour, and salty food • Milk and its products like ghee, curd, etc. • Use if honey, lukewarm water • Heavy, nutritious diet • Oil massage • Protection against cold • Physical exercise • Steam bath	• Bitter astringent food and fasting • Use of cold water • Living in cold environment

Source: CCRAS, Ministry of AYUSH, GOI, New Delhi

28.4 Concept of Yoga

Ayurveda is a way of life to learn how to live a long, healthy, and disease-free life by adopting the concept of *Ritucharya* as stated above along with Yoga and *Pranayam.* Yoga is essentially a spiritual discipline based on an extremely subtle science, which

focuses on bringing harmony between the mind and body. It is an art and science of healthy living. The word Yoga is derived from the Sanskrit root *Yuj*, meaning to join or to yoke or to unite. The history of Yoga dates back about 5000 years back and was first mentioned in *Rigveda* in form of the collection of texts that consisted of *rituals*, *mantras*, and *shaloka*s performed by the Vedic priests. Maharishi Patanjali is considered the father of Yoga who codified the Yoga concepts in a format known as *Yoga sutras* which are 196 in number. Yoga sutras of Patanjali date back from the second-century BCE. The concept of Yoga is based on five principles:[1]

- **Principle 1: Proper Relaxation:** By carrying out the various day-to-day physical activities and the mental emotion, one get tired feels fatigue in the muscles and other body organs. It is necessary to release the tension by giving the rest to the body which boosts the body performance to act and to conserve the energy.
- **Principle 2: Proper Exercise:** The human body consists of different organs which are actually a complex assembly of muscles, ligaments, and bones, interconnected with joints and over all a network of blood circulating in the system ensuring the supply of energy at cellular level. The regular exercise by adopting the different yogic postures or *Asnas* enhances the flexibility of the body parts and the blood circulation to keep the body fit and healthy.
- **Principle 3: Proper Breathing:** Breathing is a vital function of the living human being to inhale air *Vayu* by the lungs. Lungs absorb the oxygen present in the air which is transferred to the blood. Oxygen is important to every cell of the body as it converts the complex foods in to energy by the sequence of chemical changes through a process called as oxidation. Air contains about 21% oxygen and about 78% nitrogen with a very little amount of other gases like argon and carbon dioxide; traces of neon, helium, methane, krypton, and hydrogen; and traces of water vapors. As oxygen part of the air is essential for the survival of the body, and therefore it has been termed as *Pran Vayu*. Inhalation and exhalation time span plays a significant role for the amount of oxygen absorbed by the lungs. Slow deep breathing enhances the oxygen intake than superficial normal breathing. Further retention of inhaling air in lungs also increases the oxygen level in the blood. Management of inhaling and exhaling process of the air to increase the oxygen level in the blood is strongly recommended while practicing Yoga.
- **Principle 4: Proper Diet:** Food is the basic source of our energy. It is a popular saying *Jesa ann-besa mann* which means what we eat reflects to our mental status. The balanced food derived from natural sources is always idle for good health. The processed junk fast foods decrease the immunity of the body to make it prone to many disorders like obesity, diabetes, etc.
- **Principle 5: Positive Thinking and Meditation:** Meditation is the integral part of Yoga which facilitates in having a peaceful mind. It should be performed early in the morning before the sun rises in open airy atmosphere. Under the guidance of experienced *guru*, a right way of meditation training is essential. In the

[1] Source: asiatrend.org/.

struggle-full life, one should practice keeping a positive outlook of life, and no space should be given for negative thinking in the mind. Meditation and positive thinking make life more colorful and happy to live.

Yoga is a thoughtful process for building a sound body with tension-free mind. Yoga is a wide concept based upon different aspects. One of such aspects is to include some daily routine activities and self-imposed discipline rules in the lifestyle to obtain the perfection in performing Yoga. Maharishi *Patanjali* has propounded eight such elements which are mandatory for a yogi to act and live known as *Ashtanga* Yoga. These eight limbs of Yoga are:

1. ***Yama***: These are basic ethic rules or restrains like honesty, nonviolence, non-stealing, and non-possessiveness. These are meant for a person prevents him from indulging in undesirable worldly activities.
2. ***Niyama*:** This is a self-moral code of discipline for developing healthy hygienic habits to remain fit and strong. Rising up early in the morning before sunrise, going to toilet, brushing of teeth, taking bath, cutting of nails and hairs periodically, doing exercise, taking nutritious balance diet, physical working, sound sleep, etc. are good lifestyle habits.
3. ***Asana*:**
   It is a posture that one can hold for a period of time, staying relaxed, steady, and comfortable and motionless. It keeps the body healthy and balanced and helps it in maintain harmony with nature.
4. ***Pranayam*:** It is breathing exercise involves deep breathe in, retention, and finally long slow breathe out. It helps to enhance the oxygen level in blood for the effective release of energy required to perform various activities in the body.
5. ***Pratyahara*:** It is the process of withdrawing the senses from external objects of the world. It is a step of self-extraction and abstraction. It is not consciously closing one's eyes to the sensory world, but it is consciously closing one's mind processes to the sensory world, and it empowers one to stop being controlled by the external world.
6. ***Dharna*:** The practice of *Pratyahara* creates the setting for *Dharna* which means concentration. Having relieved ourselves of outside distractions, we can now deal with the distractions of the mind. The practice of *Dharna* or concentration involves to slow down the thinking process by concentrating on a single mental point or object which may be a specific energetic center in the body or an image of a deity or the silent repetition of sound. We, of course, have already begun to develop our powers of concentration in the previous three stages of posture, breath control, and withdrawal of senses. In *Asana and Pranayama*, although we pay attention to our actions, our attention travels. In *Pratyahara* we become self-observant; in *Dharna* we focus our attention on a single point. Extended period of concentration naturally leads to meditation.
7. ***Dhyana*:** It is the meditation or contemplation; the seventh stage of *Ashtanga* is the uninterrupted flow of concentration. Although *Dharna* (concentration) and *Dhyana* (meditation) may appear to be one and the same, a fine line of distinction

exists between these two stages**,** whereas *Dharna* practices one point attention, and *Dhyana* is ultimately a stage of being keenly aware without focus. The strength and stamina it takes to this state of stillness are quite impressive.

8. ***Samadhi*:** Maharishi *Patanjali* describes this eighth and final stage of *Ashtanga* as a state of ecstasy. At this stage, the meditator merges with his or her point of focus and transcends the self altogether. The meditator comes to realize a profound connection to divine, interconnectedness with all living things. With this realization comes "peace that passeth all understanding" and the experience of bliss and being at one with the universe. On the surface, this may seem to be a rather lofty, "holier than thou" kind of goal. What Patanjali has described as a completion of yogic path is what, deep down, all human beings aspire to peace. We also might give some thought to the fact that ultimate stage of Yoga enlightenment can neither be bought not be possessed. It can only be experienced, the price of which is the continual devotion of the aspirant.

In Ayurveda, it has been stressed upon to adopt an idle lifestyle for performing the day-to-day various activities so that a person may not get infected by the various diseases and live a healthy long life. It all depends upon the immunity one has developed in his body how much he is prone to be attacked by disease-causing bacteria or viruses or by other means. Adoption of the *Ritucharya* guidelines for the seasonal living style and dietary habits blended with practicing Yoga suppresses the stress hormones that adversely affect the immune system of the body. Yoga contributes to maintain the good health of various vital organs of the body and high concentration of oxygen in blood which is necessary for the overall optimum functioning of the body. One of the first chapters in the learning of Ayurveda, *Swasthavritta*, tells us how to live a disease-free long healthy life without taking any medicine. It is mainly based on the principles of *Ritucharya* and Yoga as described above.

28.5 Role of Medicinal Plants

The fast busy lifestyle, unhealthy dietary habits, lack of physical works and exercise, pollution, unhygienic environment, carrier competition and insecurity of jobs, the work tensions, lack of sound sleep, etc. are making the person more prone to various diseases in the present times. All of this is due to the fact that our immune system is not strong enough to fight against the infection-causing microorganisms. Weak immune system leads to various diseases like frequent and recurrent pneumonia, bronchitis, sinus and ear infection, skin infection, infection of internal organs, low platelet counts, anemia, loss of appetite, nausea, diarrhea, and other growth disorders.

In the light of the outbreak of COVID-19 pandemic worldwide, the situation is becoming worst day by day, and the existing healthcare systems are unable to control this fatal disease. All the efforts are being made to invent any vaccine to control the disease, but till date no country has come up with any success in this direction. Even

the most powerful country of the world the USA has been badly affected, and millions of people are infected by this virus causing the death of the population at large scale. India is also facing the similar situation with the spread of the virus at fast rate. As stated above, there is no drug or vaccine available to control COVID-19, and, therefore, the preventive measures are the only option which may play a vital role to control the spread of COVID-19 to some extent. In this regard the WHO and the National Health Departments of GOI have come up with some guidelines to follow the countrymen, how to minimize the spread of the disease. These are to maintain the social distancing, bearing mask, not to shake hands with infected person, not to go in crowds, washing hands with soap time and again, use of sanitizer, etc. But these are the temporary measures effective only to some extent to prevent the spread of the disease till the invention of some vaccine which works against the disease. Worldwide efforts are being made in this direction including India, and it is hoped that in near future we shall be able come up with such vaccine. But the protocol involved in developing such vaccines is a time-consuming process, and till that we should follow the preventive methods carefully.

Medical science correlates with the immune system of a person to fight against the attack of virus and bacteria. In the present scenario, it is strongly emphasized to develop and enhance the immunity by various ways to fight against COVID-19. As discussed above, living with *Ritucharya* lifestyle and food habits and practicing Yoga make the body healthy with strong immune system; Ayurveda has also a vital role to enhance immunity by the use of *Aushadhi Dravya* the medicinal plants. The classic texts documented by ancient Ayurvedic scholars and Rishi like *Charka*, *Sushruta*, *Bhagvatta*, and others and numerous references are available regarding the use of plants as medicine against the various ailments. According to the therapeutic time tested actions of the plants, these have been grouped variously, and one of such group of medicinal plants which enhances the immunity of the body is classified as *Rasayana.* In this group some herbs, shrubs, and trees have been kept which yield different forms of the crude drugs derived from the morphological parts of the specific plants recommended for use as food supplements either as single-herb or compound formations. These formulations are use as decoction, infusion, Avaleh, powder, raw, cooked, and the like. *Chyavanprash Avaleha* is one of the examples of *Rasayana*'s well-known Ayurvedic formulation for enhancing the immunity of the body.

In the present COVID-19 hit circumstances prevailing in country, the AYUSH Ministry, GOI, and the state departments of Ayurveda have advised the use of some herbal-based Kwath/Kadha against the corona infection. One such preparation under the name of AYUSH Kadha is said to be highly effective in the decease and is recommended by the AYUSH Ministry, GOI, for the use as safety measure against COVID-19 and to be taken as decoction. It is a mixture of four crude drugs: tulsi (*Ocimum sanctum*) 40%, Kali Marich (*Piper nigrum*) 20%, ginger (*Zingiber officinale*) 40%, and Dalchini (*Cinnamomum zeylanicum*) 20%. There is a long list of medicinal plants which can be used as immunity booster, as relevant remedy against the virus indirectly.

India is climatically diverse country that exhibits all the types of climates which make it a repository of a large number of vegetation types. It is one of the mega climatically diverse countries in the world. The central part of the country is predominantly tropical with the costal habitats along the marine line, while the northern part of the country is mainly temperate Himalayan region with varying types of habitats starting from subtropical to extremely cold desert types, and in between are the temperate subalpine to alpine types of ecosystems. Presence of the Himalayas in the north and oceans in the south greatly influences the occurrence of a variety of plants species in the different natural habitats of the country. India is perhaps one of the rare countries in the world where all types of vegetation types are found. The European countries and the countries near polar regions are dominated with the temperate vegetation types, while the countries near Mediterranean region are of tropical in nature, while India exhibits both the tropical as well as temperate types of vegetation thus making it a biodiversity-enriched destination in terms of the occurrence of plants species. It is estimated that about 50,000 plants are found in the country out of which about 18,000 are flowering plants. Many of these plants are directly utilized as a basic source of food in form of cereals, pulses, vegetables, fruits, and oil-yielding plants while indirectly fodder plants as a source of food to the animals to meet out our demand of dairy, poultry, and such other products required in the daily life. A segment of these plants possess the medicinal properties conventionally utilized as remedy to cure many ailments since time immemorial and termed as medicinal plants. Number of such plants is approx. 6000 in the country out of which about 2500 are in use in various Indian systems of medicines. The role of some plants as an effective means of healthcare in the society is well accepted and described in detail by the various Ayurveda scholars like *Charak* and *Sushruta*. About 90% preparations of the Ayurvedic drugs are herbal-based. The various morphological parts of the plants yield the basic raw material called as crude drugs derived from the morphological parts of the species which may be in form of root drugs, bark drugs, hard wood drugs, leaf drugs, flower drugs, fruit/seeds drugs, whole plant drugs, exude drugs, and the like. These crude drugs as raw or in semi-processed form constitute a drug formulation of the Ayurvedic medicine. Many Ayurvedic classic formulations are either a single-herb preparation or a combination of many herbs; sometimes the number may be more than 30 herbs as in *Chyavanprash*. Poly-herbal combinations are proven far effective than single-herb formulations. In poly-herbal formulations the constituents are combined in such a way that the formula is balanced and reproducible. One or two of the plants in these combinations will be active, and the others will play a supporting role. The supporting herbs will each have different actions, acting as catalysts to help proper absorption and transportation and to reduce or eliminate toxicity. If an ideal combination is delivered, the results can be excellent, the outcome of which requires experience and thorough knowledge.

28.6 Dosage Forms

There is a long list of prescriptions either in from of single herbs or poly-herbal combinations to act as immunity booster under the prescribed dosage by the physician in different forms. Dose of the drug prescribed by the physician varies from man to man as per the age, *Prakriti* and *Doshas* of the individual. Some of the dosage forms are:

1. **Mishran (Mixtures)**: Containing two or more than drug formulation which may be in powdered form or liquid.
2. **Ekal Dravya Aushadhi (*Monoherbal Preparations*)**: Medicinal products that contain only a single herb or an extract thereof as an active ingredient.
3. **Hima (*Cold Infusion*):** It is obtained by soaking one part of herbs in six parts of water overnight and then scoured and filtered.
4. **Phanta (*Hot Infusion*):** Powder or coarse powder is prepared of the drug material, and then it is poured in four times the quantity of hot water in boiling stage (but not heated further from heat source), and the same is retained as such for some time. Afterward in hot state itself, it is strained or filtered, and this preparation is known as *Phanta* (hot infusion).
5. **Saar (*Extracts*):** Concentrate sap of the crude drug which is a mixture of active ingredients in liquid or viscous form.
6. **Swarasa (*Juice*):** The liquid extract of fresh, green, and clean herbs collected by squeezing, using pressure, or using any instrument is known as Swarasa which has to be to be pasteurized or treated with ultrahigh temperature to enhance the shelf life.
7. **Kwath (*Decoction*):** The liquid prepared by simmering 1 part of herbs in 16 parts (or 1/8 or ¼) of water and reducing it to 1/8 parts (or ¼) of water is known as *Kwath.*
8. **Kalka (*Paste*):** The paste made by crushing and grinding fresh herbs is known as *Kalka*. It also includes paste made by dried herbs along with water.
9. **Churan (*Powder*):** It is a fine-powdered form of drugs. When made in coarse form for making the decoction, it is known as *Kashaya* or *Kwath Churna.*
10. **Sharvat (*Syrups*):** It is a viscous preparation containing more than 50% sucrose, mainly used as flavoring agents, to mask an unpleasant taste of the ingredients.
11. **Taila (*Medicinal Oils*):** *Tailas* are the preparations in which *Taila* (oil) is boiled with prescribed *Kashaya* (decoction) and *Kalkas* of drugs according to the formulation. This process ensures absorption of active therapeutic properties of the ingredients used**.**
12. **Asava and Arista (*Fermented Syrups*):** *Asavas and Arka* are medicinal preparations made by soaking the drugs, either in powder form or in form of *Kasaya* (Decoction), in a solution of sugar or jaggery, as the case may be, for a specific period of time, during which it undergoes a process of fermentation generating alcohol, thus facilitating the extraction of the active principles contained in the drugs. The alcohol, so generated, also serves as a preservative.

13. **Arka (*Concentrate*):** Arka is a liquid preparation obtained by distillation of certain liquids or drugs soaked in water using the *Arkayantra* (distilator) or any modern distillation apparatus.
14. **Avaleh** or **Leha and Paka:** These are the semisolid preparation of drugs, prepared with addition of jiggery, sugar, or sugar candy and boiled with prescribed drug juice or decoction. They are also known as Rasayana, Lek, Paka, etc.
15. **Lepa:** Medicines in form of a paste used for external application are called lepas.
16. **Vati and Gutika (*Tablets*):** These are the compressed form of the powdered drug material mixed with some inert binder, and it is ensured that the tablets rapidly dissolve in the water. These may be coated with some coloring agent and made smooth to make them easily swallowed. Although the modern times on the analogy of allopathic drugs, it is customary to take the tablet form in many Ayurvedic formulations, as it is easy to swallow and carry them, but it can be difficult to provide adequate dose of extract, especially if it contains several compounds that need a higher dosage for optimum efficacy. Old-fashioned teas or decoctions might be more appropriate dosage forms in such cases.
17. **Ghrita (*Snehakalpa*):** Ghrit is the preparation in which ghee is prepared by heating with prescribed *Kasayas* (decoction) and *Kalkas* of drugs according to the formula. This process ensures absorption of the active therapeutic principles of the ingredients used.
18. **Varti, Netrabindu, and Anjana (*Eye Drops and Applications*):** Medicines used externally for the eyes come under the category *of Varti, Netrabindu,* and *Anjana*.

(***Source: AFI, Ministry Of AYUSH, GOI***)

In addition there are other dosage forms like teas, ointments, capsules, granules, suppositories, etc. in practice. For the drugs derived from mineral, metallic, and animal source, there are different dosage forms described separately.

Out of the long list of medicinal plants under use in the various drug formulations in Ayurveda, an attempt has been made to short-list some of the important medicinal plants species that play an important role to act as immunity booster either as a single-herb preparation or compound poly-herbal formulations. The medicinal plants species included in the following table have been selected very carefully on the basis of the properties they possess to act as general tonic and antioxidant, to cure debility, to act as cardiac tonic, to act as blood purifier, to strengthen pulmonary system, to act as Nervine tonic, to fight against infections, and over all to strengthen the immune system of the body.

S. No.	Name of the species	Trade name	Part(s) in use	Indications	Vishisht Ayurvedic Yog (Principal ayurvedic drug formulations)
1.	*Abies spectabilis* (D. Don) Spach Syn. A. webbiana Wall ex D. Don	Talish Patra	Leaves (needles)	Carminative, in cough and phthisis	Talisadya Churna Talisadi Vati
2.	*Adhatoda zeylanica* medic	Adusa	Leaves, Panchang	Yield a drug Vasaka used in bronchial troubles. Juice of leaves is for diarrhea, dysentery, and glandular tumors	Vasa avaleha Vasa-aristh Vasa-panak Vasa-chandadi tail
3.	*Albizia Amara* (Roxb.) Boivin	Krishan Sarish	Stem bark	In allergy	Mahasharish Agad Shrisha Aristh
4.	*Allium sativum* Linn	Rasona, Lahsuna	Bulb	In pulmonary phthisis, gangrene of lungs, and whooping cough. Juice is for laryngeal tuberculosis, lupus, duodenal ulcers, skin troubles, and used as ear drops	Rason Vati Rason Pind Rason Astak Lashunadya Ghrita, Rason sura, Lashun Kshir
5.	*Asparagus adscendens* Roxb.	Safed Musli, Sansbai	Tuberous roots	Nervine tonic, cooling, and general tonic	Musli Pak Mushaldi Yog
6.	*Asparagus racemosus* Willd	Shatavar	Tuberous roots	Potential tonic, diuretic, galactagogue and for strengthening nervous system and immunity	Shatavari Ghrita Narayan tail

(continued)

S. No.	Name of the species	Trade name	Part(s) in use	Indications	Vishisht Ayurvedic Yog (Principal ayurvedic drug formulations)
					Vishnu tail, Shatmulyadi Loh, Shatavripanak, Shatavar Pak
7.	*Azadirachta indica* A. Juss	Neem	Leaves, stem bark, seeds, and seed oil	Bark in skin trouble. Leaves as antiseptic, applied to boils, decoction given for ulcers and eczema; dried leaves keeps away incents in books and cloths. Berries purgative and emollient. Seeds yield non-drying oil used for skin infections	Nimbadi Churan Nimba Aristh Nimb Haridra Khand
8.	*Bacopa monnieri* (L.) Wettst	Brahami, Enderya Jal Brahmi	Aerial parts	Potential nervine tonic, improves intellect; used for epilepsy, insanity, and other nervous disorders. Often confused with *Centella asiatica*	–
9.	*Berberis aristata* DC	Daruharidra, Kashmal	Roots, extract (Rasont)	Bitter tonic for intermittent fever, source of berberine hydrochloride used variously. Hepatoprotective, immune-modulatory, and antidepressant	Dravyadi Kwath Dravyadi Leha Dravyadi tail
10.	*Celastrus paniculatus* Willd	Jyotishmati, Malkangni	Seeds. Seed oil	Seed tonic and abortifacient; yield a fatty oil reputed as a nerve stimulant and brain tonic; also used for rheumatic pains	Jyotishmati tail
11.	*Centella asiatica* (L.) Urban	Mandukparni	Leaves	Brain tonic, used in leprosy. Fresh leaves in control dose recommended to kids for sharpening memory	Brahami Panak Brahami tail Sarshvat Aristh Sarshvat Gharit

12.	*Chlorophytum borivilianum* Santapau and R.R. Fern	Safed Musli	Tuberos roots	Potential tonic. Antioxidant. Used for diabetes, arthritis, cancer, for boosting vitality, immunity, and as sexual tonic	Musli Pak Mushaldi Yog
13.	*Cinnamomum tamala* (Buch.-Ham.) Nees and Eberm.	Tejpatar Tamalpatra Gudpatraj	Leaves, bark	Leaves aromatic, carminative used as spice; also used in colic and diarrheas. Bark aroma similar to *Dalchini* often a common adulterant in pure *Dalchini*	–
14.	*Cinnamomum zeylanicum* Breyn.	Dalchini	Stem bark	Bark extensively used as spice and condiment considered astringent, stimulant, and carminative. Cinnamomum oil is popular essential oil used in flavoring chocolates, liqueurs, confectioneries, and other such products	Sitopaladi Churna
15.	*Curcuma longa* Linn. Syn. *C. domestica* Valeton	Haridra, Haldi	Rhizome	Rhizomes are source of turmeric or curcuma used as condiment and coloring agent in foods. Used as stimulant, tonic, stomachic, and depurative. Contains an essential oil and crystalline coloring matter *curcumin*, the percentage of which determines the quality of *Haldi.* Curcuma is also used for sprains, bruises	Haridrakhand
16.	*Dactylorhiza hatagirea* (D. Don) Soo Syn. *Orchis latifolia* auct. non (L.)	Salam panja, Hath punja	Tuberous roots	Potential tonic, antioxidant, galactagogue, and aphrodisiac	
17.	*Emblica officinalis* Gaertn. Syn. *Phyllanthus emblica* L.	Amla	Fruit pericarp (pulp)	Fruits sour, astringent, and cooling; diuretic, laxative, eaten raw or cooked, also pickled. A rich source of vitamin C contain 20 times as much vitamin C as in orange juice. Major constituent of Chyavanprash and *Trifla*, well-known ayurvedic preparations	Chyavanprash Brahma Rasayan Dhatri Loh Dhatri Rasayan Trifla

(continued)

S. No.	Name of the species	Trade name	Part(s) in use	Indications	Vishisht Ayurvedic Yog (Principal ayurvedic drug formulations)
18.	*Ephedra gerardiana* Wall	Somlata	Tender leaves	Source of ephedrine. Liquid extract given in asthma. Juice of berries used in respiratory troubles	Som Kalp
19.	*Glycyrrhiza glabra* Linn.	Yashtimadhu, Muhlathi	Root stock and stem	Rhizome and roots are used as tonic, expectorant, demulcent, and laxative. Used for allaying cough and catarrhal affections and irritable conditions of the membranes of urinary organs	Yashtimadhu Churna Yashtimadhu Kwath Yestimadhuayadi
20.	*Hedychium spicatum* Buch. Ham	Shati	Rhizome	Aromatic rhizomes considered stomachic, carminative, stimulant, and tonic. Used variously as fragrance in colors and in skin applications as conditioner	Shatyadi Churan Shatyadi Kwath
21.	*Hippophae rhamnoides* Linn	Chharma, sea buckthorn	Berries (pulp)	One of the richest sources of vitamin C. Berries made into jelly and syrup used in pulmonary complaints. Decoction in cutaneous troubles	–
22.	*Lilium polyphyllum* D. Don	Kshirkakoli	Rhizome	Nutraceutical	Chavanprash
23.	*Malaxis acuminata* D. Don *Syn. Microstylis wallichi* Lindley	Rishbak	Pseudo-bulbils	Pseudo-bulbils are used as tonic and are antioxidants. One of the members of *Ashtavarga* and ingredient of *Chavanprash*	Chavanprash
24.	*Malaxis muscifera* (Lindley) Kuntze *Syn.* *Microstylis muscifera* (Lindley) Ridley	Jeevak	Pseudo-bulbils	Pseudo-bulbils are used as tonic and are antioxidants. One of the members of *Ashtavarga* and ingredient of *Chavanprash*	Chavanprash

25.	*Moringa oleifera* Lam	Shobhanjan, Shigru, Sehanjan	Root, stem bark, leaves, fruits (pods)	In Ayurveda it is known as Shigru, an anti-inflammatory crude drug. All parts of the tree used in the treatment of ascites, venomous bites, rheumatism and as a cardiac and circulatory stimulant. Roots are rubefacient and vesicant. Leaves are rich in vitamins A and C useful in scurvy and catarrhal affections. Flowers tonic, diuretic, and cholagogue. Seeds antipyretic. Seed oil applied for rheumatism. Tender pods and flowers eaten as vegetable and pickled	Shobhanjanadi Lep Shobhanjanadi Churan Shigru Guggulu
26.	*Ocimum sanctum* Linn.	Tulsii	Leaves, aerial part, seeds and Panchang	Two types of the plants: Green type (shri Tulsi) and purple type (Krishna or Shyama Tulsi). Leaves are aromatic and yield essential oil eugenol which possesses insecticidal and antibacterial properties. Seeds yield fixed oil. Leaves are stimulant, diaphoretic, antiperiodic, and expectorant; used in catarrh and bronchitis, ringworm, and other cutaneous diseases; infusion of leaves as stomachic. Decoction of roots given in malaria fevers. Seeds are mucilaginous and demulcent and used in genitourinary disorders	–
27.	*Picrorhiza kurroa* Royle ex Benth.	Kutki	Roots and rhizome	Bitter tonic, cholagogue and stomachic; laxative in small doses but cathartic in large doses, effective in dropsy	Arogya Vardhni Vati
28.	*Piper longum* Linn.	Pippli, Magh			

(continued)

S. No.	Name of the species	Trade name	Part(s) in use	Indications	Vishisht Ayurvedic Yog (Principal ayurvedic drug formulations)
			Fruit, root (Piplamool)	Root and fruit are used for diseases of respiratory tract; as counter irritant and analgesic for muscular pains and in inflammation; as snuff in coma and drowsiness and internally as a carminative as sedative in insomnia and epilepsy; as cholagogue in obstruction of bile duct and gall bladder and as emmenagogue and abortifacient	Trikatu Churna Talisadi Churna Gudpippali Pippali khand, Pippalyasav
29.	*Piper nigrum* Linn.	Kali Marich	Fruits	Fruits are used as condiment after drying as black pepper or after processing into white pepper; green fruits are pickled. Possess biting pungent taste due to the presence of an oleoresin. Pepper is employed as an aromatic or a stimulant and in weakness following fevers, as a stomachic, and as an antiperiodic in malarial fever, externally valued as a rubefacient and as a local application for relaxed sore throat, piles, and cutaneous troubles	Trikatu Churan Shwas Kuthar Ras Marichadi Gutika Marichadi tail Marichadi Churan Marichadya Ghrit
30.	*Pistacia integerrima* Stewart ex Brandis	Karkatshringi	Leaf galls	Leaf galls are used for dyeing and tanning. Medicinally used in asthma, phthisis, and other diseases of respiratory tract and for dysentery	Shringyadi Churan Karkatadi Churna Balchaturbhadra Churna
31.	*Polygonatum cirrhifolium* Royle	Mahameda	Rhizome		Chavanprash

				Rhizome tonic and antioxidant. A member of *Astavarga* group, constitute as an ingredient of *Chavanprash*	
32.	*Polygonatum verticillatum* all	Meda	Rhizome	Rhizome valued as a salep, a strength giving food, tonic, and antioxidant. A member of *Astavarga* group, constitute as an ingredient of *Chavanprash*	Chavanprash
33.	*Solanum indicum* Linn	Brhati	Whole plant (Panchang)	Roots are carminative and expectorant, useful in cough and catarrhal affections, dysuria, and colic. Fruits are laxative and digestive. Member of *Dashmoola* group, an immunity-enhancing group	Brihatyadi Kwath Brihatyadi ghan Dashmularishta, Dasmool Kwath
34.	*Solanum surattense* Burm. F. Syn. *Solanum xanthocarpum* Schrad. and Wendl.	Kantkari	Whole plant (Panchang)	Roots are expectorant, employed in cough, asthma, and chest pain. Stem flowers and fruits are carminative; used in burning sensation in the feet accompanied by vascular watery eruptions. Juice of berries is used for sore throat. Whole plant is used for dengue fever and bronchitis and in chest affections. Member of *Dashmoola* group, an immunity-enhancing group	Nidigdhadi Kwath Vyaghri Haritaki Kantakari Ghrita Vyaghri tail Dasmool Kwath
35.	*Swertia chirata* Buch.-Ham. ex C. B. Clarke Syn. *S. chirayita* (Roxb. Ex Flem.)	Kiratatikta Chirayata	Whole plant (Panchang)	It is bitter tonic and febrifuge; also used against asthma and liver disorders. If taken with sandalwood paste, it stops internal hemorrhage of the stomach	Sudarshan Churan Kritadi Kwath
36.	*Taxus wallichiana* Zucc. *Syn. Taxus baccata* Linn.	Talish, Rakhal	Leaves (needles) and stem bark	Leaves are antispasmodic and emmenagogue, used for nervousness, hysteria, and epilepsy. A tincture is made from young shoots used for headache, giddiness, feeble and falling pulse, coldness of extremities, diarrhea, and	

(continued)

S. No.	Name of the species	Trade name	Part(s) in use	Indications	Vishisht Ayurvedic Yog (Principal ayurvedic drug formulations)
				severe biliousness. All parts of the tree except fleshy aril are poisons. Bark and leaves yield *baccatin* processed into *taxol,* an anticancer drug	
37.	*Terminalia arjuna* (Roxb.) Wight & Arn	Arjun	Stem bark, fruits, and leaves	The bark is a cardiac tonic. It is styptic, febrifuge, and antidysenteric; pulverized bark gives relief in symptomatic hypertension and acts as a diuretic in cirrhosis of liver. Fruits are tonic and deobstruent. Juice of leaves is used in earache	Kakubhadi Churna Arjun Aristh Arjun Ghrit
38.	*Terminalia chebula* Retz.; C. B. Clarke in part	Haritiki, Harad	Pericarp, bark	Fruits are laxative, stomachic, tonic, and alterative. One of the important constituents of well-known ayurvedic formulation *Trifla.* Coarsely powdered fruit is smoked in asthma. Bark is diuretic and cardio tonic	Abhay Modak Avha Aristh Pathyadi Vati Pathyadi Kwath Vyaghri Haritaki Chitrak Haritaki Agastyaharitiki Danti Haritaki Kansa Haritaki Haritaki Khand

					Pathyadi Churna Trifla
39.	*Tinospora cordifolia* (Willd.) Miers. ex Hook. F. and Thoms.	Garuchi, Giloe	Stem	Stem is a constituent of several ayurvedic preparations used in general debility, dyspepsia, fevers, and urinary diseases. Bitter principles present in the drug show antispasmodic, antipyretic, and anti-inflammatory properties. A kind of starch *Garuchi Ghansatava* prepared from aqueous extract of dry stems used as tonic and in gout	Guduchyadi Churna Guduchyadi Kwath Guduchi Loh Amrita Aristh Guduchi Taila Guduchi Ghan Vati Amrita Guggulu
40.	(a) *Viola canescens* Wall (b) *Viola odorata* Linn (c) *Viola pilosa* Blume Syn. *V. serpens* Wall. ex Ging., non-Ridley.	Banafshah	Herb, flowers leaves, root, seeds	Expectorant, diaphoretic, and diuretic and used as laxative in bilious affections. Flowers are emollient and demulcent. Leaves are said to relieve pain due to cancerous growths particularly in the mouth and throat. Roots are emetic, used a substitute of ipecac. Seeds are purgative and diuretic	Banafshadi Kwath
41.	*Withania somnifera* Dunal	Ashwagandha	Roots	Roots have long been in use for hiccup, cough, dropsy, rheumatism, and female disorders and as a sedative in cases of	Ashwagandha Churna Ashwagandha Rasyan

(continued)

S. No.	Name of the species	Trade name	Part(s) in use	Indications	Vishisht Ayurvedic Yog (Principal ayurvedic drug formulations)
				senile debility. Antioxidant and immunity enhancer	Ashwagandha Ghrita Ashwagandha Arisht
42.	*Zingiber officinale* Rosc.	Adrak (fresh), Sunthi (dried)	Rhizome	Rhizome is highly esteemed as a spice for its characteristic odor and warm pungent taste. Dried ginger (*Sunthi*) is widely used for flavoring foods, for extraction of oleoresins, and preparation of extracts and distillation of an essential oil called oil of ginger. It is antioxidant and used as a constituent of various ayurvedic preparations	Ardraka Khand Panchsam Churna Samsharkra Churan Rasnadi Kwath Soubhagya Shunthi Shunthi sura Shunthi Pachak

28.7 Conclusion

To live a long healthy life, it is necessary that we do not fall ill and not get infected by various diseases. Strong immune system keeps away such infections and helps to fight against such disorders. In the present times, the whole of the world is under the grip of COVID-19 pandemic spreading at a fast rate. It seems that this situation is not going to be diluted in near future and will prevail for a longer time till the invention of an effective vaccine or drug. We have to learn how to live with the virus by adopting preventive measures. The right way of lifestyle, adoption of idle food habits as per *Ritucharya*, practicing Yoga, and use of selected herbal preparations as described in Ayurveda may help to build a strong immunity system to fight against the disease.

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The Pathophysiology of Liver Disorders and Pharmacotherapy Options with Special Reference to Traditional Herbal Medicines: A Comprehensive Review

29

Hasandeep Singh, Tanveer Singh, Harpal Singh Buttar, Sarabjit Kaur, Saroj Arora, Istvan G. Télessy, and Balbir Singh

Abstract

Besides synthetic drugs, a wide variety of medicinal plants have been used for the prevention and management of various liver disorders. Generally, plant therapies are well tolerated due to their lesser side effects. The aims and objectives of this review are to describe the drug therapies used for treating liver disorders as well as the most commonly used hepatoprotective plant-derived bioactive ingredients and their formulations employed for treating various liver pathologies. The extensive literature review was conducted using different databases such as ScienceDirect, SciFinder Scholar, Wiley Online Library, PubMed, ResearchGate, Google Scholar and Chemical Abstracts (until March 2021). Our literature searches showed that a wide array of plant products or plant extracts have been used in the folklore and traditional remedies for the prevention and management of liver disorders. The complex chemical structures of many isolated plant

H. Singh · S. Kaur · B. Singh (✉)
Department of Pharmaceutical Sciences, Guru Nanak Dev University, Amritsar, Punjab, India
e-mail: hasanpharma.rsh@gndu.ac.in; sarabjit.pharma@gndu.ac.in; balbir.pharma@gndu.ac.in

T. Singh
Department of Neuroscience and Experimental Therapeutics, Texas A&M University College of Medicine, Bryan, TX, USA
e-mail: tanveersingh@tamu.edu

H. S. Buttar
Department of Botanical and Environmental Sciences, Guru Nanak Dev University, Amritsar, India

S. Arora
Department of Pathology and Laboratory Medicine, Faculty of Medicine, University of Ottawa, Ottawa, ON, Canada

I. G. Télessy
Department of Pharmaceutics, Faculty of Pharmacy, University of Pécs, Pécs, Hungary
e-mail: telessyist@vnet.hu

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ingredients such as flavonoids, polyphenols, and steroid-type compounds have been determined using sophisticated analytical techniques. While the pharmacological and toxicological activities of many plant products have been tested in animal models, their underlying mechanism of action remains unknown. In this review, we will describe the hepatoprotective actions of the following plants and their bioactive components: *Allium sativum*, *Allium hirtifolium*, *Andrographis paniculata*, *Apium graveolens*, *Asparagus racemosus*, *Berberis vulgaris*, *Curcuma longa*, *Emblica officinalis*, *Glycyrrhiza glabra*, *Marrubium vulgare*, *Nigella sativa*, *Phyllanthus niruri*, *Picrorhiza kurroa*, *Solanum nigrum*, *Swertia chirayita*, *Taraxacum officinale*, oleanolic acid, Cliv-92, ursolic acid, berberine, proanthocyanidins, naringenin, silymarin, andrographolide, glycyrrhizin, curcumin, rhein, geniposide and resveratrol. There are challenges and opportunities for understanding the mechanism of action of the phytotherapies used for curing liver diseases as well as discovering new drug molecules useful for targeting different liver ailments. Combinations of traditional herbal remedies found to be safe and effective are also suggested as a possible cost-effective therapeutic tool to be tested in future researches aiming to unveil novel options to treat liver disorders in humans. However, well-designed, randomized, placebo-controlled, multicentre trials are needed to establish the long-term safety and efficacy as well the optimal dose schedules required for treating different liver disorders in humans.

Keywords

Liver disorders · Pathophysiology of liver diseases · Hepatotoxicity · Hepatoprotective medicinal plants · Drug therapy of liver diseases

Abbreviations

ALF	Acute liver failure
CLD	Chronic liver disorder
DILI	Drug-induced liver injury
e-NOS	Endothelial nitric oxide synthase
HDL	High-density lipoprotein
HMGB1	High mobility group box 1
HPS	Hepatopulmonary syndrome
HRS	Hepatorenal syndrome
ICAM-1	Intercellular adhesion molecule-1
MAT	Methionine adenosyltransferase
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NO	Nitric oxide
PPHTN	Portopulmonary hypertension
SAM	Sulfoadenosylmethionine

TAA Thioacetamide
TNF-α Tumour necrosis factor alfa

29.1 Introduction

Liver is the largest vital organ of the body associated with the detoxification of various drugs and xenobiotics and also maintains the metabolic homeostasis of the body. It also helps in regulating the physiological processes which are involved in various functions such as metabolism, storage and excretion (Pal and Manoj 2011; Samuel et al. 2012). The primary function of the liver is to control the safety of the substances that are absorbed from the digestive system before their distribution into systemic circulation. The liver has a great physiological importance in the body, and malfunctions or complete loss of the liver functions leads to morbidity and mortality (Ozougwu 2014).

29.1.1 Functions of the Liver and Types of Liver Diseases

1. The main functions of liver are discussed hereunder:
   (a) Bile secretion.
   (b) Metabolism of bilirubin.
   (c) Metabolism of nutrients such as fats proteins and carbohydrates.
   (d) Detoxification of drugs, toxins and hormones.
   (e) Storage of vitamins and minerals such as iron, copper and glycogen.
   (f) The liver is also involved in endocrine functions such as activation of vitamin D, secretion of angiotensinogen, hormone metabolism and conversion of thyroxine (T_4) to triiodothyronine.
   (g) The liver is also involved in the protection functions such as phagocytosis by kupffer cells, removes haemolysis products and filters the portal blood from bacteria.
   (h) Inactivation of drugs and toxins by oxidation, by reduction, by conjugation and by cytochrome P450 system (Moore and Dalley 2006; Guyton and Hall 2006).
2. According to Porth (2011), the main types of liver diseases are:
   (a) Hepatitis
      - Viral hepatitis
      - Autoimmune hepatitis
      - Acute fulminant hepatitis
   (b) Intrahepatic biliary disorders
      - Primary biliary cirrhosis
   (c) Drug- and alcohol-induced liver disease

- Drug-induced liver disease
- Alcohol-induced liver disease

(d) Non-alcoholic fatty liver disease
(e) Hepatic syndrome
- Cirrhosis of liver
- Portal hypertension
- Liver failure

(f) Liver cancer

Approximately 1.7% of the US population is affected by liver diseases (US Department of Health Centers for Diseases Control and Prevention Summary Health Statistics 2018). To date, prevention is the first suggestion for the general population because there is no effective and universal therapy for majority of liver diseases.

As the list of liver diseases shows, there are many different causes of liver disorders, and therefore their therapeutic interventions are also multiple. There is no "general hepatic therapy", but we have specific treatment options for viral hepatitis, for medication-induced hepatitis and for immunogenic hepatitis. We can take general hepatoprotection measures which protect the liver from toxic injury, and we have agents supporting hepatic regeneration after hepatic pathology. We also have antiviral agents against certain types of viral hepatitis and anticancer drugs slowing down the proliferation of hepatic tumours. In addition, there are special interventions to prevent or reverse drug- and/or alcohol-induced hepatic injury.

In this book chapter, we will describe hepatoprotective pharmaceuticals and bioactive phytochemicals which have been used for treating or prevention of certain liver diseases as well as for the restoration of liver tissue integrity and function for the management of liver disorders in humans. In support of our arguments, we will report the evidence obtained from studies done in animal models to investigate the hepatoprotective actions of bioactive compounds isolated from diverse plants (Table 29.1).

29.1.2 Hepatotoxicity

Hepatotoxicity is the common cause of liver failure and accounts for 10% of acute liver failure all over the world. An estimated 1000 drugs in the market have been suspected to cause hepatotoxicity more than once and are the most expected adverse drug event which leads to the discontinuation of new drugs during preclinical or clinical stages (Zimmerman 1999; Fisher et al. 2015). It is one of the most challenging disorders due to difficulties in diagnosis and management. The significant data regarding the risk and occurrence of drug-induced hepatotoxicity is rare (De Abajo et al. 2004; Ponte et al. 2017).

There are several grouping of liver disorders, including toxic hepatic injuries. Hyman Zimmerman, a hepatologist, categorized the drug-induced liver injury (DILI)

Table 29.1 Studies done in animal models to investigate the hepatoprotective actions of bioactive compounds isolated from different plant species

Plant spp. used	Dose (mg/kg), admin. route	Animal models	Possible mechanism of action	Reference
Allium sativum	300 mg/kg for 14 days, *i.p.*	Male Wistar rats, D-galactosamine and lipopolysaccharide (DGaIN/LPS)-induced hepatotoxicity	Antioxidant defence system	El-Beshbishy (2008)
Allium hirtifolium	0.1 and 1 g/kg, *i.p.*	Wistar rats, alloxan-induced diabetes and liver damage	By the reduction of liver cell damage and also through hypoglycaemic and hypolipidaemic activities and thereby preventing fatty liver formation	Kazemi et al. (2010)
Andrographis paniculata	100 mg/kg, *i.p.*	Wistar rats, CCl_4-induced hepatotoxicity	By decreasing the oxidative stress	Handa and Sharma (1990)
Apium graveolens	200 mg/kg *p.o.*	Porton albino rats, paracetamol and thioacetamide-induced hepatotoxicity	Might be due to activation of reticuloendothelial system functions or inhibition of protein biosynthesis	Singh and Handa (1995)
Hygrophila auriculata	200 mg/kg *p.o.*	Porton albino rats, paracetamol and thioacetamide-induced hepatotoxicity	Might be due to activation of reticuloendothelial system functions or inhibition of protein biosynthesis	Singh and Handa (1995)
Asparagus racemosus	150 and 300 mg/kg, *s.c.*	Wistar rats, CCl_4-induced hepatotoxicity	Due to antioxidant activity of flavonoids	Acharya et al. (2012)
Berberis vulgaris (berberine)	80, 120 and 160 mg/kg, orally	Male Sprague-Dawley rats, CCl_4-induced acute liver damage	Not known	Feng et al. (2010)
Boerhaavia diffusa	2 mLkg, orally	Male albino rats, thioacetamide-induced hepatotoxicity	Membrane stabilising effects	Rawat et al. (1997)
Crocus sativus	20 mg/kg, orally	Male Wistar rats, acetyl-para-aminophenol (APAP)-induced hepatotoxicity	Might be due to the presence of flavonoids and their antioxidant properties	Omidi et al. (2014)
Curcuma longa	250 and 500 mg/kg, orally	Sprague Dawley rats, thioacetamide-induced hepatotoxicity	Might be due to direct antioxidant and free radical scavenging mechanisms	Salama et al. (2013)

(continued)

Table 29.1 (continued)

Plant spp. used	Dose (mg/kg), admin. route	Animal models	Possible mechanism of action	Reference
Cynara scolymus	Various in vitro test concentrations	Male Sprague-Dawley rats, hydroperoxide-induced oxidative stress in cultured rat hepatocytes	By preventing oxidative modification of blood lipoproteins and cholesterol through choleretically induced elimination and inhibition of hepatic cholesterol biosynthesis	Gebhardt (1997)
Emblica officinalis	500 mg/kg, *i.p.*	Male Wistar rats, CCl_4-induced hepatotoxicity	Due to antioxidant properties	Jose and Kuttan (2000)
Flacourtia indica	1.5 g/kg, orally	Long Evans rats, paracetamol-induced hepatotoxicity	By inhibiting enzymatic oxidation	Nazneen et al. (2009)
Glycyrrhiza glabra	100, 150 or 300 mg/kg, orally	Male Wistar rats, CCl_4-induced hepatotoxicity	By scavenging free radicals, stimulating antioxidant enzyme activities and arresting the production of inflammatory cytokines and protecting the liver against CCl_4-induced damage	Huo et al. (2011)
Marrubium vulgare	500 mg of dry leaves powder/kg orally	Wistar rats, cyclophosphamide-induced liver toxicity	Antioxidant properties and the existence of phenolic acids and flavonoids	Ettaya et al. (2015)
Nigella sativa	500 mg/kg, orally; 1 mL/kg of *Nigella sativa* oil	Wistar albino rats, CCl_4-induced hepatotoxicity	Due to the antioxidant properties of flavonoids and phenolic compounds	Mohideen et al. (2003), Al-Seeni et al. (2016)
Phyllanthus niruri	50 and 100 mg/kg, orally	Swiss male albino mice, nimesulide induced hepatic damage	Due to antioxidant property	Chatterjee and Sil (2006)
Picrorhiza kurroa	0.5, 10 and 20 mg/kg	Adult swiss albino mice, alcohol-induced liver damage	Antioxidant effect	Navya et al. (2018)
Silybum marianum	Silymarin (100 mg/kg),orally	Wistar male rats, paracetamol-induced hepatotoxicity	Antioxidant properties	Ahmed et al. (2020)
Solanum nigrum	300 mg/kg, orally	Sprague-Dawley rats, cadmium chloride-induced hepatotoxicity	Due to natural compounds present in *Solanum nigrum* extract which chelate the toxic heavy metals and prevent the lethal accumulation in liver or kidney tissues	Abdel-Rahim et al. (2014)

Swertia chirayita	100 and 200 mg/kg, orally	Female swiss albino mice, paracetamol-induced hepatotoxicity	Due to free radical scavenging activity	Nagalekshmi et al. (2011)
Taraxacum officinale	500 mg/kg, *i.p.*	Male Wistar rats, sodium dichromate-induced liver injury	Due to the antioxidant properties of the phytochemical constituents present in the extract	Hfaiedh et al. (2016)
Terminalia arjuna	1, 5, 10, 25, 50 mg and 100 mg/kg, orally	Swiss albino mice, CCl_4-induced hepatotoxicity	The extract exhibits potent antioxidant activity, thereby protects the liver and kidney via alteration of cytochrome P450	Manna et al. (2006)
Tragopogon porrifolius	50 and 250 mg/kg, *i.p.*	Male Wistar rats, CCl_4-induced hepatotoxicity	Due to the antioxidant activity of high contents of phenols and flavonoids present in the extract	Tenkerian et al. (2015)
Cleome viscosa (Cliv-92)	100 and 200 mg/kg, orally	Albino rats (Druckrey strain), CCl_4-induced hepatotoxicity	Might be due to the interference of compounds with cytochrome P_{450}, which results in the interference in the formation of free radicals and thereby protecting the integrity of the membranes	Gupta and Dixit (2009)
Oleanolic acid	20, 40, 80 mg/kg, *s.c.*	Male Balb/c mice, concanavalin A-induced liver damage	Through the activation of PPARα, inhibiting the apoptosis and autophagy through inhibition of JNK signalling	Wang et al. (2018)
Ursolic acid	5, 10, 20 mg/kg, orally	Druckrey rats, paracetamol-induced hepatotoxicity	May be due to its stabilizing effect on plasma membrane	Shukla et al. (1992)
Berberine	80, 120 and 160 mg/kg, orally	Male Sprague-Dawley rats, CCl_4-induced hepatotoxicity	Preventive and curative effects of berberine	Feng et al. (2010)
Naringenin	50 mg/kg, orally	Swiss male mice, CCl_4-induced hepatotoxicity	Due to its ability to scavenge free radicals	Hermenean et al. (2014)
Proanthocyanidins	10 and 50 mg/kg, orally; 100 mg/kg, orally	Male Sprague-Dawley rats, ethanol-induced hepatotoxicity Male Wistar rats, cadmium-induced hepatotoxicity	By directly scavenging ROS, regulating ADH and ALDH enzymes and inhibiting CYP2E1 expression; Increase the aerobic capacity of muscle tissue and improve the mitochondrial function	Miltonprabu and Manoharan (2016), Bak et al. (2016)

(continued)

Table 29.1 (continued)

Plant spp. used	Dose (mg/kg), admin. route	Animal models	Possible mechanism of action	Reference
Silymarin	200 mg/kg, orally	Normotensive male Wistar rats, APAP-induced hepatotoxicity	Due to anti-inflammatory and antioxidant properties	Freitag et al. 2015, Vargas-Mendoza et al. 2014
Glycyrrhizin	25 and 50 mg/kg, *i.p.*	Adult male Wistar rats, CCl_4-induced hepatotoxicity	Glycyrrhizin in combination with silymarin helps in the healing of necro-inflammatory lesions induced by CCl_4	Rasool et al. (2014)
Curcumin	100 mg/kg, orally	Sprague-Dawley rats, dimethylnitrosamine-induced liver cirrhosis	Due to anti-inflammatory effect and suppression of HSC activity which thereby attenuate fibrosis	Kyung et al. (2018)
Rhein	20, 50 and 100 mg/kg, orally	Male Wistar rats, methotrexate-induced hepatotoxicity	Through Nrf2-HO-1 pathway activation to enhance the liver antioxidant status	Bu et al. (2018)
Geniposide	25, 50 and 100 mg/kg, orally 400 mg/kg, orally	Male Sprague-Dawley rats, high fat emulsion-induced non-alcoholic steatohepatitis Male Kunming mice, CCl_4-induced hepatotoxicity	Through free radical scavenging activity Via induction of antioxidant defence	Ma et al. (2011), Chen et al. (2016)
Resveratrol	100 and 200 mg/kg, orally	Male C57BL/6J mice, ethanol-induced liver damage	By suppression of lipid peroxidation and activation of SOD gene expression	Chen et al. (2016)

in two types such as intrinsic drug-induced liver injury (IN-DILI) and idiosyncratic drug-induced liver injury (IS-DILI) (Senior 2008), whereas the third category of liver injury consists of alcohol-induced liver cirrhosis. All these three pathologies are briefly discussed below:

1. **Intrinsic Drug-Induced liver injury (IN-DILI)**
   Hepatotoxicity by acetyl-*para*-aminophenol (APAP) is a typical example of drug-induced liver injury. It shows a dose-dependent response in rodents and human hepatocytes and is also reproducible (McGill et al. 2011, 2013; Xie et al. 2014). It is not feasible to conduct dose-response studies for APAP hepatotoxicity in humans due to ethical reasons, but the available data points towards a threshold of toxicity usually 6–10 g/day in adults (Dart et al. 2006). However, few reports demonstrated that only a few patients taking therapeutic doses of APAP develop temporary elevation in hepatic biomarkers, and they do not experience a significant liver injury and never have liver failure (Watkins et al. 2006; Kuffner et al. 2006; Dart and Bailey 2007; Heard et al. 2014). In addition, rare cases of hepatotoxicity have been reported for other drugs such as cocaine (Vitcheva 2012), amiodarone (Grecian and Ainslie 2012; Chen and Wu 2016) and methotrexate (Banerjee et al. 1988).
2. **Idiosyncratic Drug-Induced Liver Injury (IS-DILI)**
   Idiosyncratic drug-induced liver injury is a severe type of liver injury which occurs in patients who are using particular drugs at recommended doses such as antibiotics (amoxicillin-clavulanate), cardiovascular drugs (amiodarone), CNS drugs (phenytoin), NSAIDs (diclofenac), etc. Various dietary supplements and herbal products may also cause idiosyncratic DILI (Björnsson et al. 2013; Chalasani et al. 2015; Vega et al. 2017). In most of the cases, after months of asymptomatic exposure, the injury develops suddenly which resolves quickly upon discontinuation. However, there are some rare cases of very rapid and extremely latent response, and this rarity and variety of causative agents make it difficult to study idiosyncratic DILI (Uetrecht 2009).
3. **Alcohol-Induced Liver Cirrhosis**
   Alcohol drinking resulted in about 3.8% of deaths globally and global disability of 4.6% (Rehm et al. 2009). Alcohol use disorders (AUC) are most common cause of liver cirrhosis, and alcoholic liver disease (ALD) is the critical cause of death in adults due to alcohol (Rehm et al. 2013). The consumption of alcohol when exceeds a certain amount can cause a variety of liver lesions among which steatosis is present in almost all drinkers who consume in excess of 40 g/day regularly. The underlying mechanisms of ALD pathogenesis are still incompletely understood but seem to be related with complex interactions between behavioural, environmental and genetic factors (Konishi and Ishii 2007). Most recent stage of knowledge is represented by Indian authors (Namachivayam and Gopalakrishnan 2021).

29.1.2.1 Clinical Biomarkers of Hepatotoxicity

The clinical biomarkers of liver damage are represented by the increased plasma or serum levels of alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphate (ALP), bilirubin, albumin, triglycerides and total cholesterol pertinent to liver damage as they could flow into the blood due to hepatotoxicity (Gutierrezl and Solis 2009). The magnitude of the elevation and the enzymes involved depend on the quality and quantity of the impairing agent, the severity and the stage of hepatic injury. The serum levels of enzymes result from the damaged hepatocytes, as these enzymes are located in the cell cytoplasm and are released into the bloodstream from the damaged liver cells (Yousef et al. 2010).

29.1.2.2 The Pharmacometabolomic Impact of Hepatotoxicity

One of the major consequences of ALD is the progressive deterioration of hepatic structure: first the steatosis, afterwards the hepatitis and finally the cirrhosis. This cascade results in reduction in hepatic cell mass and consequent decrease in the functional capacity of the liver. This will influence pharmacokinetic parameters: reduced metabolic function cause increase in bioavailability of most drugs therefore, and, in general, decrease in dose and increase in dosage intervals are necessary. Pharmacodynamics is also affected: the central nervous system of patients with ALD is more sensitive to sedative and hypnotic effect of drugs; therefore adjustment of dose and the dosing interval are desired.

29.1.3 Xenobiotic Metabolism by the Liver

A wide range of drugs are metabolized by the liver to water-soluble or less lipid-soluble products which are further excreted in bile. Biotransformation of xenobiotics usually occurs in two steps: Phase I (oxidative, reductive or hydrolytic process) and Phase II (conjugation). Phase I metabolic reactions are mediated by various cytochrome P450 (CYP) enzymes including oxidation, reduction and hydrolysis reactions.

29.1.3.1 Hepatic Cytochrome P450 Coenzymes

Cytochrome P450 (CYP) coenzymes are a superfamily of monooxygenases that are found in all zoological kingdoms of life and which show extraordinary diversity in their reaction chemistry. In mammals, these enzymes are found primarily in the membranes of the endoplasmic reticulum (microsomes) within liver cells (hepatocytes), as well as many other cell types. They perform oxidation and reduction reactions using iron to increase the water solubility of drugs for excretion. They can also clear the body of metabolic products such as bilirubin, which arises from the breakdown of haemoglobin. There is a high concentration of CYP proteins in the liver, but these enzymes are also found throughout the body, where they often have specialized roles. These enzymes are so named because they are bound to membranes inside the cell and contain heme pigment which absorbs light at 450 nm on exposure to carbon monoxide. The various forms of CYT P450 are

classified according to their amino acid sequence. The importance of these enzymes can be seen in subgroups that lack particular genes. For example, in the case of anaesthesia, codeine is metabolized to morphine by CYP2D6; therefore, the patients having its deficiency find codeine ineffective (Vaja and Ghuman 2017). Cytochrome P450s are also found in higher plants. The evolution of P450 family of isozymes in plants is thought to occur through gene duplication and conversion due to the need of plants to acclimatize in a harsh environment and also to protect themselves from pathogens and predators (Werck-Reichhart and Feyereisen 2000).

29.2 Pharmacotherapies of Liver Disorders

The diseases associated with the liver are of major medical concern all over the world. The main causes of liver diseases in Africa and Asia are parasitic and viral infections, while alcohol abuse is a major cause in America and Europe. In addition, viral hepatitis has also increased recently. So far, there is no established drug available that could cure alcohol-associated liver disease (Subramaniyan et al. 2021). However, the currently used medications in the management of liver diseases are discussed herein:

29.2.1 Corticosteroids

Corticosteroids allegedly are helpful in case of alcoholic hepatitis as they attenuated the typical inflammatory response, decrease the production of cytokines, suppress the acetaldehyde adduct formation and also inhibit the collagen production (Zhang et al. 2005). Corticosteroids are also used for the short-term survival of patients with severe alcoholic hepatitis (Mathurin et al. 2002). Steroidal treatment caused improvement in some patients having severe alcoholic disease, and this short-term histological improvement by steroids is due to decrease in the tumour necrosis factor alfa (TNF-α), intercellular adhesion molecule-1 (ICAM-1) expression as well as changes in the soluble intercellular adhesion molecule-1 (sICAM-1) in the hepatic vein (Spahr et al. 2001). Notably, in a systematic review of Rambaldi et al. (2002), neither beneficial effects nor any clinical outcomes such as liver histology, complications or mortality of anabolic-androgenic steroids are demonstrated in patients having alcoholic liver disease. In recent scenario, corticosteroids are considered to have poor but sure future in the management of liver disorders. Similar conclusion is supported by a recent summary evaluating RCTs in this treatment modality (Crabb et al. 2020).

29.2.2 Interferons

Interferons (IFNs) belong to cytokine family having pleiotropic effects on the target cells including the antiviral state inhibition of cell growth and immune response

modulation (Muriel 2007). IFN-α is considered as a hallmark in treating viral hepatitis, the major cause of liver cirrhosis and hepatocellular cancer all over the world. IFNs are also known to cause the production of matrix proteins such as fibronectin and collagen. Muriel et al., demonstrated the effect of IFN-α_{2b} on fibrosis and cirrhosis induced by CCl_4 in rats. Furthermore, there is a recovery of hepatocyte and erythrocyte membranes to its normal composition as a result of antifibrotic effects on IFNs (Muriel et al. 1994a; Muriel 1995). A study also demonstrated that there is downregulation of collagen gene transcription and suppression of CCl_4 hepatic fibrosis in mice by IFN-α. Mukai et al., in 2006 showed that IFN-γ prevents liver fibrosis in nonobese diabetic mice. IFN-α_{2a} causes the reversal of hepatic cirrhosis induced by thioacetamide in rats (Mathew et al. 2007).

29.2.3 Nitric Oxide

Nitric oxide (NO) gas was investigated as an endogenous vasodilator (Palmer et al. 1987). It is produced from L-arginine by NO synthases, and its synthesis is regulated by the liver (Muriel 2000). NO helps in regulating the proper functioning of every organ in the body, and its alteration is involved in various hepatic disorders. NO is beneficial to maintain the integrity of microvascular function and inhibits the platelet aggregation and neutrophil infiltration to prevent apoptosis. It also acts as second messenger in harmful and helpful cytokine signalling. Hence, the modulation of NO synthesis and NO donors, especially which are liver-specific, can be a powerful tool in the treatment of liver disorders in future (Muriel 2006).

29.2.4 Sulfoadenosylmethionine (SAM)

SAM is an endogenous molecule which participates in liver health and diseases (Mato and Lu 2007). The three distinct enzymes that synthesize SAM in mammals are methionine adenosyltransferase (MAT) I, II and III. The synthesis and utilization of SAM occur mainly in the liver via MATI, MATIII and glycine *N*-methyltransferase, and these three enzymes are responsible for the synthesis and removal of hepatic SAM (Mato et al. 2002). It has been previously demonstrated that there is an alteration of methionine metabolism in patients with liver disease. A study conducted by Kinsell et al. (1948) showed that there is a significant impairment in the methionine clearance after methionine load in patients having liver disease, and this suggests the important role of liver in the metabolism of methionine. In most of transmethylation reactions, SAM is a methyl donor and is utilized by methyltransferases in synthesizing various biological compounds including plasma membrane lipids (Muriel and Mourelle 1992; Muriel 1993). MAT and SAM are significantly reduced in case of experimental and human hepatic damage (Mato and Lu 2007). Treatment of animals with SAM showed prevention in the prolonged biliary obstruction-induced cirrhosis (Muriel et al. 1994b). In another study, the administration of SAM to alcohol-fed mice, rats and baboons attenuated the

depletion of GSH and hepatic injury (Tsukamoto and Lu 2001). It also helps in the improvement of survival of animals in galactosamine-, acetaminophen- and thioacetamide-induced liver damage and also in ischemia reperfusion-induced hepatotoxicity (Mato et al. 1997). Exogenous supply of SAM also decreased the liver fibrosis in rats treated with CCl_4 and also reduced the neoplastic hepatic nodules in animal models of liver cancer (Mato et al. 1997; Pascale et al. 2002).

29.2.5 Thalidomide and Analogues

Thalidomide is derived synthetically from glutamic acid. It is also known as α-*N*-phthalimidoglutarimide (Marriott et al. 1999). It was first synthesized in West Germany by Chemie Grunenthal and sold under the name Contergan™ which was then licensed in 46 countries all over the world (Miller and Strömland 1999). It was originally used as an antiemetic and sedative but was later associated to have birth defects in the 1950s due to its teratogenic nature (Marriott et al. 1999). It has potential therapeutic applications and has been widely used in various diseases such as rheumatoid arthritis, angiogenesis, prurigo nodularis, Behcet's disease and discoid lupus (Marriott et al. 1999; Raje and Anderson 1999). It has been reported that thalidomide is reported to possess anti-TNF-α and anti-inflammatory and antifibrotic activities in the lungs and liver (Moreira et al. 1993; Raje and Anderson 1999; Fernández-Martínez et al. 2001, 2004; Muriel et al. 2003; Tabata et al. 2007; Chong et al. 2006). CCl_4-induced toxicity caused 33.3% mortality which was reduced to 13.3% by thalidomide treatment. The serum biomarkers such as ALT, γ-GTP and ALP were increased with CCl_4 which were attenuated with thalidomide treatment. Also, there was marked increased in lipid peroxides and abolished glycogen in the liver by CCl_4 which was prevented by thalidomide treatment. In addition, significant improvement in liver collagen was also observed by thalidomide treatment (Muriel et al. 2003). However, thalidomide can be a good approach for cirrhosis, but clinical studies are still lacking and require further studies.

29.2.6 Plant-Derived Hepatoprotective Agents

A wide variety of herbal remedies have been used for the treatment and management of liver disorders or diseases. Most herbal therapies were discovered through the folklore knowledge of tribal people, and some of these have laid the foundation of basic research (Son et al. 2014; Sagar et al. 2014; Ali et al. 2018). Plant-derived remedies are reported as the major source of hepatoprotection, and their isolated bioactive compounds have been tested in many rodent-type animal models. Here, we will discuss some selected medicinal plants and their isolated constituents evaluated scientifically against liver disorders along with their mechanistic studies summarized in Table 29.1.

29.2.6.1 *Allium sativum*

A. sativum (family: Alliaceae), commonly known as garlic, is one of the most known medicinal herb and spice. It is well-known for its nutritional properties due to its bioactive constituents. For healing of lifestyle-associated disorders, this plant is used as antidiabetic, anti-inflammatory, antihypertensive, antimicrobial, anti-atherosclerotic and hepatoprotective (Amagase et al. 2001). Different species of *Allium* genus have shown the presence of various chemical constituents such as sapogenins, saponins, sulphuric compounds and flavonoids (Kazemi et al. 2010). Ethanolic extract of *A. sativum* (EEAS) was administered for 14 days intraperitoneally and was investigated against DGalN/LPS-induced hepatitis in rats (El-Beshbishy 2008). It was demonstrated to possess significant hepatoprotective activity in thioacetamide (TAA)-induced hepatotoxicity in rats at the doses of 200 and 400 mg/kg orally for 21 days. EEAS treatment resulted in the significant reduction of serum liver markers and also reverts the histopathological changes due its free radical scavenging ability (Chinnala et al. 2018).

29.2.6.2 *Allium hirtifolium*

A. hirtifolium is commonly known as Persian shallot (Moosir in Persian language), endemic to Iran, and belongs to family Alliaceae (Rechinger 1984). The compounds identified in *A. hirtifolium* are sulphur-containing compounds, saponins, sapogenins and flavonoids including quercetin and kaempferol (Kazemi et al. 2010). The most common compounds present in this plant are disulphide and trisulfide compounds, and the most important biological secondary metabolites of this plant are allicin, s-allylcysteine, diallyl disulphide, diallyl trisulphide and methyl allyl trisulphide (Rose et al. 2005; Azadi et al. 2009). Alloxan monohydrate at a dose of 120 mg/kg body weight was used to study the diabetes-induced liver damage. Rats treated with hydroalcoholic extract of *A. hirtifolium* have shown to protect liver cells against oxidant effects of alloxan and consequently caused a marked reduction in serum concentration of alkaline phosphatase (ALP), alanine transaminase (ALT) and aspartate transaminase (AST). It has been found on the basis of available pharmaceutical investigations that plants possess antioxidant and hepatoprotective effects (Kazemi et al. 2010).

29.2.6.3 *Allium ursinum* L.

This medicinal plant and herb (*Allium ursinum* L.) also known as Ramson or bear's garlic or wild garlic, spreads in whole Europe with a long tradition of medicinal use. Its bioactive compounds come near to *Allium sativum* (rich composition in sulphur-containing compounds, polyphenols and steroidal glycosides), but proportions are different and contain much higher amount of prostaglandin A, B and F. From medical point of view antioxidant, antiproliferative activity is strong enough to be a potential remedy in hepatic injuries besides the well-known effect in cardiovascular diseases (Stanisavljevic et al. 2020; Pop et al. 2020).

29.2.6.4 *Andrographis paniculata*

Andrographis paniculata is a well-known plant for treating liver disorders. It belongs to family Acanthaceae and is commonly known as kalmegh. The plant is very bitter, and therefore it is known as "king of bitters". The main active constituent present in the plant is andrographolide which is responsible for the hepatoprotective activity. It is reported that the methanol extract of *A. paniculata* showed 32% recovery in CCl_4 toxicity in rats. The protective effects of andrographolide are comparable to silymarin against liver damage by various drugs by reducing the oxidative stress (Handa and Sharma 1990).

29.2.6.5 *Apium graveolens*

A. graveolens is a medicinal plant that belongs to family Umbelliferae and is commonly known as celery. It is an edible plant that grows mostly in Mediterranean areas (Rechinger et al. 1994; Kooti et al. 2014). *A. graveolens* is rich in beta carotene, folic acid, vitamin C, chlorophyll, fibre, magnesium, silica, potassium and sodium. The essential oil mainly contains δ-limonene and various sesquiterpenes: isoimperatorin, isoquercitrin, linoleic acid, coumarins, furanocoumarins (including bergapten), flavonoids (apigenin, apiin), phenolic compounds, choline and some unidentified alkaloids (Asif et al. 2011; Nagella et al. 2012). In Iranian medicine, seeds of this plant are used in the treatment of liver ailments and other disorders that affect the liver and showed hepatoprotective activity. On examining the antihepatotoxic effect of *A. graveolens* seeds, the most significant effect was shown by methanolic extract on rat liver (Asif et al. 2011).

29.2.6.6 *Asparagus racemosus*

Asparagus racemosus dried roots and leaves contain hepatoprotective ingredients. It belongs to family Liliaceae and is commonly known as shatavari. It is found throughout Australia, India, Sri Lanka, South China and tropical Africa. In India, it is found in tropical, subtropical, dry and deciduous forests and also in the Himalayas at 4000 ft height and also in plains. The main constituents present in the plant are alkaloids, tannins, saponins, diosgenin and proteins. It also contains phytoestrogen triterpene saponins known as shatavarin I to IV. The roots of the plant are used as an anti-inflammatory, anti-tumour and anti-epileptic and also in kidney problems. It has been used in Ayurveda in the treatment of liver disorders, CNS disorders and some infectious diseases (Madhavan et al. 2010). The hydroalcoholic extract of *A. racemosus* and its fractions were evaluated against CCl_4-induced hepatotoxicity. Pretreatment with various extracts resulted in significant decrease in the oxidative stress and serum markers such as glutamate oxaloacetate transaminase, glutamate pyruvate transaminase, alkaline phosphatase and bilirubin showing its potent hepatoprotective action (Acharya et al. 2012).

29.2.6.7 *Berberis vulgaris*

B. vulgaris is a shrub of Berberidaceae family and is commonly known as barberry. It is 1–3 m in height and is found in many regions of the world including Iran (Parsaee et al. 2006). The active constituents of this plant are berberine,

oxyacanthine and other alkaloids such as berbamine, palmatine, columbamine, malic acid, jatrorrhizine and berberrubine (Fatehi et al. 2005). Berberine is an isoquinoline alkaloid which has a long medicinal background and is found in roots, rhizomes and stem bark of this plant. It possesses hepatoprotective effects, both preventive and curative, on $CC1_4$-induced liver injury through scavenging the peroxidative products (Feng et al. 2010).

29.2.6.8 *Boerhaavia diffusa*

Boerhaavia diffusa is a dried herb which occurs mainly as a weed in India and is also cultivated in West Bengal. It is commonly known as punarnava and found at an altitude of 2000 m in Himalayas. The roots of the plant contains alkaloids, ursolic acid, β-sitosterol, flavones, rotenoid boeravinones A1, B1, C2, D, E and F along with borhavine, punarnavoside and dihydroisofurenoxanthin. The plant is used as an expectorant, diuretic and stomachic and also used in jaundice treatment. The roots of the plant are used by many Indian tribes for the treatment of various liver disorders. The extract of plant has been evaluated in thioacetamide-induced hepatotoxicity at a dose of 2 mL/kg (Rawat et al. 1997). *B. diffusa* extract was also investigated against CCL_4-induced hepatotoxicity in mice at doses of 100, 200 and 300 mg/kg body weight. Treatment with *B. diffusa* extract resulted in the significant reduction in serum markers such as alkaline phosphate, ALT, AST, LDH and bilirubin along with decrease in oxidative stress showing its hepatoprotective effect (Monali and Ramtej 2014).

29.2.6.9 *Crocus sativus*

Crocus sativus belongs to family Iridaceae and is commonly known as saffron. Saffron is dried stigmas and upper parts of styles of plant. It is mainly cultivated in India at 5300 ft above sea level mainly in Jammu and Kashmir. The main glycosides present in saffron are crocin and picrocrocin along with lycopene, β-carotene and g-carotene. It acts as sedative and used as emmenagogue. It is used in liver enlargement and fevers and has stimulant and stomachic properties (Sharma et al. 2008). The hydroalcoholic extract of *C. sativus* petals (CSP) was evaluated against acetyl-*para*-aminophenol (APAP)-induced hepatotoxicity in rats. CSP administration at the doses of 10 and 20 mg/kg resulted in the decreased levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and bilirubin suggesting its protective role in APAP hepatotoxicity (Omidi et al. 2014).

29.2.6.10 *Curcuma longa*

Curcumin is a main active metabolite obtained from the rhizomes of *Curcuma longa*, commonly known as turmeric and belongs to family Zingiberaceae. It is mainly grown in rainy and warm places all over the world such as India, Jamaica, China, Indonesia and Peru. Turmeric has been used traditionally for jaundice treatment, other liver disorders, ulcers, inflammation and skin diseases. The extract of turmeric has been reported to possess hepatoprotective activity against CCl_4-induced liver damage in in vitro and in vivo. Most of the activities of turmeric are due to its potent antioxidant activity (Anand and Lal 2016). The ethanolic extract of *C. longa* (CLRE)

rhizome at a dose of 250 and 500 mg/kg was evaluated against thioacetamide-induced liver damage in rats. Treatment with CLRE attenuated the altered serum TGF-β1 and TNF-α and oxidative stress as assessed by malondialdehyde, urinary 8-hydroxyguanosine and nitrotyrosine levels. The observed effect may be due to the antioxidant and anti-inflammatory activities of CLRE (Salama et al. 2013).

29.2.6.11 *Cynara scolymus*

C. scolymus belongs to family Apiaceae and is commonly known as artichoke. Since ancient times, it is used for curing liver diseases, digestive disorders and hyperlipidemia. The leaf extract of this plant possesses hepatoprotective activity (Gebhardt 1997). The leaf extract of *C. scolymus* contain certain compounds such as cynarin, luteolin, chlorogenic acid, caffeic acid, other flavonoids and polyphenol compounds, some of which possess antioxidant properties (Fallah Huseini et al. 2011). The ethanolic extract of *C. scolymus* leaves (EEA) was investigated in animals fed on high fat diet for 8 months. The EEA at a dose of 200 and 400 mg/kg significantly decreased the abnormal serum hepatic profile, attenuated the oxidative stress and reduced the fatty liver deposition in hepatic lobule as shown by histopathological studies (Ben Salem et al. 2019).

29.2.6.12 *Emblica officinalis*

Emblica officinalis, commonly known as amla is a member of family Euphorbiaceae. It is a deciduous tree found at an altitude of 350 m in India. It is mainly cultivated in Uttar Pradesh, Gujrat, Rajasthan and Maharashtra. It contains tannins (5–6%) such as ellagic acid, gallic acid and phyllemblin. It acts as an aphrodisiac, haemostatic and nutritive tonic. The aqueous fruit extract of amla significantly reduced the blood glucose levels along with the triglyceride levels and also improves the liver functions in alloxan-induced diabetic rats (Khan 2009). The aqueous extract of *E. officinalis* (EO) at 50 and 250 mg/kg was evaluated in CCL_4-induced hepatotoxicity in rats. The EO treatment showed a marked decrease in serum markers such as alkaline phosphate, glutamate pyruvate transaminase and oxidative stress suggesting its protective effects (Jose and Kuttan 2000).

29.2.6.13 *Flacourtia indica*

The aerial parts of *Flacourtia indica* were evaluated in paracetamol-induced hepatotoxicity in rats. It is commonly known as ramontchi, Indian plum, and belongs to Salicaceae family. The various extracts of aerial parts of *F. indica* (Burm. f.) Merr., such as petroleum ether, ethyl acetate and methanol extracts, were evaluated in paracetamol-induced hepatic necrosis in rat. All the extracts of the plant ameliorated the hepatic marker enzymes, but a significant reduction was observed by treatment with petroleum ether and ethyl acetate extracts at a single dose of 1.5 g/kg. Histopathological studies also showed a marked recovery in liver necrosis was also observed in petroleum ether- and ethyl acetate-treated groups. This protective effect may be due to the inhibition of drug metabolizing enzymes (Nazneen et al. 2009; Anand and Lal 2016).

29.2.6.14 *Glycyrrhiza glabra*

Glycyrrhiza glabra belongs to family Leguminosae and is commonly known as liquorice. It is most commonly used in the traditional systems of medicine. Glycyrrhizin is the main active constituent present in the plant, and it helps in the protection of liver injury in various animal models such as CCl_4 hepatotoxicity and hepatitis. The protective effect of glycyrrhizin is due to its antioxidant, immunomodulatory and anti-inflammatory activities. It is also reported to enhance the hepatic glucuronidation and activates P450 Phase I detoxification reactions (Anand and Lal 2016).

29.2.6.15 *Marrubium vulgare*

M. vulgare is most commonly known as horehound and belongs to Lamiaceae family. It is a Mediterranean plant that is used since antiquity to treat various disorders. Various activities possessed by this plant are analgesic, anti-inflammatory, antidyslipidemic, antioxidant and hypoglycaemic (Sahpaz et al. 2002; Elberry et al. 2015). Total 46 compounds, containing 96.3% of the oil, were found to be present in this plant. The important constituents present in the oil are (E)-caryophyllene, germacrene D and bicyclogermacrene (Elberry et al. 2010). The hepatoprotective effect of methanol extract of *M. vulgare* whole plant was investigated in paracetamol-induced hepatotoxicity at the doses of 100 and 200 mg/kg. Treatment of animals with methanol extract resulted in the significant reduction in the elevated serum levels of ALT, AST, ALP, albumin, total bilirubin and triglycerides and also decreased the oxidative stress showing its potential in hepatotoxicity (Akther et al. 2013).

29.2.6.16 *Melilotus officinalis*

This medicinal herb melilot or yellow sweet clover (family Fabaceae) is widely distributed around the world. It is published in the European Pharmacopoeia ed. 8, and traditionally used in Europe for hepatoprotection. The plant is distributed all over the world, native in Asia and Middle and South Europe. Its ethanolic extract contains antioxidant and anti-inflammatory compounds (Sheikh et al. 2016; Liu et al. 2018). Aqueous extract shows the highest antioxidant activity, due to phenols and tannins that dominate in it (Mladwenovic et al. 2016). Within the water-soluble compounds, carotenoids, with special emphasis on lutein, exert strong anti-inflammatory effect (Horváth et al. 2021). Hepatoprotective effect has been affirmed by Alamgeer et al. against paracetamol and carbon tetrachloride-induced liver injury (Alamgeer et al. 2017).

29.2.6.17 *Nigella sativa*

N. sativa is used since ancient times by people of the Middle East nation due to its aromatic properties. This aromatic plant is a member of Ranunculaceae family and is used for the treatment of asthma, bronchitis, cough, eczema, fever and influenza. It is reported that seeds of *N. sativa* possess antioxidant and resolution of hepatorenal toxicity (Al-Ghamdi 2001). It has been proven that the volatile oil of this plant contains monoterpenes such as p-cymene, α-pinene and thymoquinone (Kanter et al.

2005). The oil of *N. sativa* at dose of 1 mL/kg body weight was investigated in CCl_4-induced liver damage in rats. Administration of *N. sativa* oil resulted in marked improvement in hepatic enzymes, total bilirubin, creatinine, uric acid, lipid peroxide total cholesterol, triglyceride, low-density lipoprotein, very low-density lipoproteins, interleukin-6, and antioxidant enzymes showing its protective role in hepatoprotection (Al-Seeni et al. 2016).

29.2.6.18 *Phyllanthus niruri* and *Phyllanthus amarus*

Phyllanthus niruri is a well-known plant of family Euphorbiaceae and is commonly known as bhuiamlaki. It is used in folklore medicine for the treatment of—among others, like malaria, viral and bacterial infections, diarrhoea—jaundice and liver disorders. The main hepatoprotective compounds present in the plant are lignans phyllanthin and hypophyllanthin. The hexane fraction obtained from the ethanol extract showed good hepatoprotective activity. Both compounds showed remarkable liver protection in CCl_4 and galactosamine-induced cytotoxicity in cultured rat hepatocytes due to their antioxidant activity (Anand and Lal 2016). Furthermore, the ethanol extract of *P. niruri* (PN) was evaluated at doses of 100 and 200 mg/kg for 8 weeks in thioacetamide (TAA)-induced liver cirrhosis in rats. Treatment with PN ameliorated the TAA-induced elevated liver biochemical parameters, total antioxidant capacity, lipid peroxidation and oxidative stress enzyme levels and also minimized the inflammation and maintained the normal hepatic architecture (Amin et al. 2012). Very similar effect has been demonstrated with the other plant of this family *Phyllanthus amarus* (Ogunmoyole et al. 2020).

29.2.6.19 *Picrorhiza kurroa*

Picrorhiza kurroa belongs to family Scrophulariaceae, and it consists of dried rhizomes of the plant. It is commonly known as kutki and found in Sikkim, Himalayas and Jammu and Kashmir. The main glycosidal bitter principle present in the root is known as kutkin, and it is a mixture of two iridoid glycosides picroside I and kutkoside. The plant has been used mainly for the treatment of liver and lung disorders. The main constituent kutkin is used as an antioxidant, hepatoprotective and immunomodulator and also helps in the digestion of fats. Kutkin is useful in the treatment of fatty liver, and it also helps in the regulation of fats in liver (Satyapal et al. 2008). The aqueous extract of *P. kurroa* (PE) was evaluated for its hepatoprotective activity using ethanol-induced model of hepatotoxicity in mouse liver slice culture. Ethanol treatment resulted in the significant increase in intracellular enzyme levels such as lactate dehydrogenase, glutamate oxaloacetate transaminase and glutamate pyruvate transaminase along with increase in oxidative stress. Treatment with PE at the doses of 0.5, 10 and 20 mg/mL restored the levels of these enzymes and decreased the lipid peroxidation (Sinha et al. 2011).

29.2.6.20 *Silybum marianum*

Silybum marianum belongs to family Asteraceae and is mainly found in Western Himalayas at a height of 1800 m and in Kashmir at 2400 m high. It is commonly known as milk thistle, and its main active compound is silymarin which helps in the

protection of the liver. Silymarin is a group of flavonoids such as silibinin, silidianin and silicristin which helps to repair the liver cells that are damaged by alcohol or toxic compounds and also helps to keep new liver cells from being damaged by toxins. Silymarin shows its hepatoprotective effect through various mechanisms such as antioxidant, enhanced protein synthesis, toxin blockade and antifibrotic activity (Ball and Kowdley 2005). A study was conducted to evaluate the effect of silymarin (SLM) on the hypertension state and the liver function changes induced by acetaminophen (APAP) in spontaneously hypertensive rat (SHR). Treatment of animals with silymarin at a dose of 200 mg/kg resulted in the restoration of liver function markers and oxidative stress and also restored the normal architecture of the liver in histopathological analysis (Freitag et al. 2015).

29.2.6.21 *Solanum nigrum*

Solanum nigrum is commonly known as black nightshade and belongs to Solanaceae family. The plant extract (SNE) was evaluated against thioacetamide (TAA)-induced liver fibrosis in mice at a dose of 200 mg and 1 g/kg orally. SNE treatment decreased the hepatic hydroxyproline and L-smooth muscle actin protein levels and also inhibited the collagen (L1) (I), transforming growth factor-M1 (TGF-M1) and mRNA levels in the liver. The histopathological examination also revealed a reduction in fibrosis due to TAA toxicity. The protective effect of SNE may be attributed to the reduction of TGF-B1 secretion (Hsieh et al. 2008).

29.2.6.22 *Swertia chirayita*

Swertia chirayita is an entire herb of family Gentianaceae and is found at an altitude of 1200–3000 m in the temperate Himalayas from Kashmir to Bhutan and also in the Khasi Hills of Meghalaya. It is commonly known as chirata, and it contains ophelic acid, chiratin, amarogentin, gentiopicrin and swerchirin. According to Ayurveda amarogentin is the bitterest compound and is used as a bitter tonic and stomachic. It is useful in the treatment of liver and eye disorders (Joshi and Dhawan 2005). The hepatoprotective activity of ethanol extract *S. chirayita* (SCE) was evaluated against paracetamol-induced hepatotoxicity in mice at doses of 100 and 200 mg/kg. SCE treatment resulted in the attenuation of abnormal serum marker enzymes and reduction of oxidative stress showing their protection in hepatotoxicity (Nagalekshmi et al. 2011).

29.2.6.23 *Terminalia arjuna*

Terminalia arjuna is commonly known as arjuna, and it belongs to family Combretaceae. It consists of dried stem bark and is mainly grown in all parts of India and is also planted for shade and as an ornamental plant. The main constituents of the plant are arjunic acid, β-sitosterol and ellagic acid. The bark is used as cardioprotective agent and anti-hypertensive and is also used in ischaemic heart disease. It is used as a general tonic in case of liver cirrhosis (Girish et al. 2009). The ethanolic extract of *T. arjuna* bark was evaluated in paracetamol-induced liver damage in rats at a dose of 200 mg/kg for 7 days. The results demonstrated that

the plant extract showed a significant restoration of serum enzyme levels which were altered by PCM administration (Sangamithira et al. 2016).

29.2.6.24 *Taraxacum officinale*

T. officinale is a member of Asteraceae family, and it grows in many parts of the world (Molina-Montenegro et al. 2010). It is commonly known as dandelion and has a long history of ancient use in hepatobiliary diseases. It is reported that leaf of this plant contains aesculin and the roots have shown to possess active constituents such as sesquiterpene lactones (germacrane- and guaiane-type), triterpenes, carbohydrates, fatty acids (myristic), carotenoids (lutein), flavonoids (apigenin), minerals, taraxalisin, coumarins and cichoriin (Tabassum et al. 2010). The sesquiterpene lactones present in the plant have shown protective action against acute hepatotoxicity induced by the administration of $CC1_4$ in mice (Mahesh et al. 2010). Furthermore, the aqueous extract of *T. officinale* was evaluated at a dose of 1 g/kg for 8 days in ethanol-induced hepatotoxicity in mice. The treatment resulted in the marked reduction of hepatic markers and also attenuated the oxidative stress induced by ethanol which suggested its protective action (You et al. 2010).

29.2.6.25 *Tragopogon porrifolius*

T. porrifolius is commonly known as purple salsify and belongs to family Asteraceae (Mroueh et al. 2011). The active constituents of this plant prevent free radical-associated disorders. The compounds that are present in major proportion in this plant include 4-vinyl guaiacol (19.0%), hexadecanoic acid (17.9%), hexahydrofarnesylacetone (15.8%) and hentriacontane (10.7%) (Konopiński 2009; Formisano et al. 2010). It has shown substantial hepatoprotective activity against CCl_4-induced hepatic injury in rats (Mroueh et al. 2011). The methanol extract of *T. porrifolius* was evaluated in CCl_4-induced hepatotoxicity at 50, 100 and 250 mg/kg, *i.p.* in rats. The plant extract significantly attenuated the CCl_4-induced increased in hepatic markers (AST, ALT and LDH) and oxidative stress showing a protective effect (Tenkerian et al. 2015).

29.2.7 Bioactive Compounds Used in Treating Liver Disorders

Various isolated compounds evaluated against hepatotoxicity are discussed here, their mechanistic studies are given in Table 29.1 and their structures are given in Fig. 29.1.

29.2.7.1 Cliv-92

Cliv-92 is a potent hepatoprotective agent isolated from the seeds of *Cleome viscosa* Linn. of family Capparidaceae. It is a mixture of three coumarinolignoids, i.e. cleomiscosins A, B and C. It was reported to possess hepatoprotective activity similar to silymarin against carbon tetrachloride and phalloidin-induced liver toxicity (Anand and Lal 2016). The ethyl acetate fraction of *C. viscosa* was evaluated in CCl_4-induced hepatotoxicity in rats. Animals were treated with ethyl acetate fraction

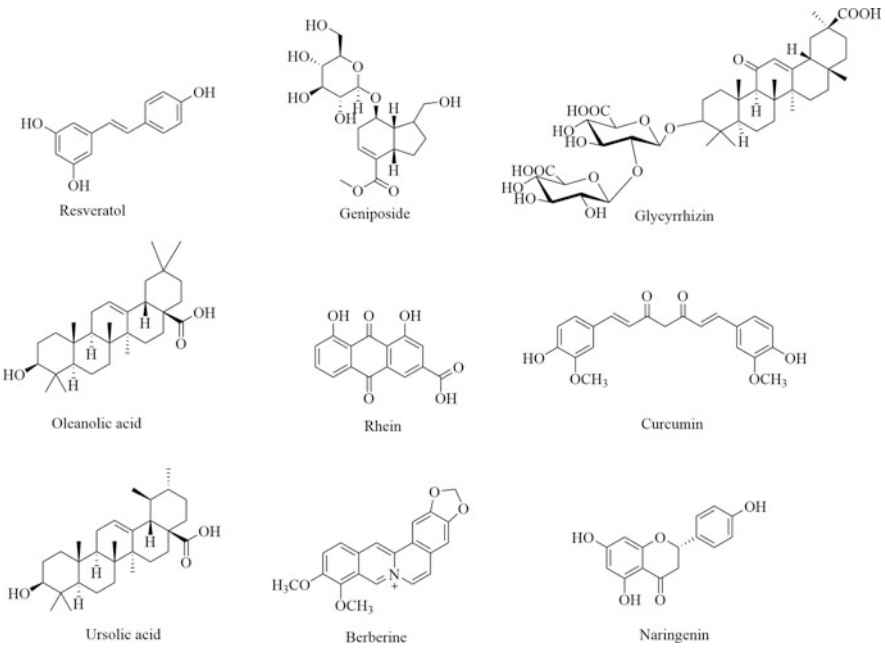

Fig. 29.1 Chemical structures of the bioactive ingredients of herbal remedies discussed in this book chapter

at the doses of 100 and 200 mg/kg for 4 days which resulted in the significant reduction of serum hepatic enzymes and oxidative stress and also showed the normal hepatic architecture in histopathological studies (Gupta and Dixit 2009).

29.2.7.2 Oleanolic Acid

Oleanolic acid is a triterpenic acid found in various plants such as *Lantana camara*, *Syzygium aromaticum*, *Ocimum basilicum*, *Salvia triloba*, etc. It is reported to inhibit carbon tetrachloride-induced liver injury. The hepatoprotective effect of oleanolic acid is due to the inhibition of carbon tetrachloride biotransformation by reduced expression of P_{450} (Anand and Lal 2016).

29.2.7.3 Ursolic Acid

Ursolic acid is a common triterpenic acid found in the leaves of various plants such as *Eucalyptus tereticornis*, *Salvia triloba, Vinca minor*, *Ocimum basilicum*, etc. It is reported to possess hepatoprotective activity comparable to silymarin in carbon tetrachloride-, ethanol-, thioacetamide- and galactosamine-induced liver damage in rats (Anand and Lal 2016).

29.2.7.4 Berberine

Berberine is an alkaloid isolated from the roots, rhizomes and stem bark of *Berberis aristata* belonging to family Berberidaceae. The plant is commonly known as

barberry. Berberine is reported to inhibit the oxidative damage induced by *tert*-butyl hydroperoxide (*t*-BHP) in hepatocytes due to its antioxidative potential and inhibition of potassium and calcium ion channels in the rat hepatocytes (Anand and Lal 2016). Berberine was reported to possess significant hepatoprotective activity in APAP-induced hepatic abnormalities (Zhao et al. 2018).

29.2.7.5 Naringenin

Naringenin is a flavonoid isolated mainly from *Citrus paradisi* belonging to family Rutaceae. Naringenin is reported to decrease hepatic triglycerides (TGs) and hepatic total cholesterol (TC). It also increases the high-density lipoprotein (HDL) which is due to its antioxidant activity (Madrigal-Santillán et al. 2014). Naringenin is also reported to show hepatoprotective activity in various models of hepatotoxicity such as carbon tetrachloride (CCl_4), alcohol, *N*-methyl-*N*-nitro-nitroguanidine, lipopolysaccharide (LPS) and heavy metals through its antioxidant, anti-inflammatory and profibrotic signalling pathways (Hernández-Aquino and Muriel 2018).

29.2.7.6 Proanthocyanidins

Proanthocyanidins are the class of phenolic compounds which are widely available in fruits, vegetables and various seeds. They are useful in the prevention of hepatic fibrosis and normalize the serum albumin and total protein levels in hepatic fibrosis. They are also used to reduce hepatic level of MDA (Madrigal-Santillán et al. 2014). Proanthocyanidins were also reported to exert their hepatoprotective effect in cadmium- and ethanol-induced hepatotoxicity in rats through their antioxidative defence mechanisms (Bak et al. 2016; Miltonprabu and Manoharan 2016).

29.2.7.7 Silymarin

Silymarin is a compound from the class of lignans and obtained from *Silybum marianum* belonging to family Asteraceae. It is a mixture of isomeric flavolignans such as silybin, silychristin and silydianin. It shows anti-hepatotoxic activity by hindering the α-amanitin to penetrate through the membrane into the nucleus cell through competitively blocking the binding of phalloidin to receptors on the hepatocyte membrane surface (Valan et al. 2010). Silymarin is also reported to possess hepatoprotective activity through its free radical scavenging ability in various models of ethanol-, acetaminophen- and carbon tetrachloride-induced hepatotoxicity (Vargas-Mendoza et al. 2014).

29.2.7.8 Andrographolide

Andrographolide is diterpenoid lactone obtained from *Andrographis paniculata* belonging to family Acanthaceae. It shows hepatoprotective activity and improves levels of various biochemical parameters like SGOT and SGPT, serum bilirubin and hepatic triglycerides in CCl_4-induced hepatotoxicity. This effect is due to its antioxidant activity (Handa and Sharma 1990).

29.2.7.9 Glycyrrhizin

Glycyrrhizin is a compound isolated from the *Glycyrrhiza glabra* belonging to family Fabaceae. It is a glycosylated saponin consisting of two molecules of glucuronic acid and one molecule of glycyrrhetinic acid. It is used in chronic hepatitis occurring from viral infections, toxin exposure and tissue perfusion injury. It decreases the concentration of high mobility group box 1 (HMGB1) by binding directly with it. HMGB1 plays important role in triggering inflammatory responses in the liver. Glycyrrhizin has strong anti-inflammatory properties which makes it useful in chronic hepatitis (El-Magd et al. 2015). Glycyrrhizin in combination with matrine which is isolated from *Sophora flavescens* attenuated the acetaminophen-induced hepatotoxicity through anti-inflammatory effect and also decreases the incidence of sodium and water retention (Wan et al. 2009).

29.2.7.10 Curcumin

Curcumin is obtained from plant *Curcuma longa* belonging to family Zingiberaceae. It is one of the most ancient compounds isolated two centuries ago. It is used to treat various diseases such as body ache, diarrhoea, rheumatism, inflammation and constipation (Pari et al. 2008). It is used in hepatic disorders due to its ability to inhibit many factors such as nuclear factor-kappa B, which helps in modulating various pro-inflammatory and profibrotic cytokines. Its antioxidant property also makes it helpful in hepatic disorders (Rivera-Espinoza and Muriel 2009). In addition, curcumin also showed protection in dimethylnitrosamine (DMN)-induced liver fibrosis in rats by attenuating the fibrosis and inflammation (Kyung et al. 2018).

29.2.7.11 Rhein

Rhein is an anthraquinone derivative obtained from *Rheum rhabarbarum* belonging to family Polygonaceae. Rhein plays a significant role in antioxidation and anti-inflammation, inhibiting the expression of TGF-beta 1 and in the suppression of hepatic stellate cells through which it protects hepatocytes from injury and also prevents the progress of hepatic fibrosis in rats (Sheng et al. 2011). It also ameliorates fatty liver disease through hepatic lipogenic regulation, negative energy balance and immunomodulation (Guo et al. 2002). Rhein also ameliorated the methotrexate (MTX)-induced hepatotoxicity by acting through Nrf2-HO-1 pathway (Bu et al. 2018).

29.2.7.12 Geniposide

Geniposide is an iridoid glycoside obtained from the fruit of *Gardenia jasminoides* belonging to family Rubiaceae (Kojima et al. 2011). Geniposides increase the expression of peroxisome proliferator-activated receptor-α (PPAR-α), inhibit the expression of CYP2E1 and effectively inhibit liver fibrosis. These effects may be shown due to increase in SOD (superoxide dismutase) and MDA (malondialdehyde). It also exhibits antioxidant, anti-inflammatory and angiogenic activities through which it shows protective effects against hepatic steatosis (Kim et al. 2005). Geniposide was evaluated in CCl_4-induced hepatotoxicity in rats. Geniposide significantly reduced the serum biochemical markers and also showed

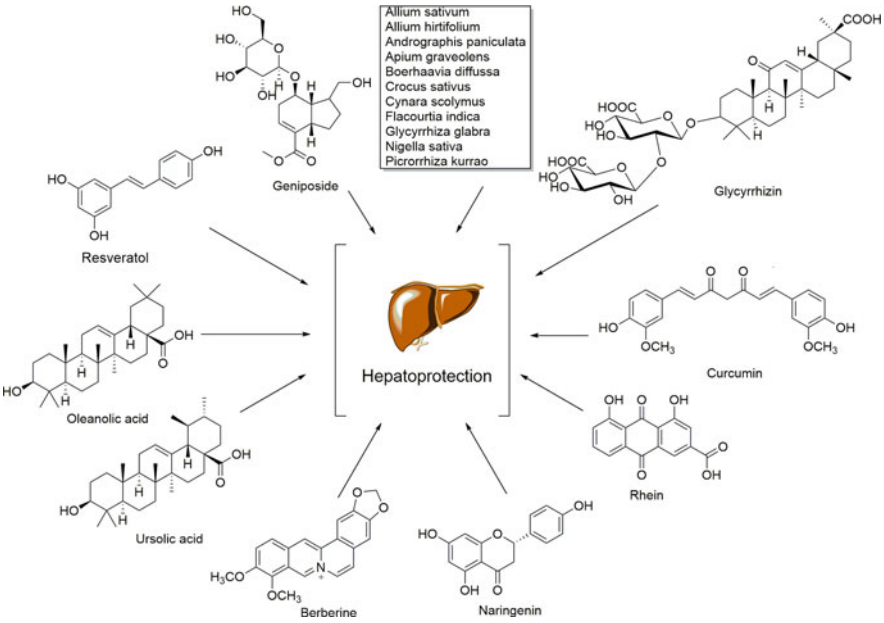

Fig. 29.2 Graphical abstract of book chapter

the normal liver architecture in histopathology. The observed effect may be due to the antioxidative potential of geniposide (Chen et al. 2016).

29.2.7.13 Resveratrol

Resveratrol is a polyphenolic compound present in berries and grapes mainly. Many studies have shown the hepatoprotective properties of resveratrol (Chan et al. 2011). It shows hepatoprotective effects in *N*-nitrosodiethylamine-induced hepatocellular carcinoma through inflammatory cytokines and free radicals, induces antioxidant enzymes, elevates glutathione content and also modulates various signal transduction pathways of liver diseases. Resveratrol reduces TNF-α IL-6 mRNA and also decreases the Kupffer cells hired in injured liver (Rajasekaran et al. 2011) (Fig. 29.2).

29.3 Conclusions

The liver is a vital organ of human body that performs diverse physiological functions and supports food digestion through bile secretion, metabolism of food and drugs, detoxification of xenobiotics, storage of glycogen and many other important functions. Hepatotoxicity or pathological liver damage can occur due to the abuse of some synthetic drugs, excessive use of alcohol, substances of abuse, idiosyncratic reactions, viral infections and exposure to environmental toxicants or

industrial chemicals. The treatment of liver disorders is a great challenge and is complicated due to various adverse effects caused by available medications. Therefore, to find the effective cure for liver disorders, we have looked back at the traditional medicinal plants used in folklore medicine. The herbal remedies and their isolated constituents mentioned in this review have been used traditionally as well as have been evaluated pharmacologically and found to be clinically effective against hepatotoxicity. This review throws some light on the hepatoprotection of drugs and plant-derived remedies. We have done compilation of Indian medicinal plants and isolated compounds, which have been found to be potent against hepatotoxicity and have been studied in animal models to discover novel therapies for treating liver failure disorders in humans.

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Polymeric Vehicles for Controlled Delivery of Ayurvedic Drugs for Wound Management

30

Arpan Biswas, Pralay Maiti, and Manoranjan Sahu

Abstract

The wound healing is a composite phenomenon as it passes through many intricate phases, where cell-to-cell and cell-to-matrix interactions are very crucial. Due to the complexity of wounds, different types of dressing materials are needed for faster healing. The traditional herbal medicines have a long history for wound management but often suffer from their low bioavailability and proper matrix materials to hold the drug. There are various dressing materials available in the market, but the efficient dressing materials using Ayurvedic drugs are one of the important aspects in the present-day scenario. The wound management using different herbal drugs loaded dressing materials will be emphasized in this chapter. The encapsulation of herbal drug in polymer matrix increases their efficiency toward wound healing. Polymeric dressing materials have been discussed in details with their efficacy in terms of ex vivo, in vivo and clinical studies. Different forms of modern dressing materials include hydrogels, patch and scaffolds which will shed light in terms of their relative efficacy. Finally, in vivo and clinical studies have been presented showing better wound management using traditional herbal drugs.

Keywords

Wounds · Herbal medicines · Dressing materials

A. Biswas · P. Maiti
School of Materials Science and Technology, Indian Institute of Technology (Banaras Hindu University), Varanasi, India

M. Sahu (✉)
Department of Shalya Tantra, Institute of Medical Science, Banaras Hindu University, Varanasi, India
e-mail: msahuvns@gmail.com

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30.1 Introduction

Dressing of a wound is very essential for its faster healing because it maintains the moisture level of any wound, protect the wound from microbes and dust, and deliver biologically active molecules to the wound site to accelerate the healing (Biswas et al. 2018a). The polymeric dressing materials draw huge attention of scientists because of their advantages as a carrier of drug molecules and growth factors (Kazunori et al. 1993; Price et al. 2006). They are capable to improve the stability of biologically active (BA) molecules and solubility of BA molecules. The worst part of wound management is to change the wound dressing frequently because it not only increases pain, swelling, and discharge but also delays the healing by damaging the healthy new tissues (Price et al. 2006). A new dressing material needs to be designed considering all these major issues. Further, the developed dressing material should be cheap in price, flexible in nature and easily detachable from the wound site without harming the newly grown tissues. Synthetic drugs, especially antibiotic-loaded dressing materials, are being used in wound management throughout the world for a long time (Chouhan et al. 2017). But, they have some serious issues like side effects, multidrug resistance, etc. that increase complications along with medical expenses which increase concerns of common people. Nowadays, the herbal products, plant extractions and traditional medicines have attracted great attentions of the researchers as a potential alternative of modern medicines (Sridhar et al. 2015). From the ancient age, different parts of plants are being used in wound management which have antimicrobial and anti-inflammatory properties (Hammer et al. 1999; Friedman 2015; Dorman and Deans 2000). The alkaloids, flavonoids, terpenoids and phenolics are the possible extract from plants. The use of various strategies to fight against pathogens includes disruption of cell walls, complex formation with cell membrane, substrate deprivation and enzyme inhibition (Cowan 1999).

Moisture level of a wound is another important factor which can decide the healing rate of any wound. The healing rate decreased in presence of both the excess moisture or under dry condition. Hence, to maintain the adequate moisture level at wound site is very essential for triggering healing of the wound by enhancing the epithelialization of superficial wounds. Scientists design different types of occlusive dressing materials to maintain the proper moisture environment over the wounds (Winter 1962; Hinman and Maibach 1963). Hydrogels and soft physiological tissues are widely used as wound dressing materials and are available in the market for advance wound management. Hydrogels have very similar physicochemical properties, viz. mechanical strength, low interfacial tension to body, air permeability, water content, etc. (Blanco et al. 1996) and are capable to release water soluble drugs in a sustained and controlled manner following the appropriate diffusion mechanism (Shivakumar et al. 2006). Further, the morphological advantages of electrospun scaffold attracted the attention of researchers. The porous structure with very high surface area of electrospun scaffolds allows it to mimic the extracellular matrix (ECM) and facilitates the cell migration, water evaporation, exudate drainage and air permeation (Heydarkhan-Hagvall et al. 2008; Han and Gouma 2006; Schneider et al.

2009). Further, it can incorporate any kind of drugs into nanofibres through electrospinning technique and is capable to deliver it locally in a faster way. The biocompatible and biodegradable polymers like chitosan, gelatin, poly(-ε-caprolactone), poly(lactic acid), poly(vinyl alcohol) and poly(lactic acid-*co*-glycolic acid) are capable to form nanofibre using electrospinning technique. These nanofibres are capable to appropriate substrate for cell proliferation and migration which trigger the repairing of the damage tissues and, thereby, encourage new tissue restoration (Shim et al. 2008; Lee et al. 2009). Polymeric dressing materials are capable to incorporate and deliver biologically active molecules. But, the most challenging part in delivering is that any therapeutic agent has to maintain the therapeutic concentration at the wound site throughout its delivery which depends on the diffusion mechanism of the drug from the dressing material and its penetration ability through skin barrier. The controlled release of therapeutic agents decreases the total amount of released therapeutic agents initially and ultimately improves its bioavailability for longer period of time which triggers the healing process of most wounds (Verreck et al. 2003).

30.2 Dressing Materials

For proper healing of wounds, whether it is a minor cut or major incision, it is necessary to take care of it. Proper dressing of wounds plays an important role in wound management, and the selection of proper dressing depends on the nature of wounds. An ideal dressing material should have the following characteristics, viz. (a) it should maintain proper moist condition surrounding the wound, (b) it should boost the epidermal migration, (c) it should allow adequate gas exchange between wound tissue and environment, (d) it should inhibit the bacterial infections, (e) it should endorse the angiogenesis and connecting tissue generation and (f) it should be non-adherent to the wound and easy to take out after healing (Dhivya et al. 2015). From ancient age (2500 BCE), wet to dry dressings have been used to promote wound debridement, and clay tablets and linen strips soaked in oil or grease covered with plasters were usually used to heal the wounds. The wounds were cleaned with milk or water before dressing with honey or resin (Carla Daunton et al. 2012). Antiseptic dressing was first introduced in the nineteenth century in which antibiotic drugs were used to prevent the infection and reduce the mortality while in early twentieth century; the modern dressing material was introduced (Shah 2011).

Generally, traditional dressing includes gauze, lint, plasters, bandages (natural or synthetic) and dry cotton wool. These traditional dressings are basically used as primary or secondary dressing for protecting wounds from contamination (Boateng et al. 2008). But, they need to change frequently to inhibit the softening of the healthy tissues. Further, these dressings get easily moistened due to excessive wound drainage which makes them adherent to the wound resulting it painful during removal. Hence, traditional dressings are better for clean or dry wounds with mild exudates, or they are useful as secondary dressings. Xeroform™ is an example of non-occlusive dressing (petrolatum gauze with 3% bismuth tribromophenate)

generally applicable for non-exudating to slightly exudating wounds. Further, Bactigras, Jelonet and Paratulle are examples of commercially available tulle dressing which are appropriate for superficial clean wounds. The limitations of the traditional dressings, like frequent changing of dressing, fail to maintain moist condition which produces appropriate situation for modern dressing to come up with more advanced formulations (Boateng et al. 2008). The modern dressings are designed to provide proper moist condition, air circulation and active components to the wound to promote the cell migration and angiogenesis for faster healing. There are numerous dressing materials available in the market depending on the cause and type of the wounds. The modern dressings can be classified as passive, interactive and bioactive, and usually the synthetic or natural polymers are used for this purpose. The interactive dressings are basically polymeric films, hydrogels and hydrocolloids which function as a blockade against dispersion of bacteria to the wound environment (Rivera and Spencer 2007; Strecker-McGraw et al. 2007). The passive dressings are non-occlusive like gauze and are applied to cover up the wound to promote the healing underneath.

30.3 Polymeric Drug Delivery Vehicle

Polymeric drug delivery vehicles allow the delivery of any therapeutically active molecules inside human body in a controlled and sustained manner. Further, it provides better protection and improves effectiveness of the drug by tuning the rate, time and site of release in the human body. Polymeric drug delivery vehicles can be nanoparticles, hydrogels, films and scaffolds in nature. Different stages of wound require different forms of dressing materials. Hence, proper selection of a delivery vehicle is a tricky job (Biswas et al. 2018b). Recently, researchers are looking for new alternative to modern medicines like antibiotics, corticosteroids, etc. for wound healing to eliminate the side effects of these medicines. Ayurvedic medicine incorporated in polymeric delivery vehicles can be a good alternative.

Hydrogels are three-dimensional polymeric networks that can hold different drugs inside network structure and release the drug in controlled manner. Hydrogels are generally used to treat dry chronic wounds, necrotic wounds, pressure ulcers and burn wounds. The starch-zeolite nanocomposite hydrogel by incorporating herbal drug chamomile is able to control the release of the drug along with greater absorption of the exudates which results in better contraction of the wound as compared to pure hydrogel. Further, the effectiveness of simvastatin in wound healing significantly improved after incorporating simvastatin-chitosan nanoparticle in poly(vinyl alcohol) hydrogel as compared to simvastatin ointment (Yasasvini et al. 2017). The hydrogel dressings have some limitations like low mechanical strength and exudate accumulation which make them difficult to handle and leads to maceration and bacterial proliferation (Martin et al. 2002). Hydrocolloids are made of two layers, inner colloidal layer and outer water-impermeable layer. It is one of the highly used interactive wound dressings. Hydrocolloid dressings are the combination of gel-forming agents like carboxymethyl cellulose, gelatin and pectin and

other materials, viz. elastomers and adhesives (Boateng et al. 2008). They allow water vapour to pass through but restrict the bacteria. These dressings are used for mild to moderate wounds like pressure sore and minor burn, and they are also recommended for paediatric wound management (Thomas 1992). They produce gels on contact with wound exudate and maintain the moist condition. The hydrocolloids are not very effective for neuropathic ulcers or high exuding wounds. The electrospun patches are highly permeable in nature and have the ability to deliver any kind of drugs to the wound site at a faster rate (Kataria et al. 2014). Further, this porous structure allows passing air or exudates easily and helps to maintain the moisture label in the wound site. Further, they have large surface area, are easy to process and are benign to the wounds. Poly(lactic acid) (PLA) and poly (ε-caprolactone) (PCL) are highly biocompatible in nature, and their electrospun scaffolds are very effective in delivering different biologically active molecules at the wound site. The wound dressing with thymol-loaded PLA-PCL composite nanofibres show better wound contraction as compared to thymol-loaded pure PCL/PLA nanofibres (Karami et al. 2013). The bioactive dressings are usually produced from natural tissue or artificial sources like collagen (Ramshaw et al. 1996), hyaluronic acid (Doillon and Silver 1986), chitosan (Ishihara et al. 2002), alginate and elastin. Sometimes, these dressing materials are incorporated with antimicrobials or growth factors to promote wound healing process depending on the nature of the wound. Collagen, one of the important structural proteins, has important role in fibroblast formation and endothelial migration in wound healing, while hyaluronic acid being a glycosaminoglycan component of extra cellular matrix (ECM) has unique biological or physiological features. Both of them are biocompatible and biodegradable and lack immunogenicity in nature (Supp and Boyce 2005). As compared to other dressings, biological dressings are reported to be superior to other types of dressings.

30.4 Characterization of Dressing Materials

Characterization of any dressing material is necessary before its application on animals and human beings. The necessary characterizations like drug loading efficiency of the dressing material, physical state of the dressing after drug loading, size of the drug in the dressing material, in vitro release kinetics of the drug from dressing material and the effect of drug-loaded scaffold on secondary or primary cells are very essential for their further applications. The polysaccharide (PAP) residue of *Periplaneta americana* has a long medicinal history which is being used in wound management. The composite hydrogel made of PAP, carbomer 940 (CBM) and carboxymethyl cellulose (CMC) shows better three-dimensional network structure, improves swelling and water retention capability and bears decent mechanical strength as compared to pure polymer hydrogels (Fig. 30.1a(i), (ii)) (Wang et al. 2020). The cytocompatibility of the hydrogels is examined with 3T3 fibroblast cell line revealing composite hydrogels are biocompatible in nature. In another study, curcumin-phospholipid complex (CPC) is incorporated into a thermosensitive

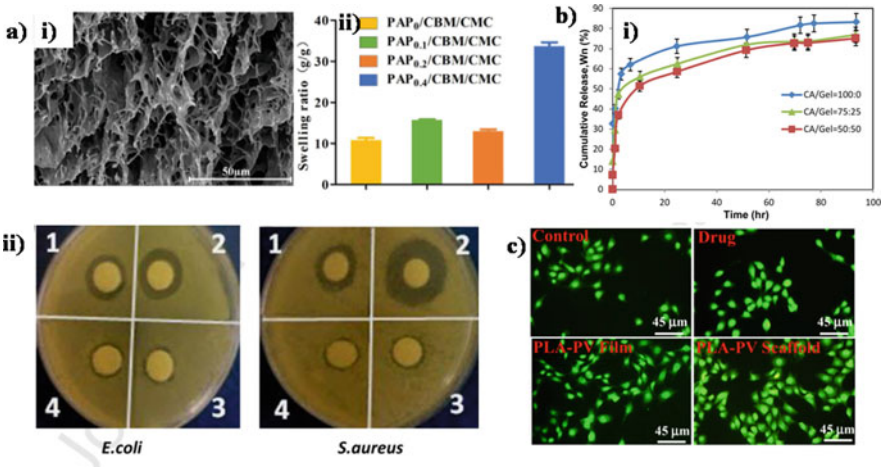

Fig. 30.1 Characterization of wound dressing materials: (**a**) (i) the SEM investigation shows 3D porous structure of composite hydrogel of PAP, CBM and carboxymethyl cellulose (CMC); (ii) the swelling ratio of the composite hydrogel in presence of different amount of herbal drug (PAP); (**b**) (i) cumulative release kinetics of the herbal drug from cellulose acetate/gelatine nanofibre revealing more control release of the drug with increasing the gelatine content; (ii) the demonstration of antibacterial activities of the electrospun nanofibres using zone of inhibition method (1). CA/gel = 100:0 with drug, (2) CA/gel = 50:50 with drug, (3) CA/gel = 100:0 without drug, (4) CA/gel = 50:50 without drug; and (**c**) fluorescent images of 3T3 fibroblast cells after staining with AO/EB after 3 days of cell proliferation in presence of different dressing materials

hydrogel of poloxamer for wound management. The formation of CPC leads to physical state conversion of curcumin from crystalline to amorphous which improves its transdermal release efficacy from the in situ forming hydrogel as compared to release of pure curcumin from in situ forming hydrogel. Further, in vitro release study shows a very sustained and controlled release kinetics for CPC, while a burst release is evident for pure curcumin (Du et al. 2016). The *Tecomella undulata* is well-known for its antibacterial property. The step bark of *Tecomella undulata* comprises tecomin which is widely used in wound management since ancient time (Ullah et al. 2008). The incorporation of 7.5 wt% (with respect to polymer) of tecomin into PCL/PVP nanofibre through electrospinning technique improves the stability of the herbal drug and increases fibre diameter and pore size in the nanofibre mat. In vitro release study exhibits a burst release of the herbal drug initially (almost 24% in 4 h) and followed by a control release of drug ~40% in 24 h which corresponds to a cumulative release of ~3.5 wt% (with respect to polymer) of the drug from the dressing mat (Suganya et al. 2011). The zone of inhibition study has been carried out against the pathogenic bacteria *P. aeruginosa* MTCC 2297, *S. aureus* ATCC 933 and *E. coli* (IP-406006), and pure PCL/PVP nanofibre is taken as control. A considerable zone of inhibition with drug-loaded nanofibre mat suggests superior antibacterial property of the drug-loaded electrospun mat. Zataria multiflora nanoemulsion is well-known for its wound healing activity. A wound

dressing nanofibrous mat is prepared incorporating *Zataria multiflora* nanoemulsion into cellulose acetate/gelatine blend and using electrospinning technique to improve wound management (Farahani et al. 2020). The fibre diameter increases with increasing the cellulose acetate/gelatine ratio, while mechanical strength of the fibre decreases with increasing the ratio. In vitro release study reveals a controlled release kinetic of the herbal drug with increasing the gelatine content in the nanofibre (Fig. 30.1b(i)). Further, the release kinetics from different nanofibres follows the Korsmeyer-Peppas model with '*n*' value below 0.45 indicating pseudo-Fickian diffusion of the drug. The zone of inhibition study exhibits the nanofibre with 1:1 ratio of cellulose acetate/gelatine which shows better antibacterial activity than the other systems against *S. aureus* and *E. coli* bacteria (Fig. 30.1b(ii)). Hence, the nanofibre mats of cellulose acetate/gelatine incorporated with herbal drug have potential as wound dressing material. The 'panchavalkala' (PV) a well-known polyherb for wound management is incorporated into PLA film and nanofibre to improve the efficacy of the polyherb. The incorporation of the polyherb increases the fibre diameter of electrospun scaffold. Further, thermal stability increases, and particle size decreases of the polyherb after incorporation into nanofibre. In vitro release kinetics shows a controlled of the drug up to 80% continuously for 5 days from the scaffold with an initial burst release from the scaffold, while a controlled release of drug (45%) is observed for the film. The release kinetics follows Higuchi model which indicates the release mechanism is highly diffusion controlled and the initial burst release is due to large surface area of the electrospun scaffold. The cell adhesion, cell viability and cell imaging studies with 3T3 fibroblast cell line reveal that the presence of the polyherb improves the cell attachment and proliferation over the scaffold indicating the developed wound dressing is biocompatible in nature (Fig. 30.1c). Further, the enzymatic degradation studies suggest the developed dressing mats are biodegradable in nature (Biswas et al. 2018a). Generally, sponges are porous and flexible in nature and are capable of absorbing huge amount of exudates. These characteristics make sponges good wound dressing materials. The sponge made of chitosan and gelatine is very effective in wound management (Nguyen et al. 2013). The porous structure is evident through morphological investigation. The folding endurance and percentage of drug release increase with increasing gelatine content, while water uptake increases with increasing the chitosan content. A greater zone of inhibition against *P. aeruginosa* is observed with curcumin-loaded sponge, and it increases with increasing chitosan content. Further, the biocompatible nature of the sponge is evident from cytotoxicity measurement.

30.5 Efficacy of Dressing Material (In Vivo Study)

The superiority of any dressing material with or without any bioactive materials can be determined through in vivo animal studies. Generally, animals like rat, mice or rabbits of any sex are chosen randomly for this purpose, and a full-thickness wound (circular or square shape) is created with the permission of Animal Experimentation

Ethics Committee. Then, those wounds are dressed on daily basis with the developed dressing materials, and healing efficacy is determined with respect to the control (wound not treated by any means). Generally, herbal drug-loaded wound dressing creams are prepared through oil/water emulsification process. The wound dressing cream incorporated with cow ghee, flax seed oil, *Phyllanthus emblica fruit*, *Shorea robusta* resin and Yashada bhasma is very effective in triggering wound contraction, improvement of tensile strength of wound, growth of hydroxyproline and collagen deposition (Datta et al. 2011). Hydrogels are very promising wound dressing materials, and recently hydrogels are modified with some specific functional groups to interact with loaded molecules in such a way that the control release of that loaded molecules can be observed through in vivo studies (Gong et al. 2013; Jeffords et al. 2015; Wang et al. 2010). Further, this kind of surface modifications improve water solubility, bioavailability and selectivity (Brandl et al. 2010). The nanohybrid hydrogel of starch-zeolite is biocompatible in nature and exhibits excellent wound closure efficacy after incorporation with chamomile extract, a herbal drug (Salehi et al. 2017). In vivo studies exhibit better efficiency of the drug-loaded nanohybrid hydrogel as compared to pure hydrogel. The histopathological studies show complete epithelialization and hair follicles after 21 days of treatment with drug-loaded nanohybrid hydrogel. Hence, the presence of zeolite nanoparticles regulates the delivery of the herbal drugs in a controlled way which helps to trigger the healing process. Another in vivo study on Wistar rats reveals better wound contraction with low dose of simvastatin-chitosan microparticle-incorporated poly(vinyl alcohol) (PVA) as compared to simvastatin ointment (1%) (Yasasvini et al. 2017). Vitexin is a flavonoid and has anti-inflammatory, analgesic and antioxidant effects (Borghi et al. 2013). The vitexin-loaded chitosan hydrogel exhibits superior healing properties in terms of re-epithelialization and vascularization as compared to pure chitosan and madecassol group in in vivo wound model. The wound area contraction is significant in 7th and 14th day with vitexin-loaded chitosan hydrogel group as compared to control or madecassol group (Bektas et al. 2020) (Fig. 30.2a, b). The anti-inflammatory and antioxidant property of chitosan and antimicrobial property of the herbal drug synergistically accelerate the healing rate in animal model.

The electrospun scaffolds have advantages as wound dressing because of its high reproducibility, large surface area, non-adherent property, biocompatibility and fast local drug releasing capability (Biswas et al. 2018a; Agnes Mary and Giri Dev 2015). Curcumin is one of the widely used traditional herbal drugs for wound healing purpose because of its antioxidant and anti-inflammatory properties which help to minimize the inflammatory stage of wound and heal faster (Lian et al. 2014). But, its poor stability and inadequate bioavailability restrict its application in wound healing. However, these demerits can be overcome by incorporating it into a suitable delivery vehicle. The curcumin-loaded PCL/gum tragacanth electrospun nanofibre exhibits excellent wound healing properties in terms of significantly faster wound contraction, collagen deposition, complete early re-epithelialization and formation of sweat glands and hair follicles in diabetic animal wound model (Ranjbar-Mohammadi et al. 2016). *Nepeta dschuparensis*, a herbal medicinal plant, is very well-known for its antibacterial, antioxidant and anti-inflammatory properties. The

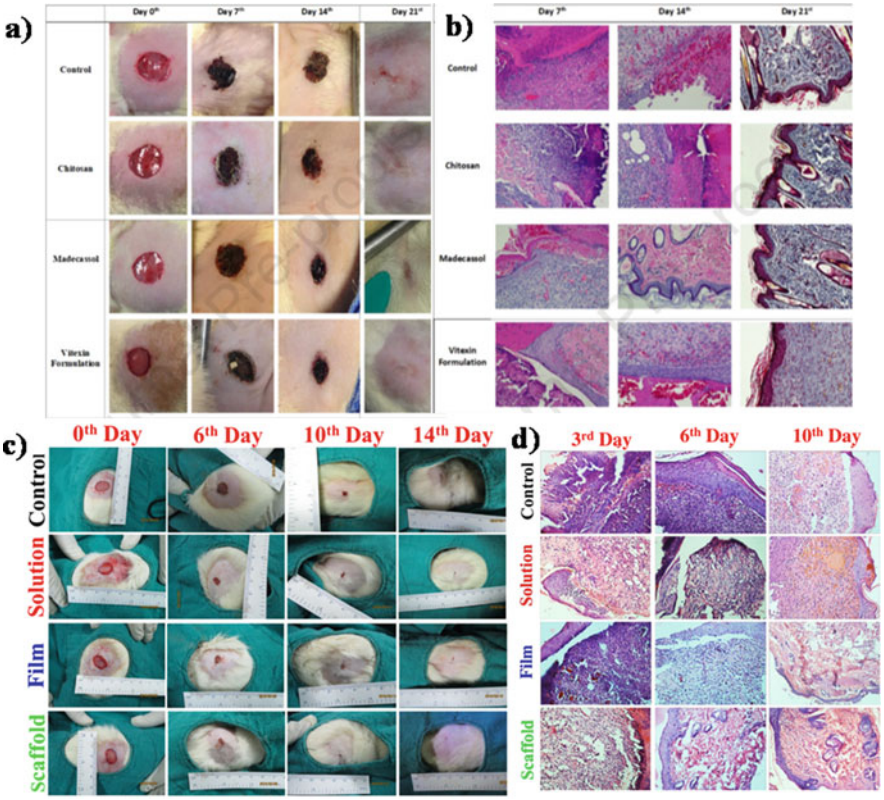

Fig. 30.2 In vivo study with different wound dressing materials: (**a**) the circular wounds on rats are treated with vitexin-loaded chitosan hydrogel, madecassol and pure chitosan hydrogel, and the wound contraction is observed with time; (**b**) the histopathological study exhibits superior healing properties of herbal drug-loaded chitosan hydrogel in terms of re-epithelialization and vascularization as compared to pure chitosan and madecassol group; (**c**) the excisional wounds on albino rats are treated with herbal drug 'panchavalkala'-loaded solution, PLA film and electrospun scaffold. The wound contraction is observed with time and compared with control; and (**d**) the histopathological studies indicates faster healing with drug-loaded scaffold in terms of greater angiogenesis, construction of granulation tissues and less inflammation

incorporation of honey and *Nepeta dschuparensis* together into chitosan/poly(vinyl alcohol) electrospun nanofibre triggers the healing rate of the wounds in animal model. In in vivo experiment, the treatment of a second-degree burn injury of rats has been carried out with developed dressing material, and the healing efficacy is determined with respect to 1% silver sulfadiazine treated group (Naeimi et al. 2020). A faster wound contraction is observed with the developed dressing material after 21 days of treatment which is further supported by the histopathological investigation. Minimal inflammation, higher angiogenesis and increased collagen deposition are observed with the developed dressing material after 21 days of treatment, while slow wound contraction, higher necrosis and less collagen deposition are

documented with 1% silver sulfadiazine. Hence, the developed herbal drug-loaded electrospun dressing mat is far better as wound dressing as compared to commercially available 1% silver sulfadiazine. 'Panchavalkala' (Anandjiwala et al. 2008) (PV) is a combination of stem bark of five trees, namely, *Ficus benghalensis*, *Ficus lacor*, *Ficus racemosa*, *Ficus religiosa* and *Thespesia populnea*, quite popular as a traditional herbal medicine for wound management. The main disadvantages of these types of polyherbs are easily washable, low bioavailability and very short shelf life which decrease its efficacy in wound management. The impregnation of this polyherb into a biocompatible and biodegradable polymer nanofibre, namely, poly(lactic acid) (PLA) through electrospinning technique not only improves its shelf life and bioavailability but also releases the drug on the wound site in a faster and controlled manner. In vivo study with a full-thickness excision wound on albino rats reveals faster wound contraction with PV-loaded PLA electrospun scaffold as compared to other dressing materials like PV-loaded PLA film or PV solution (Biswas et al. 2018a) (Fig. 30.2c). The histopathological investigation reveals complete healing in terms of prominent vascularization and intact epithelium with very minimal inflammation after 7 days of treatment with PV-PLA group, while other groups take more than 10 days to show complete epithelialization (Fig. 30.2d). Further, the immunohistochemistry investigation with CD31 shows neovascularization in PV-PLA scaffold group after 7 days of treatment. The positive staining accumulation near the periphery of small vessel and capillary in PV-PLA group indicates accelerated healing process in PV-PLA group as compared to other groups. Another dressing patch made of thymoquinone-incorporated PLA-cellulose acetate nanofibres exhibits excellent wound healing property (Gomaa et al. 2017). The thymoquinone is well-known for its antibacterial and wound management properties. In vivo studies reveal that the impregnation of thymoquinone into nanofibre promotes re-epithelialization, angiogenesis and granulation tissue formation. The freeze-dry scaffolds are also very effective as wound dressing because of its porous structure and high absorbing ability. The nanohybrid of curcumin and chitosan nanoparticle-incorporated collagen freeze-dry scaffold exhibits significant wound contraction ($p < 0.001$) in animal excision wound model as compared to control and placebo groups which is further supported by the histopathological examination. A thick granulation tissue with complete re-epithelialization is observed in nanohybrid group, while incomplete collagen deposition and inflammation are noticed in placebo and control group, respectively (Karri et al. 2016).

The phytoconstitutes like alkaloids, flavonoids and tannins are highly effective against chronic wounds and piles. The antioxidant and antimicrobial properties of phytoconstitutes of *Ficus racemosa* help to accelerate the wound healing rate. The dermal patches of Protanol LF10/60 incorporated with alkaloids, flavonoids and tannins separately are very effective in wound management. In vivo experiment reveals that flavonoid- and tannin-loaded dermal patches exhibit superior healing ability as compared to alkaloid-loaded dermal patch (Ravichandiran and Manivannan 2015).

30.6 Clinical Studies Using Drug Delivery Vehicle

The ultimate goal of any researcher behind developing any wound dressing is its application on human beings. To achieve that goal, it needs to overcome some hurdles. The clinical trial is the ultimate hurdle through which every wound dressing material needs to pass. In clinical trial, newly developed dressing materials which showed their efficacy in in vivo studies are applied on patients. The nanohybrid hydrogel of starch-zeolite impregnated with chamomile extract showed its efficacy in in vivo studies. Now, the treatment of some refractory ulcers of patients at different portion of the body with this developed nanohybrid hydrogels triggers the healing of the wound area with time. Further, the colour of the wound's surface also changes with time. Further, the formation of granulation tissue and epithelialization have been evident from histopathological data. Hence, the controlled release of the impregnated herbal drug from the developed nanohybrid hydrogel wound dressing accelerates the healing process (Salehi et al. 2017). In another report, almost 20 patients of either sex having age between 15 and 65 years are treated with herbal drug 'panchavalkala'-loaded solution, film and electrospun scaffold to understand the efficacy of the developed dressing materials (Biswas et al. 2018a) (Fig. 30.3). The dressings are changed after every 4 days, and contraction of the wound size, development in wound edges and margins, decrease in peripheral tissue edema, induration and wound necrotic tissue content are documented with time. The structural advantages of scaffold allow the exudates to come out and maintain the moisture level of the wound which in turn accelerates the healing rate. Further, better cell proliferation and faster release of the herbal drug from the scaffold allow it to heal the wounds in a faster rate. The biopsy report suggests appearance of vascularization and good re-epithelialization with time. Most of the chronic wounds delay in healing because of the imperfect revascularization and re-epithelialization. But, in this clinical trial, gradual reduction of wound exudates, improved vascularization and re-epithelialization are observed which clearly indicate toward proper wound management. Hence, the developed drug-loaded dressing materials are very promising as wound dressing materials.

30.7 Conclusion and Future Outlook

The wound dressing material is one of the key factors in management of wounds, and proper selection of dressing material is very tricky and essential for proper healing of a wound. Cream, hydrogels, scaffolds, hydrocolloids and other advance dressing materials are capable to maintain moist condition at the wound site, absorb or drain the exudates and deliver therapeutic agents. Further, they allow the cell migration, proliferation and differentiations. Natural and synthetic biopolymers are biocompatible and biodegradable in nature which makes them automatic choice for wound dressing. Further, these polymeric materials are capable to deliver the therapeutic agents in a sustained and controlled manner to trigger the healing rate as evident from in vitro, in vivo and clinical studies. The herbal drugs have some

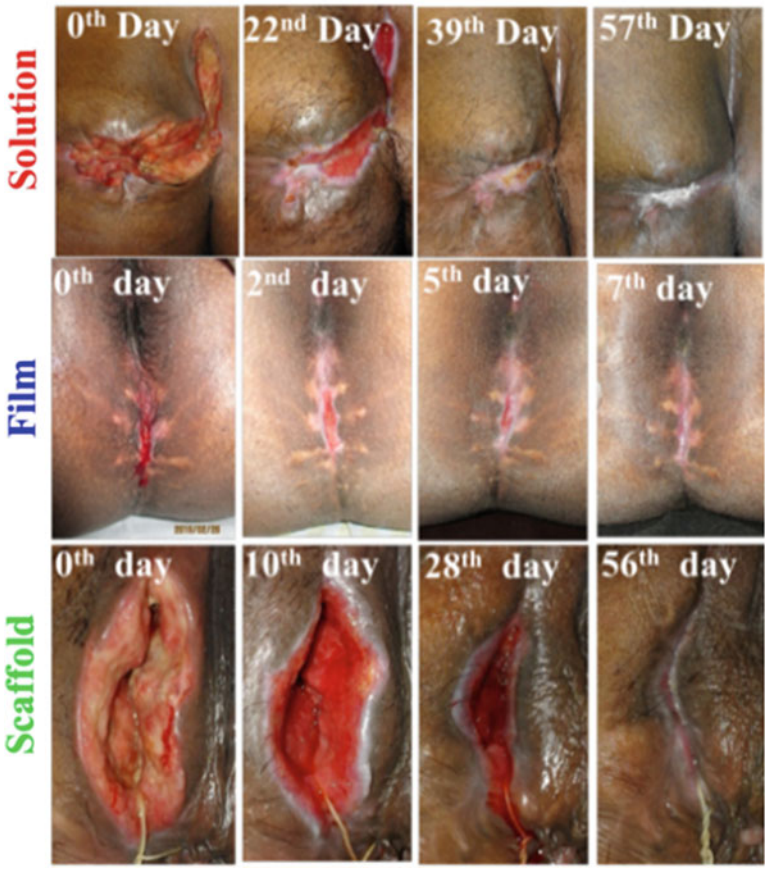

Fig. 30.3 Clinical study using herbal drug-impregnated different dressing materials. The ulcers at different parts of the body threated with panchavalkala-loaded solution, PLA film and electrospun scaffold

advantages like fewer side effects and are cheaper in price over modern drugs but have some limitations like low bioavailability. The impregnation of herbal drugs into proper delivery vehicles improves its bioavailability and sustained its release over the wound site. The herbal drug-impregnated wound dressing materials exhibit better cell attachment, proliferation, differentiation and antimicrobial properties and increase the overall quality of wound healing. The future direction of wound management includes the design of the drug-impregnated systems using shape memory polymers which will change its shape with healing of the wound and deliver the drug molecules either through controlled release, changing the shapes or combining all these therapies for effective wound management (Biswas et al. 2016, 2018c, 2019a, b).

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