Tilak Saha · Manab Deb Adhikari ·
Bipransh Kumar Tiwary *Editors*

Alternatives to Antibiotics

Recent Trends and Future Prospects

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Editors
Tilak Saha
Immunology and Microbiology
Laboratory, Department of Zoology
University of North Bengal
Siliguri, West Bengal, India

Manab Deb Adhikari
Department of Biotechnology
University of North Bengal
Siliguri, West Bengal, India

Bipransh Kumar Tiwary
Department of Microbiology
North Bengal St. Xavier's College
Rajganj, Jalpaiguri, West Bengal, India

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Foreword

Antibiotic resistance is one of the critical issues in modern healthcare, emerged due to the indiscriminate and arbitrary use of antibiotics. According to the estimates by World Health Organization (WHO), drug resistant pathogens, in absence of remedial actions can kill 10 million people each year by 2050. Zoonotic viruses including Avian Influenza, Ebola, Nipah, Sever Acute Respiratory Syndrome (SARS) and Middle East Respiratory Syndrome (MERS) were seen as global threats in the twenty-first century. Amongst these coronavirus disease 2019 (COVID-19) in December 2019 from China's Hubei province (Wuhan) spread to different parts of the globe in a short span of time leading to a pandemic. It has affected millions of people across the globe. In these unprecedented times of the Coronavirus pandemic, use of antibiotics increased manifold to avoid secondary infections.

The present book covers the most relevant and promising research on alternative antibacterial agents and endeavor to find way out of antibiotic resistance. This book comprises of 22 chapters under four main sections each of which describes and analyses the alternative approach towards the problem. The four sections cover the most relevant and promising research on alternative approaches other than conventional antibiotics to treat bacterial infection. First section of the book deals with the naturally derived compounds/agents. Traditional healing systems that rely upon plant-based antimicrobials (PBAs), bacteriophage or phage therapy, bacterial quorum-quenching and enzyme-based & antibody-based antibacterial therapeutic approaches have been discussed in this section. Second section of the book covers the chemical compounds that are putative antibacterial agents. Metal complexes, semi-synthetic modifications of available antibiotic arsenal and polymeric amphiphilic molecules are covered in detail for their antibacterial activities. Third section of the book describes the nanomaterials as antibacterial agents. Metal nanoparticles, MoS_2 nanosheets, carbon-based nanomaterial etc. are the potential approaches of antibacterial application that are discussed in detail. Fourth section of book that

covers the probiotic/prebiotic/peptides compounds/agents primarily say about immune-boosting and prophylactic use of these dietary components. All of these "-biotics" are designed to modulate the gut microbiota in a way that improves health and reduces the need to gulp antibiotics indiscriminately and thus indirect approach to fight potential bacterial threats.

Present strategies aim to minimize the use of antibiotics in the clinical practices as well as in animal farming and focus on endorsement of sustainable alternatives. The growing menace of drug-resistant pathogenic bacteria warrants the need for novel bactericidal agents that are potent, broad-spectrum, non-toxic to human cells and possess a mechanism of action that does not favour development of resistance in the target bacteria. Clinical use of antibiotics can be minimized by boosting up the immune system using prebiotics and phytobiotics that up-regulate the mucosal immunity. Probiotics can be competent alternatives to antibiotics and antibiotic growth promoters (AGP) by resisting pathogenic bacteria and serving health promoting metabolites. Efficient probiotic bacteria can boost up the immune system of farm animals and shall prove to be economically and environmentally sustainable. These prime areas of research have been adequately covered in the proposed book. Conventional therapeutic antibiotics are mostly known to act on definite physiological targets such as cell wall biosynthesis, cell membranes, ribosomes, transcription machinery etc. in pathogenic bacteria. Many bacterial pathogens have evolved mechanisms to evade the action of these conventional antibiotics. Synthetic amphiphiles, nanomaterials are conceivably attractive as bactericidal agents because of their facile synthesis, structural diversity, protease resistance and membrane targeting ability which is counterproductive to development of resistance in the target bacteria. Further, the high surface area to volume ratio of nanomaterials and cationic amphiphiles are suitable to achieve enhanced interactions with the target pathogen. Chapters that discuss these areas of research have enhanced the essence of the proposed book. The book also highlights the phytochemicals with potent antibacterial activities as alternatives to conventional antibiotics. Chemical modification to develop next generation antibiotics with enhanced efficacy as well as to overcome the inherent resistance of the parent antibiotics have also been included. Phage therapy and targeted antibodies are considered as potential alternative approaches to treat bacterial ailments and represent areas of cutting-edge research and have therefore been discussed with sufficient care. So, the proposed book highlights various approaches other than conventional antibiotics in treating bacterial infections and the scientific advancements in this regard will strengthen the 'One Health' approach benefiting human beings, animals and environment as well.

The endeavor in editing the book by the three young editors is commendable. I would like to thank the authors for their dedicated effort in taking up such important field of clinical relevance and describing the importance of alternative approaches to treat bacterial infections. I am sure that the information will be very useful for

Clinicians as well as Microbiologists because this covers all major developments in research on these fields.

The editors and the authors of this book have done a fantastic job.

I wish this effort a grand success.

Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India

Tapas K. Kundu

CSIR-Central Drug Research Institute, Lucknow, India

Preface

Microbial diseases are one of the leading causes of morbidity and mortality worldwide. Conventional antibiotics have undoubtedly rescued the world population from several life threatening infectious diseases since their development in early twentieth century. However, some pathogens have evolved mechanisms to evade the action of most commercially available drugs due to their indiscriminate and injudicious use. This has resulted in the emergence of one of the critical issues in modern biomedicine and healthcare regime that is the surfacing of multidrug resistant (MDR) pathogens. These MDR pathogens may emerge as completely incurable by the available antimicrobials in near future. Innovation gap in novel antibiotic development has severely crippled the choices for treating microbial infections. The challenge of treating diseases caused by drug-resistant pathogens hence demand progress in the development of novel and potent alternative antimicrobial agents. Search for alternative strategies having irrefutable cellular targets and which are counterproductive to resistance development, has been in the prime center of attention of the scientific community. The putative antimicrobial agents under development are with diverse chemical structures and novel mechanisms over the conventional antimicrobial agents rendering the pathogens with minimum scope to develop resistance. Last few decades have witnessed profound research on different areas for the development of alternative antimicrobial agents. These include phytochemicals, novel chemically synthesized molecules, nanomaterials and probiotic/prebiotic agents as immunity boosters etc. Such multidirectional approaches need to be collated on a unified podium enabling the academicians and upcoming researchers to develop an unbiased knowledge on potent alternative antimicrobial agents to combat microbial infections.

"Alternatives to Antibiotics: Recent Trends and Future Prospects" consists of twenty-two chapters encompassing current research and developments on alternative approaches towards naturally derived compounds, chemically synthesized compounds, nanomaterials and probiotics. Section-II of the book consists of five chapters that deal with naturally derived compounds/agents. Natural products and their derivatives are reliable sources of useful drugs for their varied structure and unique

mode of actions and most importantly for their minor side effects. As novel natural source, marine microorganisms provide numerous bioactive metabolites which are reported as effective and promising sources of new antibiotics or drugs which can also act against MDR strains.

Antimicrobial strategies that rely upon plant-based antimicrobials (PBAs), enzymes-based and antibody-based antibacterial therapeutic approaches along with secondary metabolites from plant endophytes have been discussed in these chapters. Other than natural sources, synthetic organic moieties are also being explored as novel antimicrobial compounds. Chemical synthesis enables researchers to develop target based prospective drug molecules to fight against the ever-changing microorganisms. The potent synthetic pathways are discussed in a chapter. The plant-based products have traditionally been used as natural healing systems. Although, modern scientific approaches are focusing on the active compounds. Bioactive natural compounds as well as the synthetic drug candidates are promising therapeutic agents for human health and disease management and their therapeutic efficacy can be enhanced if their bioavailability is raised to the optimum level and/or delivered to the target cells/tissue involving nanocarriers. The membrane targeting bactericidal agents are also emerging as potent antimicrobials since developing resistance against them demands extensive restoration of membrane compounds, which is conceivably formidable challenge for the bacteria. In this regard, membrane-targeting nanoscale materials, amphiphiles, antimicrobial peptides bear special merit. Two chapters discuss the potential of cationic amphiphiles as promising antimicrobial entities and amphiphilic nanocarriers as delivery vehicles. Another chapter discusses the design, synthesis and antimicrobial applications of Metal-Organic Frameworks (MOFs). Thus, amphiphiles of this new genre has enough potential to deliver several antibacterial molecules in years to come. The emergence of nanoscience and technology in recent years offers great promise in therapeutics. Nanomaterials are emerging as novel class of antimicrobial agents to overcome the challenges faced by the conventional antimicrobials. The use of nanomaterials as bactericidal agents represents is a novel approach in antibacterial therapeutics. Three chapters of this book cover the recent development, antimicrobial prospects of biogenic metal or metalloids nanoparticles, bactericidal QDs and MoS_2 based antibacterial nanocomposites.

Devastating pandemic caused by the severe acute respiratory syndrome-causing corona virus-2 (SARS CoV-2) virus has once again taught us that "Prevention is better than cure". The overburden of using xenobiotic drugs can be drastically reduced by boosting our own immune system and fighting the disease causing microbes in association with the helpful bacteria and their metabolites. Three chapters of the book uncover the probiotic/prebiotic/antibacterial peptide compounds as novel antimicrobial approaches and disorder-management therapies. All of these "-biotics" are designed to modulate the gut microbiota in a way that improves health and reduces the need to gulp antibiotics indiscriminately and thus indirectly assist to fight potential bacterial threats. A new-age approach to combat microbes, antimicrobial photodynamic therapy (aPDT) is discussed in a chapter. PDT uses a nontoxic and light-sensitive dye, photosensitizer (PS) in combination

with nontoxic visible light of the appropriate wavelength to excite the PS and oxygen that can selectively control bacterial infections by generation of highly cytotoxic reactive oxygen species (ROS).

The most challenging resistance phenomena of the recent years compelling the medical professionals are diseases caused by protozoans like *Plasmodium*, fungi like *Candida* sp., MDR bacteria like *Mycobacterium* and even the viruses which are evolving as mutated pathogens and warrant novel strategies to treat these deadly diseases. This book incorporates specific chapters that details the history of drug development against Malaria and postulates novel therapeutic approaches towards antimalarial drug. Emerging novel drugs effective against MDR-TB is discussed in a separate chapter. Recently, the world is facing a threatening challenge by the emerging novel strains of viruses including the present SARS-CoV2. Frequent mutations of the viral strains and growing resistance to the available antiviral drugs warrants the discovery of new drug targets and novel strategies to mitigate the deadly viral pandemic. Viruses use host genes for their proliferation. So, host factors comodulate their functions and thus also could impact viral pathogenesis. This aspect of antiviral drug development is in its early phase. However, this field is believed to have immense potential as antiviral drug targeting. A chapter discusses this outlook regarding the host proteins' implications in viral biology and how they could be exploited for treating viral diseases. So, this book will provide significantly expanded overview and updated research to a broader context regarding development of alternative approaches against microbial infections. We believe that the book will cater the professionals as well as learners in the academia, industry and health services who are aiming to learn the most significant approaches towards alternatives to existing antimicrobial therapy.

Darjeeling, West Bengal, India — Tilak Saha
Darjeeling, West Bengal, India — Manab Deb Adhikari
Jalpaiguri, West Bengal, India — Bipransh Kumar Tiwary

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Immunology and Microbiology Laboratory, Department of Zoology, University of North Bengal, Darjeeling, West Bengal, India — Tilak Saha

Department of Biotechnology, University of North Bengal, Darjeeling, West Bengal, India — Manab Deb Adhikari

Department of Microbiology, North Bengal St. Xavier's College, Rajganj, Jalpaiguri, West Bengal, India — Bipransh Kumar Tiwary

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Editors and Contributors

About the Editors

Tilak Saha is an Assistant Professor (Senior) in the Department of Zoology, University of North Bengal, Siliguri, West Bengal, India. He completed Post-Graduation in Zoology and Ph.D. in Biotechnology from the University of North Bengal. His main areas of research are medical microbiology, Immune system and host microbe interaction. He has published research papers in scientific journals such as Scientific Reports, International Journal of Systemic and Evolutionary Microbiology (Microbiology Society), New Journal of Chemistry, ACS Physical Chemistry Au, Current Science etc. He also has edited a book entitled "Microbes, Environment and Human Welfare" ISBN- 978-1-53617-945-3, NOVA Science Publisher, USA.

Manab Deb Adhikari completed Ph.D. in Biotechnology from IIT-Guwahati, India. He worked as Research Professor in the Department of Chemical and Biological Engineering, Korea University, Seoul, South Korea during 2014–2018. He is currently working as Assistant Professor at the Department of Biotechnology, University of North Bengal, Siliguri, India. His current research interest includes Nanobiotechnology, Antibacterials, Drug Delivery, Antimicrobial Peptides, Probiotics and Prebiotics. He has published research works in Chemical Communications, Advanced Healthcare Materials, Biomacromolecules, Journal of

Materials Chemistry B, Inorganic Chemistry, etc. He is a permanent member of Society for Biotechnologists (INDIA) and Association of Microbiologists of India (AMI).

Bipransh Kumar Tiwary is Assistant Professor and Head in the Department of Microbiology, North Bengal St. Xavier's College, Rajganj, Jalpaiguri, West Bengal, India. He received M.Sc. in Microbiology and Ph.D. in Biotechnology from University of North Bengal, Siliguri, India. He is a lifetime member of Association of Microbiologists of India (AMI). His areas of research are Medical Microbiology, CADD, Antimicrobials and Quorum Sensing Inhibitors. He has published research works in Plos one, RSC Advances, BMC complementary and Alternative Medicine and Letters in Applied Microbiology. He has edited a book entitled "Microbes, Environment and Human Welfare" ISBN-9781536179453, NOVA Science Publisher, USA.

Contributors

Yash Acharya Antimicrobial Research Laboratory, New Chemistry Unit and School of Advanced Materials, Bengaluru, India

Manab Deb Adhikari Department of Biotechnology, University of North Bengal, Siliguri, India

Satadal Adhikary Post Graduate Department of Zoology, ABN Seal College, Cooch Behar, India

Shadab Ahmed Institute of Bioinformatics and Biotechnology (Merged to Department of Biotechnology), Savitribai Phule Pune University, Pune, Maharashtra, India

Farwa Arshad Department of Chemistry, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Punuri Jayasekhar Babu Biomaterials and Bioengineering Research Laboratory, Department of Biotechnology, Pachhunga University College Campus, Mizoram University (A Central University), Aizawl, Mizoram, India

Arghya Basu Amity School of Applied Sciences, Navi Mumbai, Maharashtra, India

Basudeb Basu Department of Chemistry, Cotton University, Guwahati, India

Suchandra Bhattacharya Department of Chemistry, ABN Seal College, Cooch Behar, India

Nilotpal Borah Surface Chemistry, Oil and Natural Gas Corporation Ltd., Sivasagar, Assam, India

Bidya Leima Chanu Department of Vegetable Science, College of Horticulture and Forestry, Central Agricultural University, Pasighat, Arunachal Pradesh, India

Abhrajyoti Roy Chowdhury Department of Biotechnology, University of North Bengal, Siliguri, India

Koyeli Das Department of Chemistry, Indian Institute of Technology Madras, Chennai, India

Susmita Das Department of Chemistry, Amity Institute of Applied Sciences, Amity University Kolkata, Kolkata, India

Sujoy Deb CSIR-Centre for Cellular and Molecular Biology, Hyderabad, India

Poulomi Dey Kusuma School of Biological Sciences, Indian Institute of Technology Delhi, New Delhi, India

Sandeep Kumar Dey School of Chemical Sciences, Goa University, Taleigao, Goa, India

Suhrid Ranjan Dutta Department of Microbiology, Midnapore College (Autonomous), West Bengal, India

Austin Fernandez Institute of Bioinformatics and Biotechnology (Merged to Department of Biotechnology), Savitribai Phule Pune University, Pune, Maharashtra, India

Ramesh L. Gardas Department of Chemistry, Indian Institute of Technology Madras, Chennai, India

Amlan Jyoti Ghosh Immunology and Microbiology Laboratory, Department of Zoology, University of North Bengal, Darjeeling, West Bengal, India

Supriyo Ghosh Immunology and Microbiology Laboratory, Department of Zoology, University of North Bengal, Darjeeling, West Bengal, India

Abhijit Gogoi Toxicology Division, Directorate of Forensic Science, Guwahati, Assam, India

Satendra Singh Gurjar Department of Biosciences and Bioengineering, Indian Institute of Technology, Guwahati, Assam, India

Jayanta Haldar Antimicrobial Research Laboratory, New Chemistry Unit and School of Advanced Materials, Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru, India

Nazmul Haque Department of Oral Biology and Biomedical Sciences, MAHSA University, Selangor, Malaysia and TotiCell Limited, Dhanmondi, Dhaka, Bangladesh

Punabati Heisnam Department of Natural Resource Management, College of Horticulture and Forestry, Central Agricultural University, Pasighat, Arunachal Pradesh, India

Rejuan Islam Immunology and Microbiology Laboratory, Department of Zoology, University of North Bengal, Siliguri, West Bengal, India

Abhishek Jaiswal Department of Microbiology, Viral Research and Diagnostic Laboratory Midnapore Medical College and Hospital, Midnapore, West Bengal, India

Suman Kumaria Plant Biotechnology Laboratory, Department of Botany, North-Eastern Hill University, Shillong, Meghalaya, India

Chanchal Mandal Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna, Bangladesh

Keshab Chandra Mondal Department of Microbiology, Vidyasagar University, Midnapore, West Bengal, India

Sandipan Mukherjee Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Mumbai, India

Vanita Mulay Institute of Bioinformatics and Biotechnology (Merged to Department of Biotechnology), Savitribai Phule Pune University, Pune, Maharashtra, India

Swagata Nag Plant Biotechnology Laboratory, Department of Botany, North-Eastern Hill University, Shillong, Meghalaya, India
Siliguri Paramedical College, Jalpaiguri, India

Rina Ningthoujam Department of Vegetable Science, College of Horticulture and Forestry, Central Agricultural University, Pasighat, Arunachal Pradesh, India

Anirban Pandey Department of Zoology, A B N Seal College, West Bengal, India

Priyanka Pisalwar Institute of Bioinformatics and Biotechnology (Merged to Department of Biotechnology), Savitribai Phule Pune University, Pune, Maharashtra, India

Kiran Pradhan Department of Chemistry, University of North Bengal, Siliguri, West Bengal, India

Amit Ranjan Center for Infection and Immunity, Mailman School of Public Health, Columbia University, New York, NY, USA

Tilak Saha Immunology and Microbiology Laboratory, Department of Zoology, University of North Bengal, Darjeeling, West Bengal, India

Jiban Saikia Department of Chemistry, Dibrugarh University, Dibrugarh, Assam, India

Sagar Sarkar Immunology and Microbiology Laboratory, Department of Zoology, University of North Bengal, Darjeeling, West Bengal, India
Department of Zoology, Siliguri College, Darjeeling, West Bengal, India

Dhanashri Satav Institute of Bioinformatics and Biotechnology (Merged to Department of Biotechnology), Savitribai Phule Pune University, Pune, Maharashtra, India

Nutan Singh Plant Biotechnology Laboratory, Department of Botany North-Eastern Hill University, Shillong, Meghalaya, India

Vickramjeet Singh Department of Chemistry, Dr B R Ambedkar National Institute of Technology, Jalandhar, Punjab, India

Yengkhom Disco Singh Biomaterials and Bioprocessing Research Laboratory, Department of Post-Harvest Technology, College of Horticulture and Forestry, Central Agricultural University, Pasighat, Arunachal Pradesh, India

Md Palashuddin Sk Department of Chemistry, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

Divya Sriram CSIR-Centre for Cellular and Molecular Biology, Hyderabad, India

Fahim Rejanur Tasin Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna, Bangladesh

Akriti Tirkey Biomaterials and Bioengineering Research Laboratory, Department of Biotechnology, Pachhunga University College Campus, Mizoram University (A Central University), Aizawl, Mizoram, India

Bipransh Kumar Tiwary Department of Microbiology, North Bengal St. Xavier College, Rajganj, Jalpaiguri, West Bengal, India

Part I
Introductory Chapter

Chapter 1
Quest for Alternatives to Antibiotics: An Urgent Need of the Twenty-First Century

Manab Deb Adhikari, Tilak Saha, and Bipransh Kumar Tiwary

Abstract Antibiotics have saved uncountable lives from many infectious diseases caused by bacteria. But their inappropriate and indiscriminate use has given enough clues to some pathogenic bacteria to evolve as resistant microbial populations. Hence, there is a pressing need to develop new and innovative antibacterial agents with diverse chemical structures and mechanisms. Novel approaches ranging from naturally sourced to chemically synthesised compounds, nanomaterials and even symbiotic bacteria or their beneficial by-products are being investigated as potential alternatives to curb the menace of resistance development. Naturally sourced alternatives like phytochemicals, enzyme-based composites, antibodies, phage therapy, use of vaccine etc. have potential to reduce the load of antibiotic treatment. Chemical modification of antibiotics like semisynthetic modifications of vancomycin also has given the alternative routes to bypass resistance. Additionally, metal complexes, cationic amphiphiles and organic frameworks are also promising antibacterial entities which are synthetically designed in the chemical laboratories as alternative approaches. Polymeric biocides, dendrimeric entities and carbon-based and metal-based nanoparticles (NPs) also have been developed with potential antibacterial applications. Interestingly, the use of probiotics, prebiotics and synbiotics is being explored as alternatives to antibiotics because of their implications on enhancing mucosal immunity, thereby lessening the need of using antibiotics. There is ample scope to review the recent developments in these areas of research and postulate the future directives to overcome the crisis of drug resistance and implement effective antibacterial treatment. This chapter aims to present a cumulative introductory note

Manab Deb Adhikari and Tilak Saha contributed equally with all other contributors.

M. D. Adhikari
Department of Zoology, University of North Bengal, Darjeeling, India

Department of Biotechnology, University of North Bengal, Darjeeling, India

T. Saha
Department of Zoology, University of North Bengal, Darjeeling, India

B. K. Tiwary (✉)
Department of Microbiology, North Bengal St. Xavier College, Rajganj, Jalpaiguri, West Bengal, India

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for these promising alternative approaches and encourage the required scientific temperament to usher in the next-generation therapeutics.

Keywords Antimicrobial resistance · Antibiotic · Quorum sensing · Phytochemicals · CRISPR-Cas · Probiotics · Prebiotics · Synbiotics · Nanoparticles · Antimicrobial peptide · Antibodies · Phage therapy · Antivirulence · Heterocycles · Nanocomposites · Vaccine · Pathogenic bacteria · Antibacterial · Multidrug resistant · Antibiotic resistance genes

Abbreviations

ARGs	Antibiotic resistance genes
MDR	Multidrug resistant
AMR	Antimicrobial resistance
WHO	World Health Organisation
TB	Tuberculosis
CRE	Carbapenem-resistant Enterobacteriaceae
FLs	Flavonoids
EOs	Essential oils
MICs	Minimum inhibitory concentrations
AgNPs	Silver nanoparticles
QS	Quorum sensing
QQ	Quorum quenching
QSIs	QS inhibitors
AMPs	Antimicrobial peptides
SAR	Structure–activity relationship
CNMs	Carbon-based nanomaterials
ROS	Reactive oxygen species
PDT	Photodynamic therapy
FDA	Food and Drug Administration
AAD	Antibiotic-associated diarrhoea
FOS	Fructo-oligosaccharides
GOS	Galacto-oligosaccharides
XOS	Xylo-oligosaccharides
WGS	Whole genome sequencing
AV	Antivirulence

1.1 Introduction

Bacteria-caused infectious diseases are serious and growing clinical issues in recent years and causing major economic burden and public health menace (de Kraker et al. 2016). The emergence of antibiotic resistance to almost all currently available antibiotics and the lack of progress in the development of novel antibacterial drugs are contributing to a global health crisis (Solomon and Oliver 2014).

Antibiotic resistance problem is augmented by the evolution and transfer of genes within bacterial population that confer resistance to antibiotics (San Millan 2018; Peterson and Kaur 2018; Munita and Arias 2016). Acquisition of antibiotic resistance genes (ARGs) by pathogens leads to treatment failure, increasing morbidity and mortality rates and simultaneously healthcare costs also. Resistance development in bacteria is a continuous phenomenon regularly making the antibiotics of even the last resort to become ineffective.

The complex factors that have led to the antibiotic resistance in bacteria are revealed when potential solutions to reduce or prevent this problem are being examined. First of all, more than 80 years of antibiotic use have created a selection pressure on the bacteria to evolve certain mobile ARGs particularly in pathogenic bacteria. These resistant bacteria in the environmental "hot spots" spread the resistance phenomenon horizontally to other related species. Therefore, strategies have to be implemented to cut the routes of dissemination of ARGs from bacteria (Prestinaci et al. 2015; Larsson and Flach 2021). Second, horizontal transfer of ARGs can be stimulated by antibiotics themselves. Hence, careful use of antibiotics is the need of time to slow down the spread of ARGs among bacteria. Furthermore, ecological food chain has been identified as another major factor in the development of multidrug-resistant (MDR) bacteria (Founou et al. 2016; Rousham et al. 2018; Ma et al. 2021), which then affects both commensal and pathogenic bacteria in humans via dietary interaction. Aside from these, treating persistent infections, which are frequently caused by internal germs or extracellular microorganisms capable of forming biofilms, is a significant issue in antimicrobial therapy (Lahiri et al. 2021; Bowler et al. 2020; Uruén et al. 2021). Due to the limited availability of drugs inside the cells, intracellular infections are more difficult to cure than extracellular infections. Some microorganism's intracellular position protects them both from the host's defence mechanisms and from the antibiotics which are unable to infiltrate the cells effectively.

Recently, propagation of multidrug-resistant (MDR) bacteria has been accelerated during the COVID-19 pandemic due to the overuse and misuse of existing antimicrobial drugs (Pelfrene et al. 2021; Manohar et al. 2020), in addition to other driving factors of antimicrobial resistance (AMR) (Fig. 1.1). So, there is a huge need to discover novel strategies and identify new antimicrobial drugs in order to maintain the existing capability to treat infectious diseases, especially those caused by MDR pathogens. The use of alternatives to antibiotics aims to promote health and decrease the overuse of antibiotics, thereby decreasing selective pressure for the emergence and spread of ARGs. The current rate of drug discovery is not fast enough to combat the serious threat of drug-resistant diseases. To limit the rate of resistance, researchers must look at emerging bacteria, resistance mechanisms and antimicrobial drugs. Multidisciplinary approaches are required in the healthcare, environmental and agricultural sectors.

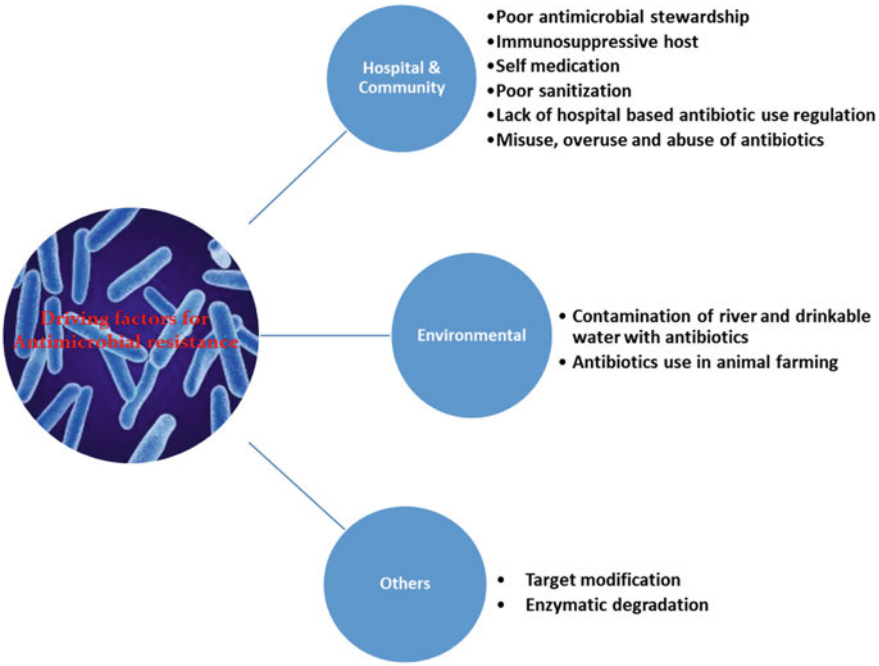

Fig. 1.1 Possible driving factors for antimicrobial resistance

1.2 Historical Perspectives of Antibiotic Resistance

Antibiotics, which have previously revolutionised medical science, are now being seriously encountered due to the emergence of ARGs among pathogenic microorganisms. MDR pathogens have emerged as global health concern, affecting detection of infection, treatment and prevention. Such high priority pathogens according to the World Health Organisation (WHO) are enlisted in Table 1.1 (World Health Organization 2017). The scientific community has found three instances of antibiotic misuse that have been linked to antibiotic resistance. The first example of misuse is the overuse of antibiotics to treat viral illnesses. Antibiotics are only effective against bacteria and have no effect on viral infections; however, they help in controlling the secondary infections. Misdiagnosis is the second type of antibiotic usage that contributes to antibiotic resistance. The third scenario of antibiotic misuse that leads to antibiotic resistance is the inappropriate usage of antibiotics. Inadequate use relates to dosages, durations and frequencies that are incorrect. This could be linked to erroneous prescriptions or poor patient care. Drug resistance in pathogenic bacteria is caused by a number of molecular mechanisms, including enzyme-catalysed target change, drug impermeability, drug inactivation, the presence of efflux pump and others (Lewis 2013).

Table 1.1 List of high priority pathogens as reported by World Health Organization (2017)

Priority	Organism	Resistance
Critical	*Acinetobacter baumannii*	Carbapenem resistant
	Pseudomonas aeruginosa	Carbapenem resistant
	Enterobacteriaceae	Carbapenem resistant, ESBL producing
High	*Enterococcus faecium*	Vancomycin resistant
	Staphylococcus aureus	Methicillin resistant, vancomycin intermediate and resistant
	Helicobacter pylori	Clarithromycin resistant
	Campylobacter spp.	Fluoroquinolone resistant
	Salmonellae	Fluoroquinolone resistant
	Neisseria gonorrhoeae	Cephalosporin resistant, fluoroquinolone resistant
Medium	*Streptococcus pneumoniae*	Penicillin non-susceptible
	Haemophilus influenzae	Ampicillin resistant
	Shigella spp.	Fluoroquinolone resistant

Infections due to the resistant bacteria have a twofold higher chance of having detrimental consequences than infections caused by susceptible bacteria. Persister cells are becoming more well recognised as a key contributor to the recurrence of chronic infectious diseases, and they may also play a role in the development of antibiotic resistance. The persistence phenomena were initially described in the 1940s, although it remained poorly understood for decades. Only recently has a flurry of groundbreaking research begun to shed light on persister physiology and the molecular and genetic basis of persister development. The discovery of penicillin-resistant *Staphylococcus* in the 1940s sparked the first concern about antimicrobial resistance (Ventola 2015). In hospitals and other healthcare settings, *S. aureus* is the most common resistant bacterium. When sulphonamides were used, this bacterium quickly developed resistance to them. Penicillin was initially effective to this bacterium; however, resistant strains that produce penicillinase boomed in the 1950s. Soon, penicillinase-stable methicillin was marketed in 1960. However, as early as the next year, in 1961, methicillin-resistant *S. aureus* (MRSA) was identified in the United Kingdom (Jevons 1961) and in the United States, in 1968 (Sengupta et al. 2013; Grema et al. 2015). The bacterium *Pseudomonas aeruginosa* is the most common cause of cystic fibrosis (CF) lung infections and resistant to antibiotic treatment, resulting in deterioration of pulmonary function and, eventually, death in CF patients (Pang et al. 2019). The most prevalent cause of death from an infectious pathogen is *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB). In 2015, around 1.5 million individuals died from tuberculosis (TB), and the rise of multidrug-resistant TB and extensively drug-resistant TB strains has made TB eradication an enormously difficult task (Kerantzas and Jacobs Jr 2017). Carbapenem-resistant Enterobacteriaceae (CRE) is a global public health threat that continues to grow (Meletis 2016).

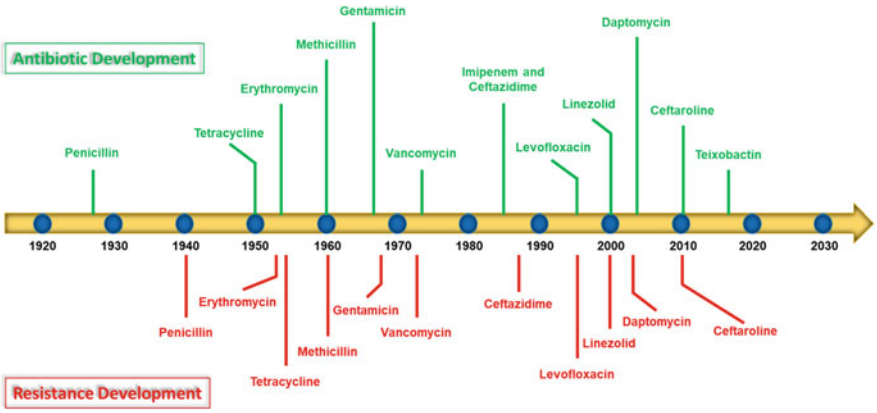

Fig. 1.2 Timeline of antibiotic development and the emergence of antibiotic resistance

According to the WHO, several bacterial pathogens have evolved high levels of resistance over the world. The WHO recently updated the *Enterococcus faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *Enterobacter* spp. (ESKAPE) list and it includes carbapenem-resistant *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, carbapenem-resistant and third-generation cephalosporin-resistant Enterobacteriaceae, clarithromycin-resistant *Helicobacter pylori* and fluoroquinolone-resistant *Campylobacter* spp. (Tacconelli et al. 2018). A timeline depicting the discovery of antibiotics and evolution of antibiotic resistance is shown in Fig. 1.2.

1.3 Alternative Strategies to Overcome Antibiotic Resistance

AMR is spreading across the globe, threatening to undermine the incredible progress made in human health. To deal with this health crisis, multi-arm approach such as phytochemicals, antivirulence (AV), antibodies, phage therapy, polymeric biocides, dendrimeric entities, nanoparticles and probiotics are all promising alternatives. The different approaches of the alternative therapeutic strategies to treat bacterial infection are shown in Fig. 1.3.

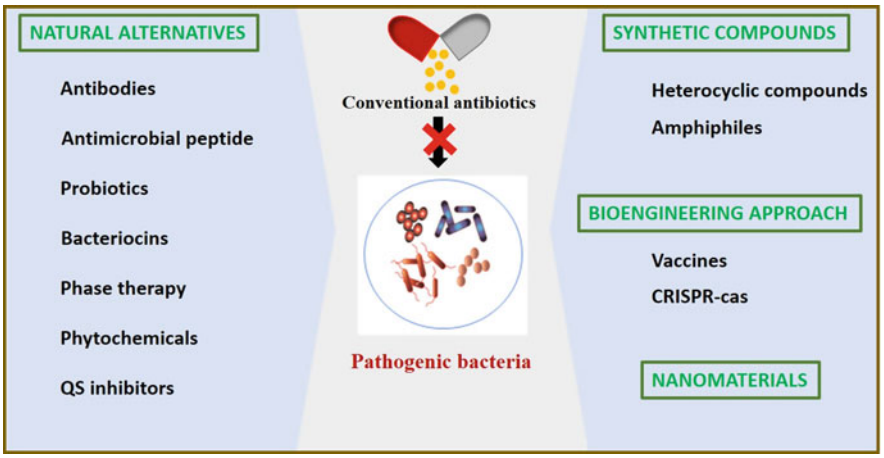

Fig. 1.3 Various alternatives that can be used as replacement of conventional antibiotics

1.3.1 Natural Alternative Approaches

1.3.1.1 Phytochemicals

Since the earliest times, natural products have been used as effective remedies against pathogenic bacteria. The plants have been known to be powerful therapeutics against bacterial infections due to their chemical diversity and structural complexity. Over the past decade, much attention has been placed on the study of phytochemicals for their antibacterial activity, especially against multidrug-resistant bacteria (Barbieri et al. 2017).

Plant-derived compounds are mostly secondary metabolites, maximum of which are phenols or their oxygen-substituted derivatives. These secondary metabolites possess various benefits, including antimicrobial properties against pathogenic microbes (Patra 2012; Khameneh et al. 2019). Major groups of compounds that are responsible for antimicrobial activity from plants include phenolics, phenolic acids, quinones, saponins, flavonoids (FLs), tannins, coumarins, terpenoids and alkaloids (Hochma et al. 2021). The essential oils (EOs) of *Carum carvi*, *Carum sativum* and *Cuminum cyminum* have been shown to exhibit antibacterial properties against *Escherichia coli* and *S. aureus* (Khalil et al. 2018). Essential oils (EOs) from plants such as oregano, thyme and tea tree had a remarkable antibacterial activity against drug-resistant strains of *P. aeruginosa*, *E. coli* and *Enterobacter cloacae* with remarkably low minimum inhibitory concentrations (MICs) (Chouhan et al. 2017; Puvača et al. 2021). EOs from *Coriandrum sativum* reduced MDR uropathogenic *E. coli* via disrupting cell membrane permeability (Scazzocchio et al. 2017). Alibi et al. (2020) used EOs from *Cinnamomum verum*, *Thymus vulgaris* and *Eugenia caryophyllata* to successfully limit the development of 105 MDR clinical isolates through substantial anti-biofilm and anti-quorum sensing

(QS) capabilities. Recently, Azizi-Lalabadi et al. (2020) synthesised silver nanoparticles (AgNPs) using the extract of the influorescence of a medicinal plant (*Tridax procumbens*) and studied the antibacterial activity against multidrug-resistant uropathogens such as *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and Gram-positive *Staphylococcus saprophyticus* isolated from urinary-tract-infected persons that are resistant to more than ten antibiotics. In another study, the in vitro antibacterial and anti-biofilm activity of extract from various Indian medicinal plants was studied against MDR clinical isolates, resistant to five classes of antibiotics and biofilm-forming *Staphylococcus* (Panda et al. 2020). Many phytochemicals have been reported to act as resistance-modifying agents when combined with existing antibacterial agents, and the right combinations may be able to retain the efficacy of life-saving antimicrobials.

1.3.1.2 Bacteriophage

Phages are potential antibacterial therapeutic agents against the multidrug-resistant pathogens (Burrowes et al. 2011). Phages were used as antibiotics to treat bacterial infections (commonly termed phage therapy) even before the discovery of penicillin. In the early 1900s, Frederick Twort first mentioned (Twort 1915) and d'Hérelle (1917) used phage suspensions to treat infections such as dysentery caused by *Shigella dysenteriae*, which at that time had no other consistently effective treatment. His success proved as pioneering for the phage therapy for bacterial infections in human (Abedon et al. 2011). Conventionally, phage therapy commonly involves lytic phages, which are phages that infect and lyse bacteria at the site of infection, thus resulting in the death of the bacterial cells (Chanishvili 2012). Compared to other therapeutic agents, a very small dose contains large populations of phages which can be administered easily, and the bacteria causing the infection will replicate and produce more of the phages in cells. Further, phages are highly specific for bacteria, so there is no serious side effect on mammalian cells. However, specificity is also their major limitation in their potential. One phage will naturally infect only a limited number of bacterial strains, so treatment against one species requires multiple different phages (Principi et al. 2019).

In the last 15 years, phage therapy has been under development in laboratories and hospitals. Phage therapy has been successfully used for the treatment of infectious diseases such as urinary tract infections, osteomyelitis, septicaemia, wound infections, skin infection and middle ear infections (Kutter et al. 2012). Phage therapy is rapidly growing and also reflected in the cases of multiple clinical trials (Furfaro et al. 2018). The Eliava Institute reported the use of phage in preclinical and clinical treatment of common bacterial pathogens such as E*nterococcus* spp., *S. aureus*, *E. coli*, *Streptococcus* spp., *Salmonella* spp., *Proteus* spp., *Sh. dysenteriae* and *P. aeruginosa* (Kutateladze and Adamia 2008). The patients with diabetic foot ulcers infected by *S. aureus* strains are treated by *Staphylococcus* bacteriophage Sb-1 (Jault et al. 2019). In England, a cystic fibrosis patient infected with drug-resistant *Mycobacterium abscessus* was reported to cure by the use of natural and

synthetically modified phages (Dedrick et al. 2019). In the United States, a patient infected by a toti-resistant strain of *Acinetobacter baumannii* was cured by phages at the University of California San Diego (Strathdee and Patterson 2019).

Phages have been studied for their potential as alternatives to antibiotic for decades, but no treatment has been approved by the Food and Drug Administration (FDA) till date. However, researchers are searching for a novel approach that could confront this challenge and efficiently open this strategy for exploiting. In the face of the current issue of increasing reports of antimicrobial resistance and limited new antibiotic discoveries and development, there is a need to establish phage therapy as a reliable antibiotic alternative. However, that potential remains to be validated by significant proof-of-efficacy results from well-designed clinical trials.

1.3.1.3 Antivirulence Therapy

Pathogenic bacteria produce both extracellular and cell surface molecules, known as virulence factors, regulated by quorum sensing (QS) to establish the infection in host. The interference or inhibition of expression of virulence factors, which inhibits with the interaction between the bacterium and its host, is considered another potential approach for controlling bacterial infection. Since the strategy does not directly target bacterial growth, the probability to develop resistance is minimised (Rémy et al. 2018).

Bacterial behaviour such as the synthesis of virulence factors relies on QS, and at high population density, pathogens can switch their expressions of genes to an invasive phenotype, including genes related to biofilm formation, antibiotic tolerance and virulence determinants and thus cause disease in hosts. So QS inhibitors (QSIs) are reported to inhibit cell-to-cell communications and consequently interfere bacterial colonisation and control an established infection. This antimicrobial control relies on reducing the burden of virulence rather than killing the bacteria (Jiang et al. 2019). The rapid emergence of bacteria resistant to antibiotics has pushed research towards the action of these substances against the QS to limit this resistance. It is also suggested that the development or searching of anti-QS molecules could be a potential approach for fighting infections *via* biofilms (Musk Jr and Hergenrother 2006).

The natural phytomolecules are capable of inhibiting the QS processes linked to human pathogens (Damte et al. 2013), which is a particularly attractive property. For these reasons, researchers are increasingly focusing their studies on medicinal herbal products to identify novel therapeutic and antipathogenic agents that could act as non-toxic QS inhibitors, thus controlling infections without encouraging the development of bacterial resistance (Hentzer and Givskov 2003). On the other hand, phytochemicals may represent the richest available reservoir of new therapeutic products (Kumar et al. 2006). Medicinal substances are present in the whole plants or in one of their particular organs (leaves, flowers, roots and seeds). The most common plants in discussed area are *Glycyrrhiza glabra*, *Terminalia chebula*, *Fragaria nubicola*, *Astilbe rivularis*, *Osbeckia nepalensis*, *Piper bredemeyeri*,

Syzygium aromaticum, *Bauhinia acuruana*, *Pityrocarpa moniliformis*, *Commiphora leptophloeos*, *Cocos nucifera, Terminalia catappa* and *Pisum sativum* represent medicinal plants that have been used as anti-QS agents with type of extracts and major compounds (Bouyahya et al. 2017; Tiwary et al. 2017; Ghosh et al. 2014).

The disruption of QS signaling, also termed quorum quenching (QQ), and quorum quenching enzymes are also capable to interfere or inhibit the QS-related phenotypes and thus control bacterial infections. The enzymes are observed to degrade AHL molecules, which may be of three distinct groups of enzymes: lactonases hydrolyse the HSL ring, acylase hydrolyses amide bond of AHL and oxidoreductase modifies the activity of AHL (Rehman and Leiknes 2018). In a study, the hydrogel-formulated recombinant Ahl-1 lactonase was found to control the infection of MDR *P. aeruginosa* infected burn using a murine model (Sakr et al. 2021). In another study, the combined treatment of lactonase along with antibiotic efficiently controls the burn wound infection (Gupta et al. 2015).

Various literature published in the last 5 years clearly demonstrates that QS is a possible target to find alternative therapeutic approaches to reduce the rapid emergence of antibiotic resistance. The QS inhibitors (QSIs) have a great potential as antivirulence agents. However, several authors proposed for combined treatment to increase the efficacy of existing drugs, orienting the way for alternative treatment (Cegelski et al. 2008).

1.3.1.4 Antibodies

Antibodies, which are proteins that recognise and neutralise specific pathogen components, are produced by the immune system to battle pathogen invasion. Monoclonal antibodies have been offered as an alternative for the treatment of MDR infections in recent years, owing to their high specificity against the bacterial pathogen, low risk of resistance and capacity to operate synergistically with antibiotics. Kollef and Betthauser (2021) studied the therapeutic efficacy of suvratoxumab, a human monoclonal antibody against the pore-forming toxin of *Staphylococcus aureus* that is found in patients with ventilated support colonised with *S. aureus*. Recently, Domenech et al. (2018) summarised the research work showing the combined effect of vaccine or monoclonal antibodies with antibiotics which could be a viable alternative to combating MDR respiratory infections. In another study, Guachalla et al. (2017) showed high prophylactic efficacy of A1124, a humanised monoclonal IgG targeting the O25b O antigen of *E. coli* ST131-H30. Targeted antibacterial formulations have the potential to improve the efficiency of existing antimicrobials for the treatment of MDR bacterial infections, limiting adverse effects and reducing the likelihood of antibiotic resistance. Ivanova et al. (2020) generated a novel nanosystem loaded with antibacterial oregano essential oil for effective elimination of Gram-positive *Staphylococcus aureus*. They have chemically immobilised *S. aureus* targeting antibodies onto their developed nanosystem for specifically targeting the *S aureus* strain. Antibodies against Staphylococci, *Pseudomonas aeruginosa*, *Bacillus anthracis* and *Clostridium difficile* are in various phases of

Table 1.2 Antibacterial antibodies currently under clinical trials (Streicher 2021; Rios et al. 2016; DiGiandomenico and Sellman 2015)

Antibody name	Pathogen	Target	Clinical status
MEDI4893	*Staphylococcus aureus*	Alpha toxin	Phase IIb
Tosatoxumab (AR-301)	*Staphylococcus aureus*	Alpha toxin	Phase IIa
514G3	*Staphylococcus aureus*	Immunomodulator	Phase II
Pagibaximab	*Staphylococcus aureus*	Lipoteichoic acid	Phase II/III
1C11	*Staphylococcus aureus*	Glucosaminidase subunit of autolysin	Preclinical
Anthim (ETI-204)	*Bacillus anthracis*	Protective antigen	FDA approved
Raxibacumab	*Bacillus anthracis*	Protective antigen	FDA approved
Valortim (MDX-1303)	*Bacillus anthracis*	Protective antigen	Phase I
GS-CDA1	*Clostridium difficile*	*C. difficile* toxin A	Phase II
MDX-388	*Clostridium difficile*	*C. difficile* toxin B	Phase II
Bezlotoxumab	*Clostridium difficile*	*C. difficile* toxin B	FDA approved
ShigamAbs	*Escherichia coli*	Shiga toxin (tx1 and tx2)	Phase II
Urtoxazumab	*Escherichia coli*	Shiga-like toxin II B subunit	Phase 1
	Pseudomonas aeruginosa		
Panobacumab	*Pseudomonas aeruginosa*	O-antigen (serotype O11)	Phase IIa
Anti-*Pseudomonas* IgY	*Pseudomonas aeruginosa*	PsI and PcrV	Phase II
MEDI3902	*Pseudomonas aeruginosa*	Psl and PcrV	Phase II

clinical testing, with several already approved by the FDA (Saylor et al. 2009; Oleksiewicz et al. 2012; Ghosh et al. 2019). The possible scope of antibody therapy for the treatment of bacterial infections is shown in Table 1.2 (Streicher 2021; Rios et al. 2016; DiGiandomenico and Sellman 2015).

1.3.2 Chemical Alternative Approaches

Resistance to antibiotics generated from nature is unavoidable, and it occurs spontaneously as a result of their prevalence in the natural environment (Ribeiro da Cunha et al. 2019). It is now necessary to return to the traditional strategy of drug design, where substrate analogues are inspired by existing natural ligands. As a result, developing a totally synthetic class of compounds may be a preferable

alternative technique for discovering antibacterial drugs. Synthetic antibacterial drugs have only been used in clinical settings a few times so far. This is because over the last few decades, significant work has been focused on semisynthetic approaches, while not enough emphasis has been paid to the creation of entirely synthetic antibacterial medications (Tiwary et al. 2015).

To find new antibacterial medicines, researchers have spent a lot of time screening synthetic chemical libraries, either targeting the proteins required for bacterial life or the entire bacterial cell. A majority of scaffolds used in target-based screening were tuned for human eukaryotic targets, whereas compound libraries for whole-cell screening were produced by nonantibiotics programmes (Fair and Tor 2014). Several ways have been used to widen the chemical space used for antibacterial screens. The diversity-oriented synthesis technique, for example, has been employed to develop promising antibacterial candidates that closely resemble bioactive natural compounds (Galloway et al. 2009). A library of compounds with identified scaffolds was also created using combinatorial chemistry. Furthermore, intriguing lead compounds were discovered via unbiased diversity-oriented synthesis and a subsequent structure–activity relationship (SAR) (Kim et al. 2018).

Heterocyclic compounds have already paved the way for quick collaboration among researchers in organic, pharmacological, analytical and medicinal chemistry. These heterocyclic compounds, particularly those with five members, piqued the scientific community's interest not only because of their natural occurrence but also because of their diverse biological activity. More than 75% of the top two hundred branded medications in the pharmaceutical business feature heterocyclic fragments in their structures (Joule and Mills 2007).

Imidazoles comprising the bisazetidinones were found to have action against the Gram-negative ESKAPE pathogen *K. pneumoniae* (Himo et al. 2005) that was nearly similar to ampicillin (Pagadala et al. 2015), an antibiotic used to prevent and treat a variety of bacterial illnesses. The activity of 1,8-naphthalimide-derived imidazole 302b against *E. coli* and *P. aeruginosa* was found to be comparable to that of chloramphenicol (Damu et al. 2013). Antibiotic amoxicillin was shown to be significantly less effective against Gram-positive *B. subtilis* than 1,10-[(tosylazanediyl)bis(ethane-2,1-diyl)]bis(3-cyanomethyl-1H-imidazol-3-ium) chloride (Al-Mohammed et al. 2015). Quinoline-based hydroxyimidazolium hybrid (Meth-Cohn 1993) was found to have potent anti-staphylococcal activity and activity against *M. tuberculosis* H37Rv. The activity of C-12 substituted berberine–imidazole hybrid against the Gram-negative bacterium *Salmonella typhi* was shown to be higher than that of the reference medications berberine, chloramphenicol and norfloxacin (Wen et al. 2016). GI262866A and GI261520A are quinazoline-based lead compounds that selectively inhibit PhoQ histidine kinase activity and hence down-regulate PhoP-activated genes in *Sa. typhimurium*. The recognition of metal compounds' potential for antibacterial applications encourages more research into their development (Carabajal et al. 2019).

Morinidazole [(R)-1-(2-methyl-5-nitro1H-imidazol-1-yl)-3-(morpholin-4-yl) propan-2-ol], a 5-nitroimidazole antimicrobial drug developed by Jiangsu Hansoh Pharmaceutical, was approved in China in 2019 for the treatment of anaerobic

bacterial infections, including appendicitis and pelvic inflammatory disease caused by anaerobic bacteria (Cao et al. 2017).

Cationic amphiphiles have attracted scientific and commercial interest due to their ease of synthesis, ability to break cell membranes and ability to modulate their activity by changing their hydrophobicity and mimic the action of antimicrobial peptides (AMPs). Interestingly, a majority of these compounds have been proven to exhibit strong antibacterial action against drug-resistant microorganisms. Their antibacterial activity is strongly determined by the cationic charge and the molecule's hydrophobicity, which is a common motif in their mechanism of action. Cationic amphiphiles are thought to promote significant electrostatic interactions with negatively charged bacterial cell surfaces, resulting in a multivalent effect on target bacterial membranes (Uday et al. 2014; Vudumula et al. 2012; Goswami et al. 2013). Traditional medicinal drugs and synthetic amphiphiles could be used in combination therapy because the amphiphile has a multivalent targeting effect via cell surface contacts followed by membrane rupture.

In the not-too-distant future, current considerable research efforts in the field of synthetic chemicals may allow for the identification and production of novel antibacterial medicines, which will be tested in clinical trials and subsequently utilised to treat patients. They'll pave the way for major advancements in this field.

1.3.3 Nanomaterial-Based Alternative Approaches

Nanoscience and technology have matured, resulting in significant advances in biology and medicine. As a growing and all-encompassing discipline of research, it has shown the ability to create a wide range of materials with structural differences between atoms and macroscopic materials, as well as at least one dimension in the nano range. The utilisation of nanoscale materials as antibacterial agents constitutes a paradigm shift in antibacterial medicine. Nanomaterials having a high surface-area-to-volume ratio can achieve improved interactions with the target pathogen and thus be investigated for the development of effective bactericidal drugs. They not only use methods of action that are fundamentally different from those reported for classical antibiotics, demonstrating efficacy against bacteria that have already evolved resistance, but they also target several biomolecules, posing a threat to the formation of resistant strains. Because of their higher membrane permeability, ability to operate as efflux pump inhibitors and potential for various antibacterial activities, nanomaterials have been discovered to be less likely to induce bacterial resistance than standard antibiotics (Hajipour et al. 2012; Rudramurthy et al. 2016; Slavin et al. 2017). Nanoparticles (NPs) are useful as antimicrobial cargo, and they also give key pharmacological benefits such as enhanced drug solubility and half-life, prolonged and stimuli-responsive drug release, site-targeted delivery and combination therapy (Spirescu et al. 2021; Colilla and Vallet-Regí 2020; Naskar and Kim 2019).

Many nanostructures, such as liposomes, nanoparticles and dendrimers, have proved their ability to improve antibiotic treatment efficacy and combat infectious

illnesses (Lee et al. 2019; Colilla and Vallet-Regí 2020; Singh et al. 2019). Antibacterial polypeptides, noble metal nanoparticles (NPs), nanocomposites, semiconductor NPs, polymeric nanostructures and carbon-based nanomaterials (CNMs) are among the nanomaterials that have been explored in vitro and in vivo to regulate and battle bacterial infection (Mba and Nweze 2021; Adhikari et al. 2013; Yeon et al. 2019). Membrane-acting nanoparticles are thought to be particularly tempting and counterproductive to the development of resistance since significant membrane damage would necessitate large-scale membrane component regeneration, which would be physiologically demanding for target cells. Polymeric nanoparticles with state-of-the-art physicochemical properties have been shown to be a therapeutic revolution against human bacterial illnesses (Spirescu et al. 2021). This class of nanocarriers has been demonstrated to be safe, biodegradable, biocompatible, quickly removed and non-toxic to tissues and organs, and they have a number of advantages over currently accessible compounds. Furthermore, the targeting of a specific organ, the reduction of many antibiotics' side effects and the sustained accumulation in the infected area over time all represent a breakthrough in this type of therapy. Various inorganic nanoparticles, such as gold, silver and others, have shown antibacterial potential (Gharpure et al. 2020; Sánchez-López et al. 2020). Silver is a noble metal with excellent thermal stability, low toxicity and antimicrobial properties. AgNP surface functionalisation of polyurethane and plastic catheters was demonstrated to exhibit anti-biofilm efficacy against a wide spectrum of pathogenic bacteria (Prasher et al. 2018).

Gold nanoparticles (AuNPs) have received a lot of attention as antimicrobial agents due to their biocompatibility and simplicity of surface functionalisation (Okkeh et al. 2021). The use of CNMs has gained widespread acceptance in the scientific community due to their unique physicochemical properties, but its clinical use has yet to be proven. Carbon nanostructures with tunable morphologies help to overcome the insoluble property of these materials, which makes them difficult to reach biological systems. Carbon nanomaterials, in general, are prospective antibacterial candidates with a variety of biological uses due to their ability to kill harmful bacteria and prevent their adherence and biofilm formation (Xin et al. 2019; Azizi-Lalabadi et al. 2020; Mocan et al. 2017). The release of positive ions such as Ag^{+}, Cu^{2+} and Zn^{2+} from the oxidation of metal or metal oxide nanoparticles can adsorb on the bacterial cell membrane, causing cell wall destruction and pit formation in bacteria (Gupta et al. 2019). It has previously been discovered that silver nanoparticles produce free radicals and reactive oxygen species (ROS), which are responsible for the oxidation of cellular DNA and proteins and lipid peroxidation, which damages bacterial cell membranes (Baptista et al. 2018; Natan and Banin 2017). Nanostructures can be used as photosensitisers or carriers in photodynamic therapy (PDT) because of their inherent potential to photogenerate several types of cytotoxic species. Photothermal treatment, which generates local heat from nanoagents under near-infrared (NIR) radiation, is a potential technique for treating bacterial infections (ranging from 700 to 1100 nm). Recent research has focused on the development of nanomaterials with inherent photocatalytic capabilities, such as graphene-based materials, MoS2, black phosphorus nanosheets and metal oxide

nanomaterials, to create ROS under laser photoexcitation and act as the PS for PDT applications (Bayir et al. 2018; Bekmukhametova et al. 2020). Despite the fact that nanomaterials are one of the most effective tools for combating MDR bacteria, practical translation of nanoparticle-based medicines remains a hurdle for the pharmaceutical industry because nanotechnology has yet to reach mainstream clinical practice. Martínez et al. (2020) demonstrated the ability of photoactive metallated porphyrin-doped conjugated polymer nanoparticles to eradicate pathogenic bacterial strains, including antibiotic-resistant bacteria of the *En. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *Enterobacter* spp. (ESKAPE) pathogens group in planktonic strain and mature biofilms. MRSA has evolved resistance to practically all antibiotics and can cause serious infections with significant morbidity and mortality in both the community and the hospital. MRSA can be killed by functional nanomaterials and nanoparticles, which can be employed as anti-MRSA therapy (Gao et al. 2021).

Many proof-of-concept studies with nanomaterials in cell culture and small animal models for medical uses are underway, and a few nanoparticles are in clinical trials or have already been approved for use in humans by the Food and Drug Administration (FDA) (Ventola 2017; Anselmo and Mitragotri 2016). To make the transition from the bench to the clinic, strict validation processes for in vitro and in vivo protocols are required. Similarly, large-scale production issues necessitate chemists and engineers innovating and regulatory policies that ease access to trials and patients. Despite significant success in preclinical research, translating these drug delivery methods into clinical practice remains one of the most challenging tasks in this discipline and will be a key focus of most nanomedicine studies in the coming decade. Nonetheless, as described above, major obstacles specific to each technology must be addressed. In general, future research in this field will benefit from employing well-considered design processes to synthesise metal nanomaterials that fill specific gaps in the literature and investigations into the exact pathways that induce cell death. There are notable variations in the published research, with some authors reporting antimicrobial efficacy as a percentage while others report a log reduction. Furthermore, the concentrations of nanomaterials employed in antimicrobial testing are very changeable, and they may be evaluated against pathogenic microorganisms for various timescales utilising various light and magnetic stimulation parameters, such as wattage and frequency. Finally, various general issues for therapeutic applications should be addressed, including high antibacterial efficiency, pathogenic microbe selectivity, low cytotoxicity, localisability to diseased areas and a simple and relevant method of delivery in clinical settings.

1.3.4 Probiotics, Prebiotics, Synbiotics and Other Alternative Approaches

Food is not only meant for the source of energy for today's health-conscious population, but they quest for food components and nutrients that enhance health benefits or prevent from chronic diseases (Webb et al. 2006). So, the focus of nutritional biology circles around 'functional foods' that primarily comprises of probiotics, prebiotics and synbiotics.

1.3.4.1 Probiotics

Probiotics are live microorganisms and act when administered in adequate amounts. Probiotics may contain either a single species or a mixture of two or more bacterial species of *Bifidobacterium*, *Lactobacillus*, *Saccharomyces boulardii*, *B. coagulans* etc. (Chapman et al. 2011). The effects of probiotics vary depending on the combination of strains used (Doron and Snydman 2015; Prado et al. 2015; Soccol and Machado 2015). Many of the probiotic bacteria are reported to produce a variety of antimicrobial compounds, organic acids and bacteriocins that can inhibit the growth of multidrug-resistant bacteria (de Melo Pereira et al. 2018; Tang et al. 2022). Probiotics can generally increase epithelial barrier and inhibit microbial adhesion to the gut line along with synthesis of antimicrobial substances and even can modulate the immune system of the host (Bermudez-Brito et al. 2012; Silva et al. 2020). Probiotics are reported to prevent infectious diseases which are commonly managed with antibiotics (Yang et al. 2019). Current research in this area suggests that probiotic use is beneficial particularly in eliminating or limiting antibiotic-associated diarrhoea (AAD), disruptions of the epithelium of the lower intestine tract due to *Clostridium difficile* and even yeast infections which are secondary to antibiotic treatment. The use of probiotics is an emerging new alternative in antimicrobial therapy (Silva et al. 2020) which is being practised in some Asian countries, Latin America, Europe and even America. Concurrent probiotic use along with antibiotic is being advocated globally, though, presently, there is no scientific documentation or medical rules guiding the application strategy of probiotics together with antibiotics regarding the probiotic dose and the combination of probiotics and the antibiotic to be used in a particular disease condition (Nicholas 2018).

General approaches of probiotic antimicrobial research have scope to be critically reviewed for pointing out future directions in the field which is particularly imperative when there have been a global surge of studies on the efficacy of probiotics as alternative antimicrobial therapy. Research on antibacterial properties of probiotics has grown manifold during the last decade. Compared to 2018 research articles published during 2010–2015, more than 6200 research articles were published during 2016–2021 on '*Antibacterial Properties of Probiotic Bacteria*'. However, application of probiotic formulations for antimicrobial therapy and maintenance of

intestinal health warrant proper standardisation of 'probiotic delivery systems'. WHO guidelines on evaluating potential probiotics in food should be followed to evaluate health benefits claimed in studies and clinical trials (Pandey et al. 2015). This would assist in filling the existing gaps in knowledge regarding the safety of claimed probiotic strains in terms of optimum doses, clinical efficacy and mechanisms of action in humans (Markowiak and Śliżewska 2017).

Probiotic products as drugs or dietary supplements should be regulated by appropriate authority. To end users, it generally is difficult to understand how a commercially available probiotic is marketed, what are the safety standards and which precautions should be taken before using the probiotic products. As food supplements, probiotics should be planned as per food safety guidelines (Venugopalan et al. 2010). But for drug/biologic use, the probiotics must undergo systematic evaluation with in vitro, in vivo and clinical experiments before approval as drug. Once marketed, labelling and beneficial claims in probiotic usage need to be appropriated. Many probiotic drugs or dietary supplements have been marketed worldwide with the adjusted number of either single species or in combination of few types of bacteria (Table 1.3). Few studies have reported that many of the probiotics marketed (not shown) are with potentially unsafe contents in contradiction to the labelling (Yeung et al. 2002; Metras et al. 2021). Another issue is insufficient information to the healthcare providers regarding the modalities for using probiotic products in the clinical practice (Reid et al. 2019). Therefore, a more consistent approach would be beneficial for regulation, testing and labelling of probiotic products.

1.3.4.2 Prebiotics

Prebiotics, on the other hand, are selectively fermented dietary fibres such as inulin/oligosaccharides which help to enrich beneficial microorganisms in the intestine of host. Prebiotics like fructo-oligosaccharides (*FOS*) and galacto-oligosaccharides (*GOS*), xylo-oligosaccharides (XOS), inulin, fructans etc. are primarily used. Whereas *synbiotics* are a selected mixture of probiotics and prebiotics that promote the growth of healthy microflora in the hosts' intestine (Markowiak and Śliżewska 2017). Prebiotics are the dietary fibres generally used together with probiotics to improve their viability. These combinations are often termed as synbiotics. Extensive research has been carried out to investigate the beneficial effects imparted by probiotics, prebiotics and synbiotics in the past few decades. These functional foods are reported to modulate the intestinal microenvironment and the biological functions thereon, leading to health benefits for the host. They facilitate smooth functioning of the intestine including antimicrobial activity against pathogens. Consensus regarding the composition and roles of probiotics, prebiotics and synbiotics in human health is of utmost importance. Besides controlling the growth of drug-resistant pathogens, these provide other immunomodulatory functions which have been found to be beneficial in allergy, cancer, obesity and type 2 diabetes, ageing, fatigue, autism, osteoporosis etc. (Harish and Varghese 2006). Health

Table 1.3 Probiotic drugs (PD) and dietary supplements (DS) marketed worldwide

Product	Category	Manufacturer	Country of origin	Composition	Reference
Acidolac	DS	Polpharma	Poland	*L. rhamnosus* GG	Zawistowska-Rojek et al. 2016
Pearls Acidophilus	DS	Enzymatic	United States	*Bifidobacterium longum* Bb536, *L. acidophilus* NCFM	Drago et al. 2010
ANTEDIA® DUO	DS	Will-Pharma	Belgium	*Sa. boulardii*; *L. rhamnosus* and *L. acidophilus*	Vanhee et al. 2010
Bifilac GG	PD	Tablets India Ltd	India	*L. rhamnosus*	Kesavelu et al. 2020
BioGaia	DS	Ewopharma	Poland	*Limosilactobacillus reuteri* DSM 17938	Zawistowska-Rojek et al. 2016
Biogermin	PD	Union Health S.r.l.	Italy	*Bacillus clausii*	Celandroni et al. 2019
Boulardi-Sanifort	DS	Sanifort Pharma	Belgium	*Sa. Boulardii*	Vanhee et al. 2010
Codex-ing	PD	Zambon	Italy	*B. coagulans*	De Vecchi et al. 2008
Combiflora	PD	Medopharm	India	*Bifidobacterium lactis* Bl-04 + *Bifidobacterium lactis* Bi-07 + *Lactobacillus acidophilus* NCFM + *L. paracasei* Lpc-37	Kesavelu et al. 2020
Dicoflor	DS	Dicofarm	Italy	*L. rhamnosus*	Vanhee et al. 2010
Econorm	PD	Dr. Reddy's Laboratories Ltd.	India	*Sa. boulardii*	Kesavelu et al. 2020
Enteriphar	DS	Teva	Israel	*Sa. boulardii*	Vanhee et al. 2010
Entero Plus	PD	Glaxo India Ltd	India	*L. rhamnosus*	Kesavelu et al. 2020
Enterogermina	PD	Sanofi	Italy	*B. clausii*	De Castro et al. 2019
Enterol capsules	PD	Biodiphar	Belgium	*Sa. boulardii*	Vanhee et al. 2010
Enterolactis Plus	DS	SOFAR	Italy	*L. paracasei* CNCM I-1572	Vecchione et al. 2018
Entromax	PD	Mankind Pharma	India	*B. clausii*	Patrone et al. 2016

(continued)

Table 1.3 (continued)

Product	Category	Manufacturer	Country of origin	Composition	Reference
Ercéflora Supra	DS	Sanofi Aventis	France	*Sa. boulardii*	Vanhee et al. 2010
GNorm	PD	Nouveau Medicament	India	*Sa. boulardii*	Kesavelu et al. 2020
GutPro	PD	Riata Life Sciences Pvt. Ltd.	India	*Pediococcus acidilacticii*, *B. subtilis*, *B. coagulans*, *L. acidophilus*, *Sa. cerevisiae*	Kesavelu et al. 2020
Imutis	DS	Trenker Pharmaceutical	Belgium	*Sa. boulardii*, *L. rhamnosus*, *L. helveticus* and *Bifidobacterium longum*	Vanhee et al. 2010
Lakcid	PD	Biomed	Poland	*L. rhamnosus*	Zawistowska-Rojek et al. 2016
Phillip's Colon Health	DS	Bayer	United States	*L. gasseri*, *Bifidobacterium bifidum*, *Bifidobacterium longum*	Drago et al. 2010

benefits like immune modulation, cancer prevention, remediation of inflammatory bowel disease etc. as claimed in many research reports should be investigated under proper guidelines and reviewed. The use of Probiotics, prebiotics and synbiotics, if implemented appropriately, has the potential to offer sustainable alternatives for antibiotics.

1.3.4.3 Vaccines

Vaccines are used prophylactically as cost-effective bio-secure alternative to reduce the number of infectious disease cases (Micoli et al. 2021). Although vaccines are not often considered as direct remedy for antimicrobial resistance, vaccination renders the pathogen with less time to evolve as resistant. Vaccines, therefore, present an opportunity to reduce the burden of using antibiotics both in human and animal farming (Kennedy and Read 2017; Hoelzer et al. 2018). Vaccines can be developed for the bacteria highlighted as 'urgent' hazard of developing resistance (e.g. *C. difficile*, *carbapenem-resistant* Enterobacteriaceae or *CRE* and *drug-resistant Neisseria gonorrhoeae*). Vaccination against *Lawsonia intracellularis*, the causative agent of ileitis, can reduce oxytetracycline consumption by nearly 80% in Danish pig herds (Bak et al. 2009). Vaccines are one of the most promising

alternatives to disease prevention, but their implementation is somewhat challenging. As for example, most of the vaccines are administered via injection which increases labour expenses (Meeusen et al. 2007). Furthermore, some vaccinations are only effective against a limited number of bacterial or viral strains, while others pose the threat of unexpected consequences (Cheng et al. 2014). Many of these issues are still being investigated, such as the possibility of mass vaccination delivery or the development of ways for eliciting more protective immune responses. In summary, immunisations have scope to develop as a better alternative to antibiotics (Mutwiri et al. 2011).

1.3.4.4 CRISPR-Cas9

CRISPR-Cas9 gene editing technology is one of the modern techniques that have the potential to reverse antibiotic resistance in certain harmful bacteria (Kim et al. 2016). CRISPR-Cas9 has significant improvement over existing gene-editing methods in terms of ease of use, speed, efficiency and cost. CRISPR-Cas9 systems have been used successfully to target the virulence factors and antibiotic resistance genes in bacteria for the development of programmable and sequence-specific antimicrobials (Bikard and Barrangou 2017).

CRISPR-based antimicrobials have a distinct advantage over all other techniques in that they can destroy bacteria depending on their genetic sequence. Because CRISPR-guided RNAs are highly specific, they may be tailored to target specific chromosomal and virulence genes, eliminating the need for broad-spectrum antibiotics to treat illnesses in animals (Shabbir et al. 2019). CRISPR-Cas can be utilised for selectively eliminating AMR genes of the virulent bacterial populations with ABR. It can also be used to reverse resistance to antibiotics by specifically targeting antibiotic resistance genes located on plasmids (Gholizadeh et al. 2017; Zhang et al. 2017; Dong et al. 2019; Wang et al. 2019). Concurrent delivery of CRISPR with a probiotic can even be applied to eliminate environmental reservoirs of AMR restricting them before they can reach human pathogens (Pursey et al. 2018). However, it may not be a suitable approach to reach a broad range of species together. The conjugative delivery speed of the probiotic and CRISPR duo is slow in comparison to other approaches like use of bacteriophage which is fast and rapid even for a large pool of bacterial population (Roach and Debarbieux 2017; Cairns et al. 2018). Nevertheless, with more suitable adaptations, CRISPR-Cas9 systems have the potential to be a possible treatment option in the era of antibiotic resistance.

1.4 Future Prospects

Global antibiotic consumption is significantly increasing which is estimated to double in a decade. It is crucial to reduce the global use of antibiotics and thus to manage the growing burden of antibiotic resistance since antibiotics are

manufactured in large quantities each year and utilized in both healthcare and agriculture. Environmental niches play a key role in persistence and dissemination of resistant microorganism. For example, many antibiotics are released into the water as a result of insufficient sewage and pharmaceutical waste treatment, which serves as a primary source for the spread of resistance genes or resistant microorganisms. Risk assessment, environmental monitoring and effective control measures to limit pollution from agricultural, industrial and residential sources are all feasible strategies to address drug resistance in this area.

Antibiotic resistance can be addressed in a variety of ways, including lowering antibiotic use through the use of alternative approaches. Because disease prevention and treatment require a combination of specific and general strategies, no single approach will be able to replace all antibiotic use. Innovative strategies like application of metagenomics provide insights into pathogen association with various ARGs. The whole genome sequencing (WGS)-based genetic relatedness among the isolates from various sample sources is helpful in understanding the possible transmission routes. The reported ARGs such as blaTEM, blaCTXM, blaKPC, blaNDM, blaVIM and mcr-1 are found responsible for the emergence of MDR strains of *E. coli*, *P. aeruginosa* etc. So genomic technologies are critical for better understanding both the host and the pathogen and the development of more targeted diagnostics and treatments. Rapid genome-level diagnostics, in particular, are critical for the delivery of individualised treatments, including antibiotics, microbiota and immune modulators, vaccinations and phages. Moreover, high-throughput screens are also effective tools for identifying critical pathogen and host factors that can be used to produce vaccines and inhibitors, as well as markers to speed up breeding programmes or targets for engineering resistance. Although many chemical and nonchemical agents have antibacterial potential, it is clear that vaccines, better health professional education, strict antimicrobial stewardship, global improvements in infection control and sanitation and more sophisticated rapid diagnostics are all needed for the future of human and animal medicine. It was reported that there were around 41 novel antimicrobials in clinical trials at the time, but only 20% of them were authorised. Factually, the low success rate for the development and approval of new antibiotics, alternative research strategies such as the development of nucleic acid–based antibacterial treatments, antimicrobial peptides, bacteriocins, antibody therapy, antivirulence compounds, bacteriophage therapy etc. should be further studied for practical implementation to control infections caused by resistant 'superbugs'. The current One Health approach which is associated with human, animal and environmental health to manage antimicrobial resistance rely on the concrete understanding of the interactions and interrelationships between these components in the transmission of AMR to humans. At present, specified and targeted research is required to determine the mechanisms of resistance and frequency of emerged resistant in various ecologies such as soil and water, to search economic and effective alternatives to antibiotics and eventually to conquer the battle against drug-resistant pathogens.

1.5 Conclusion

Antibiotics are still inevitable in regulating bacterial infections, but it is vital to reduce their usage significantly by adopting alternative approaches to restrict the pathogens. Antibiotic-free approaches are a promising new strategy to address the present antibacterial challenge, as antibiotic resistance is primarily caused by the misuse of antibiotics. The antimicrobial resistance can be tackled in a variety of ways, including lowering antibiotic use by adopting alternative products. A single alternative cannot replace uses of antibiotics, so, a variety of methods are needed to both prevent and treat microbial diseases. Nanomaterials, phage therapy, synthetic compounds, antivirulence compounds, vaccines and probiotics could be among the most promising approaches. Antimicrobial coatings on the surface of medical implants using polymer-based NPs and metal NPs could be used for a variety of biomedical purposes. NP-based methods have the potential to work alone or in combination with antibiotics for treating many bacterial infections, paving the way for future nanomedicine. Vaccination is an important component of the multi-tiered approach to restrict antibiotic resistance since it is so crucial in illness prevention. The introduction of new vaccines may also help to reduce the prevalence of infectious diseases and thereby cut the need for antibiotics. Thousands of phytochemicals which have inhibitory effects on bacterial growth under in vitro conditions have been found. Additionally, better understanding the potential of phytochemicals to inhibit QS activity is of great relevance. Recent research aims to identify and develop novel anti-QS compound capable of preventing bacterial infections in humans. Modulation of microbiome through the appropriate use of probiotics, faecal transplantation or enhanced nutrition are being considered in human and animal healthcare. Gene editing (using CRISPER-Cas9) and transgenic techniques have ushered additional alternatives to control resistant pathogens. Although such antimicrobial alternatives have significant advantages over conventional chemical antibiotics, more in vivo research into the pharmacokinetics and pharmacodynamics of these treatment techniques is required. Scientists from different fields are investigating plants for searching of natural antimicrobial agents due to their biological properties. Collective efforts from various disciplines, including microbiology, chemistry, biomedical science, animal and plant science and materials science are vital for the development of antibiotic-free antibacterial technology.

Outlining the feasibility and efficiency of various alternatives in terms of economic, technological, behavioural and social aspects is key to fight AMR. Robust political will, promotion, management and responsibility are obligatory to effectively contain antimicrobial resistance. The challenges of antimicrobial resistance are complex and multifaceted, but they are not insurmountable. Implementation of these effective measures will save millions of lives and will minimise the global crisis in management of drug-resistant bacteria.

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Part II
Natural Alternative Approaches

Chapter 2
Phytochemicals as Antibacterial Agents: Current Status and Future Perspective

Swagata Nag, Nutan Singh, and Suman Kumaria

Abstract The global emergence of multidrug-resistant (MDR) bacteria has severely compromised the efficacy of current antibacterial drugs and significantly increased the frequency of therapeutic failure. The development of novel and innovative antibacterial drugs with various chemical structures and processes that can combat harmful bacteria is urgently needed. Many studies have recently concentrated on identifying possible answers to these issues. There is a growing interest in medicinal plants for the potential sources of new therapeutic compounds, due to the structural and functional diversity existing in the specialized metabolites found in these plants. So far, many phytochemicals with varied biological activity, such as antibacterial, antifungal, and anti-carcinogenic, have been reported with low toxicity and adverse effects. Anti-quorum sensing (AQS) is a promising strategy for cell–cell communication which plays a vital role in the regulation of various bacterial physiological functions such as pathogenicity, luminescence, mobility, sporulation, etc. A variety of novel plant-based compounds have been discovered with the potential to disrupt bacterial quorum sensing (QS). The present chapter deals with the current developments in the field of plant extracts/phytochemicals, which are being used as the potential antibacterial and antimicrobial agents. Plant-derived molecules, which have antibiofilm or anti-quorum sensing activities, and the various mechanisms involved in their actions are also discussed.

Keywords Alkaloids · Antibacterial · *E. coli* · Flavonoids · *H. pylori* · Medicinal plants · Multidrug resistant · Multidrug-resistant (MDR) bacteria · Methicillin-resistant *S. aureus* · N-acyl-homoserine lactones · Phenolic compounds · Phytochemicals · *S. aureus* · Sulfur-containing phytochemicals · Secondary

S. Nag
Plant Biotechnology Laboratory, Department of Botany, North-Eastern Hill University, Shillong, Meghalaya, India

Siliguri Paramedical College, Jalpaiguri, India

N. Singh · S. Kumaria (✉)
Plant Biotechnology Laboratory, Department of Botany, North-Eastern Hill University, Shillong, Meghalaya, India

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metabolites · Tannins · Terpenoids · Quinones · Quorum sensing · Quorum sensing inhibition

Abbreviations

AHL	N-acyl-homoserine lactones
MDR	Multidrug resistant
MRSA	Methicillin-resistant *S. aureus*
SAR	Structure–activity relationship

2.1 Introduction

Infectious diseases are the second greatest cause of death worldwide, after cardiovascular disorders, with 13.3 million people dying each year, being a primary source of morbidity and mortality. Every year, 700,000 people die as a result of bacterial resistance to drugs among the two million people who are sick with several types of bacteria around the world (Adrizain et al. 2018). The number of multidrug-resistant bacterial strains is continuously increasing, as is the advent of bacteria that are less susceptible to antibiotics. The indiscriminate and inappropriate uses of antibiotics have hastened the establishment of drug-resistant bacteria. Furthermore, unsanitary circumstances and improper food handling contribute to the spread of antibiotic resistance. Nosocomial infections with highly resistant bacterial pathogens have developed from a combination of extremely susceptible patients, extensive and sustained antibiotic usage, and cross infection. Hospital-acquired illnesses that are resistant to antibiotics are costly to treat and difficult to eliminate. Drug-resistant bacteria are responsible for up to 60% of hospital-acquired illnesses worldwide. Therefore, a hunt for new antibacterial compounds from a variety of sources, including medicinal plant, has sparked in recent times.

Since the time plants evolved, their important and protective roles have been well known for the mankind. Plants have been known to have anti-infective properties due to the presence of secondary metabolites such as tannins, alkaloids, terpenoids, and flavonoids, since ancient times. An evolving effective demand of natural plant-based products day by day lays an emphasis on the different medicinal plants used traditionally and in modern medicine system. A great majority of modern medicines have their roots in ancient herbal traditions. There are a variety of natural plant compounds with antifungal, antibacterial, and antiprotozoal properties that can be utilized systemically or locally (Dias et al. 2012a, b; Savoia 2012). The remedies obtained from the plants have given an insight for various afflicting human disorders. Plants are known for the production of various chemicals that are diverse in structures and have been adequately well utilized for various purposes. The diverse chemical compounds that have been reported to be useful as raw materials have led

to the discovery of drugs for chronic disorders (Yuan et al. 2016; Dias et al. 2012a, b; Bariş et al. 2006). The increasing demands of plants in the present scenario make the plants more superior to other living organisms present on earth. A majority of medications in clinical use today are derived from naturally occurring compounds, primarily secondary metabolites. Traditional medicine is used by majority of the people worldwide to maintain their health. The earliest records on Indian traditional medicine prescribed the uses of plants in treatment of various ailments with focus on herbal medicines. The demands of herbal-based medicines are at an alarming rate in both developed and underdeveloped countries due to their safety measures and reduced costs (Cragg and Newman 2001). The current chapter discusses recent advances in the field of plant extracts/phytochemicals, which are being investigated as antibacterial and antimicrobial agents. Plant-derived compounds with antibiofilm or anti-quorum sensing properties and the diverse mechanisms behind their actions are also reviewed.

2.2 Secondary Metabolites Acting as Antimicrobial Agent

The secondary metabolites are organic compounds that are indirectly involved in the developmental processes of the plants. Metabolites play an important role in protecting the plants and conferring color, flavor, aroma, texture, and plant defense against various biotic and abiotic agents (Molyneux et al. 2007). The plants with high secondary metabolites such as alkaloids, phenols, tannins, terpenoids, saponins, and flavonoids are reported to have enhanced medicinal properties (Singh and Kumaria 2020a; Edema and Alaga 2012). The growing demands of plant-based metabolites have given insights to the discovery and utilization of the bioactive metabolites in bio-therapeutic uses (Rao and Roja 2002). The research works carried out over the years imply their efficiency and effectiveness against multidrug-resistant bacteria in both planktonic and biofilm forms. However, some phytochemicals show limited solubility in aqueous media, which further limits their medical usefulness. The use of surfactants, nanoparticles, and polymers can serve as an effective delivery vehicle to overcome this constraint.

2.2.1 Phenolic Compounds

Phenolics are diverse groups of plant secondary metabolites that are considered as evolving natural biomolecules due to their known effective bioactive properties. They are aromatic compounds synthesized in plants through shikimate/phenylpropanoid pathway, which leads to the production of phenols and polyphenols (Randhir et al. 2004). Plant phenolics are known to have significant roles in growth, development, and reproduction and also play a defensive role against the various biotic and abiotic stresses such as UV, chitosan, cold, dark, nutrient

deficiency (Lattanzio 2013; Nag and Kumaria 2018; Singh and Kumaria 2020b). Phenolics are reported to participate in defense role against the predators and help in the development of color at different developmental stages of plants (Bravo 1998; Alasalvar et al. 2001). The phenolics are not only predominant in plants but are also reported from the bacteria, fungi, and algae (Harborne 2013). Also, it has been reported that the phenolics exhibit various biological properties such as antimicrobial, antiallergenic, and antioxidant activity (Balachandran et al. 2021). Many different types of phenolic compounds are known to be synthesized in plants and have been reported to be localized in different plant parts and serve as the potential agents for the action of various chronic diseases such as cancer, cardiovascular disease, and diabetes. The phenolics are also know to eliminate the foodborne bacteria and reduce the formation of biofilm. Due to the various useful applications of phenolics, therefore, their interest toward the food industry has increased day by day (Takó et al. 2020; Zambrano et al. 2019; Gyawali and Ibrahim 2014; Del Rio et al. 2013).

The various phenolics such as cinnamic acid, caffeic acid, p-coumaric acid, catechol, ferulic acid, pyrogallol, and eugenol have been reported to be effective against viruses, bacteria, and fungi (Kumar and Pandey 2013). Caffeic acid has been reported to have antimicrobial potential, synergistic effects with antibiotics against *Staphylococcus aureus*, *S. epidermidis*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Pseudomonas mirabilis*, *Escherichia coli*, *P. aeruginosa*, *Bacillus cereus*, and *Mycobacterium luteus* (Santos et al. 2018; Loes et al. 2014; Cushnie and Lamb 2005). Caffeic and p-coumaric acid have been reported to have synergy with the conventional antibiotics such as ampicillin and amikacin and increase their effectiveness against various gram-positive and gram-negative bacterial pathogens (Hemaiswarya and Doble 2010).

Another investigation has shown that the caffeic acid has anti-staphylococcal action, with minimum inhibitory concentrations (MICs) ranging from 62.5 to 250 g/mL (Luís et al. 2014). Catechol (two-hydroxyl group) and pyrogallol (three-hydroxyl group) are hydroxylated phenols that show toxic effects on microorganisms. More hydroxylation of catechol results in high toxicity. Mechanisms of phenol toxicity to microbes have been shown through enzyme inhibition processes with the oxidized compounds' interaction with sulfhydryl groups or nonspecific interaction with proteins (Ciocan and Bara 2007).

Catechin is a polyphenol that acts on different bacterial strains by producing the hydrogen peroxide or by modifying the microbial membrane permeability (Kumar et al. 2013). Gallic acid has additionally been proven to have antibacterial activity against *S. aureus*, *S. epidermidis*, *E. coli*, *Shigella flexneri*, *Salmonella* spp., *P. aeruginosa*, and *A. baumannii* with MICs ranging from 630 to 5000 g/mL (Fu et al. 2016). Gallic acid is proven to be more effective against *Campylobacter jejuni* and *Campylobacter coli* with MICs ranging from 15.63 to 250 g/mL, the mechanism being the loss of calcium ions (Sarjit et al. 2015). According to another research, gallic acid–conjugated gold nanoparticles have shown a much better antibacterial activity than gallic acid alone against the foodborne pathogens *Sh. flexneri* and *Plesiomonas shigelloides* (Randhawa et al. 2016).

2.2.2 Quinones

Quinones are aromatic (hexacyclic saturated) di-one or di-ketone compounds, ubiquitous and highly reactive in nature. They are derived from the oxidation of hydroquinones, namely, anthraquinones, benzoquinones, naphthoquinones, and polyquinones. The browning reaction seen in cut vegetables is due to the accumulation of quinones. This also forms the intermediates in the synthesis of melanin and provides basis for the formation of stable free radicals. Quinones are also found accumulating irreversibly with nucleophilic amino acids in protein, which lead to functional loss of the proteins. Quinones have a wide range of antimicrobial effects (Jali 2021; Balachandran et al. 2021; Liu et al. 2017; Jung et al. 2016; Jiang et al. 2007).

2.2.3 Flavonoids and Their Derivatives

Flavonoids are a large, low molecular weight, and structurally diverse group of natural bioactive products. They are hydroxylated diphenylpropanes (C6–C3–C6) in structural skeleton. The reported flavonoids differ in the degree of oxidation, which leads to the diverse flavonoid derivatives (Kumar and Pandey 2013). Diverse group of flavonoids such as flavonols (quercetin, kaempferol, and myricetin), flavanones (naringin), flavones (luteolin), chalcones (licochalcone A, licochalcone E), catechins, anthocyanin, and isoflavonoids (sophoraisoflavone A) have been reported in plants (Farhadi et al. 2019; Patra 2012).

Flavonoids and their derivatives are synthesized in plants in response to different microbial attack. According to the report of Kumar and Pandey (2013), flavonoids have been reported to be effective against the wide array of microorganisms in in vitro studies. Flavonols such as quercetin, myricetin, morin, galangin, entadanin, rutin, piliostigmol, and their derivatives are among the most important class of flavonoids that show potent antibacterial activities (Siriyong et al. 2017; Geoghegan et al. 2010). The catechins present in green tea, epigallocatechin gallate (EGCG), have been found to be active against *B. cereus* in nanomolar concentrations (Friedman et al. 2006). Antibacterial activities of EGCG alone and in combination with various antibiotics have been studied extensively against a variety of bacteria, including multidrug-resistant strains such methicillin-resistant *S. aureus* (MRSA; Steinmann et al. 2013). The addition of long alkyl chains to EGCG dramatically increased its in vitro activity against a variety of bacteria and fungi, particularly *S. aureus* (Matsumoto et al. 2012). Another study demonstrated the action of EGCG on *E. coli*'s outer membrane and reported that the substance interacted with the membrane at many sites (Nakayama et al. 2013). Polyphenon E is prominent in distinguishing at least five distinct catechins, wherein EGCG is the most abundant component reported which is widely used clinically (Clark and You 2006). Another flavanol with antibacterial potential is a flavin, which has been shown to work

against a variety of bacteria, including *A. baumannii*, *B. cereus*, and *Shigella* spp. (Betts et al. 2017; Friedman et al. 2006).

Many studies have shown that flavonoids are responsible for the inhibition of biofilm formation by disrupting the quorum sensing (QS). Flavonoids appear to impair the interaction between acyl-homoserine lactones (signal molecules used by gram-negative bacteria) and their receptors. It has been reported that baicalein inhibits the cytoplasmic membrane-associated receptor TraR (Zeng et al. 2008; Qin et al. 2000). Quercetin is a well-known flavonoid with a wide range of biological activities that include antioxidant, antibacterial, anti-inflammatory, antiviral and anticancer properties (Wang et al. 2016). Quercetin has been demonstrated to suppress *E. coli* growth under in vitro conditions and to have antibacterial effect against *S. aureus* and *K. pneumoniae* (Ohemeng et al. 1993). Mirzoeva et al. (1997) demonstrated that quercetin and other flavonoids reduce the bacterial motility considerably. Quercetin's antibacterial activity was boosted in vitro when it was coupled with different antibiotics (Sakharkar et al. 2009; Hirai et al. 2010) and also had synergistic effects when combined with sulfamethoxazole, rifampicin, and fusidic acid against methicillin-resistant *S. aureus* (MRSA) strains and clinical isolates (Sahyon et al. 2019; Kyaw and Lim 2012). By hindering QS, quercetin was found to have antibiofilm activity against *K. pneumoniae*, *Pseudomonas aeruginosa*, and *Yersinia enterocolitica* (Gopu et al. 2015). A more recent research has revealed that quercetin can boost the antibacterial action of metals, with silver nanoparticles made from polyphenol having more antibacterial activity as compared to quercetin or silver nitrate alone against gram-negative and gram-positive infections (Jain and Mehata 2017).

Kaempferol, another flavonol, is reported to have a wide range of biological and pharmacological properties. It is known to impede the growth and survival of antibiotic-resistant *S. aureus* by inhibiting the activity of the PriA helicase (SaPriA) and bacterial efflux pumps, hence improving antimicrobial effectiveness and blocking the growth and survival of antibiotic-resistant *S. aureus* (Brown et al. 2015; Huang et al. 2015).

2.2.4 Tannins

Tannins are polymer of phenolic substances and are found in almost all the plants. Tannins are divided into condensed and hydrolyzable tannins. Hydrolyzable tannins are gallic acid based and contain multiple esters of D-glucose. The condensed form of tannins is often called proanthocyanidins and is derived from flavonoid monomers. It has been reported that tannins may have formed by condensations of flavan derivatives or by polymerization of quinine (Ciocan and Bara 2007; Karou et al. 2007). Studies have reported that tannins can be toxic to bacteria, yeast, and fungi (Cowan 1999). Punicalagin suppressed violacein synthesis in *Chromobacterium violaceum* and swarming motility in *Sa. typhimurium* SL1344 (Li et al. 2014).

2.2.5 Alkaloids

Alkaloids are the diverse group of chemical organic nitrogen-containing heterocyclic compounds. They are one of the structurally diverse groups of the metabolites effectively used as therapeutically important plant substances. Based on their core chemical structure, alkaloids are grouped into indoles, isoquinolines, piperidine alkaloids, quinolines, etc. The in-depth research on alkaloids from different plants have revealed their potential to acquire the properties of natural antibiotic with a wide antibacterial spectrum with low propensity to make drug resistant. When co-administered with ciprofloxacin, piperine, a piperidine-type alkaloid produced from *Piper nigrum* and *P. longum*, decreased the growth of a mutant *S. aureus* and considerably reduced the MIC values for *S. aureus* (Khan et al. 2006). Piperine and gentamicin co-administration was found to be effective in the treatment of MRSA infections (Khameneh et al. 2015). Tomatidine is a steroidal alkaloid found in solanaceous plants such as tomato, potato, and eggplant that has been shown to have significant antibacterial action against *S. aureus* whether used alone or in combination with aminoglycosides (Jiang et al. 2016).

The quinolone alkaloid evocarpine, isolated from *Fructus evodiae*, was found to have significant antimicrobial action against MRSA (Pan et al. 2014). The steroidal alkaloid tomatidine was isolated from the tomato plant. It has a high vulnerability to MRSA, according to the findings (Chagnon et al. 2014). Two guanidine alkaloids were found from the *Pterogyne nitens*. These two guanidine alkaloids, galegine and pteridine, possessed high anti-MRSA activity. The presence of a side chain observed in guanidine alkaloids was thought to impart the antibacterial property (Coqueiro et al. 2014). Because of the significant implications and need for conventional therapy following antibiotic failure against MRSA, there has been a huge push to develop novel compounds that can slow the progression of bacterial infections and enhance patient quality of life. Isolation of 6-methoxydihydrosanguinarine (6-MS), 6-acetonylhydrosanguinarine, and dihydrosanguinarine from *Hylomecon hylomeconoides* paved the door for medication sensitivity against MRSA to be regained. These alkaloids prevent MRSA strains with MICs ranging from 1.95 to 250 ug/mL (Choi et al. 2010). Plant alkaloids' capacity to intercalate DNA may explain their ability to inhibit MRSA activity. It has also been proposed that alkaloid components impede or degrade beta-lactamase action (Zoraghi et al. 2011). Plant alkaloids such as berberine (found in *Berberis* sp.) and piperine (found in *Piper* sp.) can interact with the bacterial cytoplasmic membrane, intercalate with DNA, and inhibit efflux pumps in *S. aureus* (Khan et al. 2006; Jennings and Ridler 1983).

Berberine is an isoquinoline-type alkaloid used in the treatment of dental infections. Several investigations utilizing a multispecies biofilm tooth model have showed the efficacy of berberine against oral streptococcal growth and certain endodontic pathogens (Dziedzic et al. 2015; Xie et al. 2012). Berberine has been demonstrated to improve the inhibitory activity of antibiotics against clinical multidrug-resistant isolates of methicillin- resistant *S. aureus* (MRSA) (Chu et al. 2016). Berberine was found to have antibacterial activity against *Streptococcus*

agalactiae by disrupting the membrane and reducing protein and DNA production (Peng et al. 2015). Dusane et al. (2014) studied the effect of reserpine and piperine from *P. nigrum* against *E. coli*, which causes urinary tract infections in humans. Piperine improved the action of the antibiotics azithromycin and ciprofloxacin in dispersing biofilms by increasing their penetration into *E. coli* biofilms.

2.2.6 Terpenoids

Terpenoids are a wide class of chemicals produced by plants that have antibacterial properties. Several terpenes and their derivatives have been demonstrated to be effective defenses against herbivores and infections. Gram-positive bacteria are usually more sensitive to terpenes than gram-negative bacteria. Terpenes' antibacterial action is closely linked to their lipophilic properties. Monoterpenes preferentially affect membrane structures by increasing fluidity and permeability, modifying protein architecture, and causing disruptions along the respiration chain. Togashi et al. (2010) studied the effects of linalool, geraniol, nerolidol, plaunotol, farnesol, geranylgeraniol, and phytol on the growth of *S. aureus*. Among all of the compounds examined, only farnesol and nerolidol, with MBC of 20 and 40 g/mL, demonstrated a significant antibacterial effect. Two diterpenoids, salvipisone and aethiopinone, were extracted from *Salvia sclarea* roots and tested as antibacterial and antibiofilm agents against gram-positive and gram-negative bacteria. These diterpenoids inhibited the growth of *S. aureus*, *S. epidermidis*, and *Enterococcus faecalis* at a concentration of 37.5 g/ml, and *S. aureus* and *S. epidermidis* pre-formed biofilms were disturbed by at least 85% (Rózalski et al. 2007). Chung et al. (2014) extracted and identified three known triterpenoids (amyrin, betulinic acid, and betulinaldehyde) from the bark of *Callicarpa farinosa.* These compounds were found to have antibacterial activity against MRSA and methicillin-sensitive *S. aureus* (MSSA) and could be used to combat antibiotic resistance in *S. aureus*. Dehydroabietic acid, a resin acid, is reported to be another terpene molecule with antibacterial action against *S. aureus*. Broniatowski et al. (2015) investigated the antimicrobial mechanism of two pentacyclic triterpenes, ursolic acid and amyrin, which are natural chemicals with broad antibacterial activity. The other well-known terpenoids are eugenol and cinnamaldehyde, which are found in the essential oils of a variety of plants and have been shown to be effective against a variety of infections. Eugenol has been reported to have a lot of bioactivity against MRSA and MSSA clinical strain biofilms. According to the findings of Yadav et al. (2015), eugenol inhibits biofilm formation, disrupts cell-to-cell communication, eradicates pre-existing biofilms, and kills bacteria in biofilms, and this is true for both MRSA and MSSA. Essential oils including thymol, carvacrol, eugenol, and vanillin demonstrated antibacterial action against *E. coli* O157:H7, *Sa. typhimurium*, and *Listeria monocytogenes* when mixed with soy sauce (Moon and Rhee 2016). Knezevic et al. (2016) tested the antibacterial activity of essential oils from *Eucalyptus*

camaldulensis against multidrug-resistant (MDR) *Acinetobacter baumannii* wound isolates.

2.2.7 Sulfur-Containing Phytochemicals

Organosulfur compounds such as allicin, ajoene, sulfasalazine, and isothiocyanates have been shown to have antibacterial activity against a variety of bacteria, including MDR strains. Park et al. (2013) investigated the antibacterial efficacy of horseradish root isothiocyanates against oral microbes. Dias et al. (2012b) investigated the antibacterial activity of isothiocyanates in the presence of antibiotics such as gentamicin and vancomycin against both gram-positive and gram-negative bacteria. Garlic's main component allicin has been found as having antibacterial activity against a wide range of microorganisms. Allicin was responsive to vancomycin-sensitive and vancomycin-resistant clinical isolates and standard strains of *Enterococcus* species (Jonkers et al. 1999). When compared to diallyl sulfide, allicin had the best anti-*Helicobacter pylori* action against three strains (O'Gara et al. 2000). According to a meta-analysis of clinical data, combining allicin with standard therapy promotes the eradication of *H. pylori* infections (Si et al. 2019). Allicin was proven to be active against *Clostridium difficile* and other commensal gut bacteria in a recent study, and there was no substantial synergy between allicin and conventional antibiotics (Roshan et al. 2017).

A list of plant secondary metabolites such as alkaloids, flavonoids, tannins, terpenes, quinines, resins, coumarins, organosulfur, terpenoids, phenols, lactones, benzoic acid, diarylheptanoid, phenolic acids, polyphenol, iridoid lactone, and sesquiterpene lactone with their reported antibacterial activities is shown in more details in Table 2.1.

2.3 Quorum Sensing (QS)

QS, as a mechanism of bacterial cell-to-cell chemical communication, plays a key role in pathogen biofilm development, antibiotic resistance, survival, proliferation, and toxin synthesis. Targeting quorum sensing has emerged as a viable technique for fighting against bacterial infections since it does not put any selection pressure on pathogens, making it unlikely to develop multidrug resistance. Quorum quenching phytochemicals may be a potential non-antibiotic therapy method for pathogenic bacteria by inhibiting bacterial communication and making them less virulent. Extracts and specific compounds from several fruits, herbs, and spices have been displayed to inhibit QS. Polyphenols, for example, are QS-inhibiting phytochemicals that can impact biofilm development in some bacteria, because their chemical structure is comparable to that of QS signals N-acyl-homoserine lactones (AHL) and/or their capability to degrade signal receptors (LuxR/LasR) (Santos et al. 2021;

Table 2.1 Plant secondary metabolites with their reported antibacterial activities

Secondary metabolites and plant families	Antibacterial activity	References
Alkaloids		
Amaryllidaceae	*Staphylococcus aureus*	Savoia 2012
Apiaceae	*Enterococcus faecalis*	Basile et al. 2009
Apocynaceae	*Acinetobacter baumannii*	Siriyong et al. 2017
Berberidaceae	*P. aeruginosa*	Boberek et al. 2010
Capparaceae	*Mycobacterium tuberculosis*	Agbafor et al. 2011
Compositae	*Salmonella typhi*	Munyendo et al. 2011
Fabaceae	*Escherichia coli*	Carson and Hammer 2011
Mimosaceae	*Pseudomonas aeruginosa*	Ramawat 2007
Piperaceae	Methicillin-resistant *S. aureus* (MRSA)	Khameneh et al. 2015 Birdi et al. 2012 Hochfellner et al. 2015
Rubiaceae	*Mycobacterium kansasii*	Mariita et al. 2011
Flavonoids		
Adoxaceae	*E. coli* Methicillin-resistant *S. aureus* (MRSA)	Wu et al. 2008 Randhawa et al. 2016
Amaryllidaceae	*Mycobacterium fortuitum*	Munyendo et al. 2011
Apiaceae	*Helicobacter pylori*	Wu et al. 2008
Asphodelaceae	*Mycobacterium fortuitum* Methicillin-resistant *S. aureus* (MRSA)	Randhawa et al. 2016
Asteraceae	*Helicobacter pylori* Methicillin-resistant *S. aureus* (MRSA)	Zhang et al. 2008 Hong et al. 2006 Stermitz et al. 2003
Capparaceae	*E. coli* Methicillin-resistant *S. aureus* (MRSA)	Wu et al. 2008
Fabaceae	*P. aeruginosa*	Agbafor et al. 2011 Gutiérrez et al. 2017 Hong et al. 2006
Labiatae	*S. typhi*	Zhang et al. 2008
Moringaceae	Methicillin-resistant *S. aureus* (MRSA)	Randhawa et al. 2016
Rubiaceae	*S. aureus*	Sibi et al. 2012
Rubiaceae	*Salmonella typhi*	Zhang et al. 2008
Rutaceae	Methicillin-resistant *S. aureus* (MRSA) *E. faecalis*	Munyendo et al. 2011 Sibi et al. 2012
Theaceae	*E. coli*	Gutiérrez et al. 2017 Li et al. 2006

(continued)

Table 2.1 (continued)

Secondary metabolites and plant families	Antibacterial activity	References
Tannins		
Fabaceae	*St. faecalis*	Mariita et al. 2011
Mimosaceae	*Bacillus subtilis*	Sibi et al. 2012
Myrtaceae	*S. aureus*	Abdulhamid et al. 2014
Rubiaceae	*E. coli*	Oboh 2010
Terpenes		
Compositae	*Staphylococcus epidermidis*	Munyendo et al. 2011
Fabaceae	*S. aureus*	Togashi et al. 2010
Labiatae Rutaceae	Methicillin-resistant *S. aureus* (MRSA)	Korir et al. 2012
Lamiaceae	*Pseudomonas aeruginosa E.coli*	Althunibat et al. 2016 Gutiérrez et al. 2017
Myrtaceae	*Streptococcus faecalis* *Staphylococcus epidermidis*	Sibi et al. 2012 Rathinam et al. 2017
Rubiaceae	*Pseudomonas aeruginosa*	Abdulhamid et al. 2014
Quinones		
Boraginaceae	*S. aureus*	Papageorgiou et al. 2008
Plumbaginaceae	*S. epidermidis*	Carson and Hammer 2011 Periasamy et al. 2019
Polygonaceae	*Helicobacter pylori*	Khalil et al. 2019
Resins		
Fabaceae	*Shigella dysenteriae*	Mariita et al. 2011
Labiatae	*P. aeruginosa*	Oboh 2010
Coumarins		
Apiaceae	*Salmonella typhi* *Enterococcus faecalis*	Tan et al. 2017 Basile et al. 2009
Fabaceae	*E. coli*	Mun et al. 2014 Jeong et al. 2009
Lauraceae	*S. aureus*	Ali et al. 2005
Rutaceae	Methicillin-resistant *S. aureus* (MRSA)	Basile et al. 2009
Organosulfur		
Alliaceae	*Acinetobacter baumannii* *P. aeruginosa*	Reiter et al. 2017
Amaryllidaceae	Methicillin-resistant *S. aureus* (MRSA)	Reiter et al. 2017
Brassicaceae	*Helicobacter pylori*	Haristoy et al. 2005
Liliaceae	*Klebsiella pneumoniae*	Reiter et al. 2017
Resedaceae	Methicillin-resistant *S. aureus* (MRSA)	Reiter et al. 2017
Tropaeolaceae	*St. pneumonia*	Reiter et al. 2017

(continued)

Table 2.1 (continued)

Secondary metabolites and plant families	Antibacterial activity	References
Terpenoids		
Lamiaceae	Methicillin-resistant *S. aureus* (MRSA)	Althunibat et al. 2016 Qiu et al. 2010
Lamiaceae	*E. coli*	Althunibat et al. 2016
Myrtaceae	*P. aeruginosa* Methicillin-resistant *S. aureus* (MRSA) *E. coli*	Althunibat et al. 2016 Togashi et al. 2010 Althunibat et al. 2016 Gutiérrez et al. 2017
Rutaceae	*H. pylori*	Ali et al. 2005
Phenols		
Ericaceae	*E. coli*	Gutiérrez et al. 2017
Scrophulariaceae	*E. coli*	Gutiérrez et al. 2017
Vitaceae	*Campylobacter* spp.	Klancnik et al. 2017
Lactones		
Apocynaceae	*M. tuberculosis*	Kumar et al. 2013
Asteraceae	*M. tuberculosis*	Kalani et al. 2019
Benzoic acid		
Scrophulariaceae	*P. aeruginosa*	Gutiérrez et al. 2017
Diarylheptanoid		
Asteraceae	*P. aeruginosa*	Wu et al. 2008
Zingiberaceae	*P. aeruginosa*	Tyagi et al. 2015
Polyphenol		
Myrtaceae	*E. coli*	Gutiérrez et al. 2017
Vitaceae	*E. coli*	Gutiérrez et al. 2017
Diterpenoid		
Acanthaceae	*M. tuberculosis*	Prabu et al. 2015
Euphorbiaceae	*M. tuberculosis*	Jung et al. 2016
Iridoid lactone		
Apocynaceae	*M. tuberculosis*	Kumar et al. 2013
Sesquiterpene lactone		
Asteraceae	*M. tuberculosis*	Kalani et al. 2019

Hossain et al. 2017). Gamma-aminobutyric acid (GABA), which is generated by some plants, promotes lactonase (AttM) degradation of the OHC8HSL AHL signal in *Agrobacterium tumefaciens*, limiting the QS-dependent infection process. The extracts from the Annurca apple having various polyphenols, such as hydroxycinnamic acids, rutin, and epicatechin, revealed anti-quorum sensing (AQS) activity against *Chromobacterium violaceum* (Fratianni et al. 2013). Cinnamaldehyde and its derivatives have an impact on a range of QS-regulated activities, including biofilm formation in *P. aeruginosa* and AI-2-mediated QS in various *Vibrio* species (Brackman et al. 2008; Niu et al. 2006). Garlic extracts have

been shown to suppress QS in *P. aeruginosa*, reducing biofilm formation and thereby aiding the bacteria's clearance (Bjarnsholt et al. 2005). Similarly, vanilla extracts have been reported to hamper with QS in *C. violaceum*, suggesting that eating vanilla-flavored meals may be helpful (Choo et al. 2006). Many plants produce polyphenol chemicals with a gallic acid moiety, such as epigallocatechin gallate, ellagic acid, and tannic acid, which can particularly interfere with AHL-mediated signalling by preventing bacteria-to-bacterium transmission (Hao et al. 2021; Bouyahya et al. 2017; Slobodníková et al. 2016).

It was reported that in case of a clinically import ant strain, i.e., *S. aureus*, baicalein (5,6,7-trihydroxyflavone) was found to lower levels of enterotoxin A (SEA) and hemolysin (hla) (Chen et al. 2016). At sub-inhibitory concentrations (32 and 64 g/ml), baicalein treatment significantly reduced the expression of the quorum sensing regulators agrA, RNAll, and sarA and the expression of the ica gene.

Quercetin, a common flavonoid, interacts to the QS receptor protein LasR and inhibits its capacity to bind promoter regions of DNA, lowering total QS gene production. The presence of two hydroxyl groups in the flavone A ring is required for suppression of QS-related self-regulatory proteins in *P. aeruginosa*, according to structure–activity relationship (SAR) analyses of various flavonoids. Among the plant-derived pigments, zeaxanthin was tested for QS inhibitory action using two *P. aeruginosa* fluorescent monitor strains, lasB-gfp and rhlA-gfp. The levels of gene expression of lasB and rhlA were shown to decrease in a concentration-dependent way (Gökalsın et al. 2017). Quorum sensing is known to influence the expression of numerous virulence factors. Attenuating pathogenicity in bacteria through QS interference is predicted to result in disease control, especially where antibiotics are ineffective owing to the development of multidrug resistance.

2.4 Future Prospects

Nature provides a rich supply of bioactive substances that are readily available, inexpensive, and simple to extract with little risk to humans. Phytochemicals have emerged as a possible alternative to conventional antibacterial medicines. A majority of antimicrobial phytochemicals lack thorough structure–activity relationship (SAR) data, which has been done for many classes of microbial antibiotics. The variety in extraction procedures and antibacterial assays utilized is a key barrier for identifying new antibacterial compounds from plants. Depending on their ability to suppress the growth of microorganisms, different phytochemicals have different antimicrobial properties. They come in a variety of forms, each with improved efficacy against a variety of diseases and pathogens. Modern approaches have been employed with traditional ways for extracting phytochemicals to boost yield and productivity. The key barrier in the development of novel phytochemicals has been translating in vitro investigations into in vivo experiments and then into human clinical trials. The challenge is particularly difficult in the case of natural antimicrobial drugs/

antibacterial phytochemicals, because a variety of parameters, such as tissue penetration, maximum plasma concentration, and bioavailability, might affect their activity. For example, phenolic natural compounds are easily glucuronidated by hepatic enzymes, which have a significant impact on tissue penetration and plasma levels. To boost phytochemical antibacterial activity, various nanoformulations can be developed using liposomes, dendrimers, micelles, and polymers. The development of new antimicrobial metabolites from medicinal plants is a promising approach to combating to human diseases' increasing treatment resistance. Scientists have conducted studies on many plant families to identify antibacterial properties of phytochemicals, and experimental investigations to evaluate the biological activities of numerous plants should be conducted in the future. Another appealing application of phytochemicals is their potential in combination with other antibacterial products, which merits more investigation.

2.5 Conclusion

Plant-derived chemicals or herbal medicine offer a significant contribution to primary healthcare and have shown considerable promise in modern phytomedicine for a variety of diseases and ailments in today's world. Scientists have looked to nature for solutions to the fast growth of bacterial resistance to conventional antibiotics. As a source of novel antimicrobials, plants offer a lot of promise. They are commonly available, inexpensive, and almost without negative effects. Numerous investigations have been conducted, and the medicinal potential of plant-derived substances has been established. Thousands of phytochemicals have been discovered all over the world that exhibit antibacterial, antifungal, and antiviral action against a variety of diseases. When used with antibiotics, the MIC values of the antibiotics are reduced, and synergistic effects are observed.

Phytochemicals, in general, damage the bacterial membrane, reduce some virulence factors such as enzymes and toxins, and hinder the production of bacterial biofilms. However, a lot of significant work needs to be done in vitro and in vivo to assure the identification of active and nontoxic antimicrobial phytochemicals. Antibacterial agents with a new mechanistic approach should be sought quickly to tackle the problems of antibiotic resistance.

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Conflict of Interest None to declare.

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Chapter 3
Quorum Quenching Enzymes: A Potent Alternative to Conventional Antibiotics

Manab Deb Adhikari, Abhrajyoti Roychowdhury, and Bipransh Kumar Tiwary

Abstract Antibiotic resistance has emerged as a serious problem in the twenty-first century. The inefficacy of conventional antibiotics for the treatment of bacterial infections has been a major concern in recent years. This led to a situation where most of the diseases caused by bacteria will become incurable, thus necessitating the development of innovative alternative antibacterials. Promisingly, the study of bacterial intercellular communication and its relation to pathogenesis has provided a direction to develop a novel strategy to treat bacteria-mediated infections, known as anti-virulence therapy. The intercellular communication between bacterial cells using diffusible signalling molecules (autoinducers) is known as quorum sensing (QS). Currently, quorum sensing inhibitors (QSIs), which interfere with bacterial pathogenicity and/or cell-to-cell signalling pathways, have been selected as the promising alternatives to conventional antibiotics for controlling infection without any selective pressure among bacterial pathogens. Quorum quenching (QQ) is the silencing of quorum sensing signals by enzymatic degradation or modification. Quorum quenching enzymes are common in bacteria and have also been discovered in eukaryotes. Lactonases and acylases that hydrolyze N-acyl homoserine lactone (AHL) signalling molecules have received the greatest attention as QQ enzymes. However, oxidoreductases are reported to target AHLs or 2-alkyl-4(1H)-quinolone. So far, the research on quorum quenching enzymes, which interfere with the production of virulence factors, strengthens the submission for the development of potential antibacterial strategies alternatives to traditional antibiotics. In this chapter, the basic mechanism of QS in both Gram-positive and Gram-negative bacteria, the promising anti-virulence strategies and quorum quenching enzymes for the future treatment of bacterial infections has been discussed. Further, modification of QQ enzymes to enhance their therapeutic efficiency and medical applications have also been discussed.

M. D. Adhikari · A. Roychowdhury
Department of Biotechnology, University of North Bengal, Darjeeling, West Bengal, India

B. K. Tiwary (✉)
Department of Microbiology, North Bengal St. Xavier's College, Rajganj, Jalpaiguri, West Bengal, India

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Abbreviations

QS	Quorum sensing
QQ	Quorum quenching
QSIs	Quorum sensing inhibitors
AHL	Acyl homoserine lactones
MLLs	Metallo-β-lactamase-like lactonase
MNPs	Magnetic nanoparticles
PLLs	Phosphotriesterase-like lactamases
cPANFs	Carboxylated polyaniline nanofibers
PVDF	Polyvinylidene fluoride

3.1 Introduction

Antibiotics are traditional choices for the combat of bacterial infections and diseases. Since the discovery of penicillin in 1940, antibiotics have saved the lives of millions of people all over the world (Theuretzbacher et al. 2020). However, the indiscriminate and overuse of antibiotics augmented the rate of emerging antibiotic-resistant bacteria and have led to a "post-antibiotic era" (Walsh 2003). The emergence of multiple antibiotic-resistant bacteria creates a fear that the bacterial diseases may become incurable in the near future. Hence, there is a dire need to discover novel drugs and alternative approaches to minimize and prevent the spread of resistant microorganisms. The conventional approaches to control bacterial infection are primarily identified as antibiotics, which are derived from other microorganisms. The modes of action of antibiotics mainly rely on the inhibition of the growth by inhibiting or interrupting the bacterial processes such as DNA replication, protein synthesis and cell wall synthesis, which are required for their growth (Dong et al. 2007), though they are incredibly effective, but they have put the bacteria under a lot of evolutionary pressure. As a result, the bacteria that were targeted quickly began to select for resistant variants (Werner et al. 2008). So various alternative approaches are being studied that focus on the inhibition or interruption of virulence rather than of bacterial growth. The virulence factors are essential for the pathogenesis of infections because they assist bacteria to evade the host's immune response and cause diseases (Jiang et al. 2019), whereas most virulence factors are not required for bacterial survival. So such approaches impose minimal "life or death" stress to the target bacterium, resulting in minimum possibility for emergence of

resistant variants (Rasko and Sperandio 2010). Quorum sensing (QS) is a type of bacterial communication system that relies on secreted signalling molecules, which regulate coordinated responses across a bacterial population (Tiwary et al. 2017). It is reported that the production of virulence factors in different bacterial strains is regulated by quorum sensing (Santhakumari and Ravi 2019; Saurav et al. 2016).

In recent years, many studies have been published that disruption of QS plays a vital role in controlling the production of virulence factors in bacteria. This explores the novel approaches to disrupt the QS system for the discovery of novel antimicrobial therapies (Hemmati et al. 2020; Jiang et al. 2019). It seems to be potential broad-spectrum therapeutic strategy needs to be discovered. However, multiple approaches have been considered for this strategy, including the use of QS inhibitors (QSIs) and quorum quenching (QQ) enzymes. The quorum quenching enzyme interrupts the quorum sensing system by interfering with the expression of the genes of virulence factors rather than killing or inhibiting the cell growth (Sikdar and Elias 2020). It seems a potential strategy as alternatives to conventional antibiotics, and if the signal communication that coordinates these pathogenic traits is blocked, bacteria might lose their ability to produce virulence factors to attack host. Consequentially, the emergence of antibiotic resistance would be minimized (Cegelski et al. 2008) because their capability to form organized community structures within the host is interrupted due to lack of communication signals.

3.2 Quorum Sensing: The Bacterial Way of Communication

The phenomenon of quorum sensing was first discovered by Nealson et al. in 1970 in Gram-negative *Vibrio fischeri*, a marine bacterium, in a free living or in symbiotic relationship with squids and fishes (Waters and Bassler 2005).

The discovery of Nealson et al. illuminates that bioluminescence of *V. fischeri* is regulated by regulatory molecules or autoinducers that work in a density-dependent manner. QS is a bacterial cell-to-cell communication that is monitored by small diffusible signalling molecules, known as autoinducers, and, in turn, regulates the expression of multitudinous genes (Ghosh et al. 2014). The QS has found to be participating in the regulation of various physiological processes of bacteria, e.g., biofilm formation, motility, bioluminescence, virulence factor secretion, etc. on a density-dependent manner. However, different types of QS signalling can be observed in Gram-positive and Gram-negative bacteria. The autoinducers identified in bacteria are mainly classified into three major types such as homoserine lactones (HLs), autoinducing peptides (AIPs), and autoinducer-2(AI-2). Gram-negative bacteria mainly produce and sense N-acetyl homoserine lactones (AHLs), whereas Gram-positive bacteria produce and sense AIPs, while both Gram-positive and Gram-negative bacteria are reported to produce and sense AI-2 (Fig. 3.1) (Li and Nair 2012).

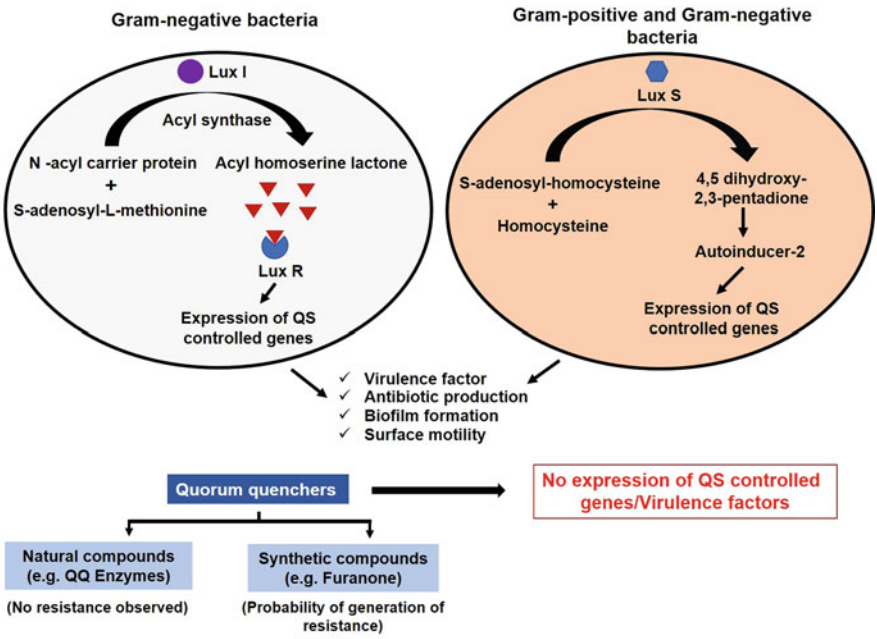

Fig. 3.1 Quorum sensing and quorum quenching in Gram-positive and Gram-negative bacteria

3.2.1 Homoserine Lactones

Acyl homoserine lactones (AHLs) are the most common autoinducers found among Gram-negative bacteria. They are composed of a core N-acyl homoserine lactone ring and a 4–18 carbon acyl chain with a C3 modification. AHLs are produced from S-adenosyl-L-methionine (SAM) and acylated acyl carrier protein (acyl-ACP) by the enzyme LuxI AHL synthase (Watson et al. 2002). In the presence of AHL synthase, the carbonyl C1 of acyl chain of acyl-ACP gone under a nucleophilic attack by amine group of SAM followed by lactonization, which results in the production of homoserine lactone. When the secreted AHL attained the threshold concentration, the complex of AHL and LuxR receptors regulates the transcription of target genes. The structure of acyl chain present in AHLs determines the specificity of receptor (LuxR)–AHL binding. AHL receptors varies from species to species, for example, in the case of *Vibrio* sp., where LuxIR system was observed, whereas in *Agrobacterium tumefaciens*, TraIR has been found to play the same role. Other examples like LasIR and RhlIR in *Pseudomonas aeruginosa*, CviIR in *Chromobacterium violaceum*, ExpIR in *Erwinia carotovora*, SmaIR in *Serratia* sp., and AhyIR and AsaIR in *Aeromonas* sp. have been discovered (Hauser 2011).

3.2.2 Autoinducing Peptides (AIPs)

AIPs are found especially in Gram-positive bacteria. After reaching the threshold concentration, the peptides trigger the quorum sensing cascade that led to induction of their own biosynthesis; hence, they are referred to as autoinducing peptides (Kavanaugh et al. 2007). Most of the Gram-positive bacteria possess membrane-bound two-component system that identifies autoinducing peptides (Waters and Bassler 2005). AIPs are produced inside the bacterial cell as pro-AIPs and further undergo different modification, inside or outside the cell such as posttranslational modifications. Being impermeable to cell membrane, AIPs are carried out by cell membrane–bound histidine kinases. Similar to the AHL, when the AIP concentration reaches the threshold level, they are detected by the kinases that results in initiation of signalling processes. It triggers QS-regulated physiological processes such as competence, sporulation, and virulence initiation in *Bacillus subtilis* (sporulation), *Staphylococcus aureus* (virulence), *Listeria monocytogenes*, *Clostridium perfringens*, and *Enterococcus faecalis* (Vadakkan et al. 2018).

A classic example for two-component quorum sensing system is observed in *Staphylococcus aureus* (Thoendel and Horswill 2010). A study carried out by Thoendel and Horswill (2010) suggests that QS is regulated by Arg locus, which is a combination of two transcripts RNA II and RNA III. In brief, initially pro-AIP is produced by *Arg D* (47 amino acid residues), which is then processed to 9 residue peptide and further modified and transported by Arg B. At high bacterial density, the activated transmembrane histidine kinase phosphorylates after binding with AIP (Le and Otto 2015). The available phosphate group interacts with Agr A and phosphorylates this response regulator. These together activate the two-component system comprised of Agr C and Agr A, which activates the transportation of RNA II. However, RNA II continues the quorum sensing circuit, but transcript RNA III is responsible for the virulence (Vadakkan et al. 2018).

3.2.3 Autoinducer-2

AI-2 molecule is synthesized and detected by both Gram-positive and Gram-negative bacteria and archaea (Miller and Gilmore 2020). AI-2 is synthesized by LuxS synthase in the S-adenosyl-methionine (SAM) recycling pathway (Nichols et al. 2009). AI-2 is imported and bound by its cognate receptor leading to a cascade of phosphorylation signalling pathways regulating phenotypes such as virulence and bioluminescence. AI-2 signalling systems can be observed in various bacterial species, e.g., LuxPQ system in *Vibrio* sp and Lsr system in *Salmonella* sp. and *Escherichia coli* (Pereira et al. 2013). In *E. coli*, LuxS produces AI-2, during active growth, and their accumulation takes place in the extracellular space. After attaining the threshold value, it triggers the activation of the receptors of Lsr system. LsrACDB and other proteins encoded by the genes of the Lsr operon are involved

in the regulation of gene expression and internalization of AI-2. They are also involved in intracellular metabolic degradation of AI-2 (Quan et al. 2017).

3.3 Inhibition of Quorum Sensing

The detailed knowledge of circuit and signalling of QS provides the opportunities to increase the number of therapeutic approaches that can sustain the efficacy of current antibiotics and minimize further pressure for the evolution of new drug-resistance mechanisms. In this regard, targeting bacterial virulence can be used to disarm pathogens in the host. Hence, interfering with QS systems has been considered an innovative strategy for the development of alternative antimicrobials to prevent or attenuate bacterial infections. QS systems can be interfered or interrupted by blocking the interaction of autoinducer with the cognate receptors without disturbing the signal integrity. The process of inhibition of QS is referred to as quorum quenching (QQ), and the agents that cause inhibition is known as quorum sensing inhibitors (QSIs) or quorum quenchers (Sikdar and Elias 2020). Although QQ can be achieved by various means, in this chapter, the basics and potentials of enzyme-mediated QQ have been discussed. Because of lesser cytotoxicity, a wide range of target and natural enzyme-mediated QQ are the major factors that are attracting the interest of researchers. Such enzymes, through the interference in QS, can effectively behave as virulence inhibitors. It is required to demonstrate the variety of QS interference strategies and the numerous enzymatic ways to inactivate signalling molecules.

3.4 Quorum Quenchers Against AHL-Mediated Signalling

AHL-mediated QS systems are the mostly studied QS system among bacteria. They can control virulence gene expression in multiple pathogens, including agricultural and human pathogens and marine habitats. There are numerous enzymes studied till now, which have shown inhibiting or quenching activity against quorum sensors (Table 3.1). According to their mode of action, they can be classified into three main groups; those are lactonases, acylases, and oxidoreductases (Fig. 3.2) (Reina et al. 2021).

3.4.1 Quorum Quenching Activity of Lactonases

AHL-degrading enzymes can be found in organisms beyond the bacterial world that are in plants, fungi, archaea, and mammals. Among them, the most widely used and studied QQ enzymes are lactonases. Lactonases degrade the AHLs by hydrolyzing

Table 3.1 Quorum quenching enzymes against AHL-mediated signalling

Type of enzyme	Enzyme family	Enzyme	Substrate	Source (references)
AHL lactonase	Metallo-B-lactamase-like lactonases (MLLs)	AiiA	C4-12-HSL	*Bacillus* sp. (Huma et al. 2011)
		AttM	C6-10-HSL	*Agrobacterium tumefaciens* (Haudecoeur et al. 2009)
		AiiB	OC8-HSL	*Agrobacterium tumefaciens* (Haudecoeur et al. 2009)
		AhlK	C6-8-HSL	*Klebsiella pneumoniae* (Chan 2013)
		Aii20J	Broad	*Tenacibaculum* sp. (Mayer et al. 2015)
		AhlS	C-10HSL	*Solibacillus silvestris* (Morohoshi et al. 2012)
		AhlD	C6-C10HSL	*Arthrobacter* sp. (Park et al. 2003)
AHL lactonase	Phosphotriesterase-like lactamases (PLLs)	Qsd A	C6-14-HSL with or without C3-substitution	*Rhodococcus erythropolis* (Barbey et al. 2018)
		GKL	C10-12-HSL	*Geobacillus kaustophilus* (Go et al. 2018)
		MCP	C4-3OC12-HSL	*Mycobacterium avium* (Rao et al. 2000)
		SsoPox	C8-12-HSL	*Sulfolobus solfataricus* (Rémy et al. 2020)
	Paraoxonases	PON1	3OC12-HSL	Liver (Camps et al. 2009)
		PON2	3-Oxo-C12-HSL	Various tissues of the brain, heart (Mackness et al. 2010)
		PON3	3-Oxo-C12-HSL	
				Kidney (Schweikert et al. 2012)
AHL acylases	Ntn hydrolases	PvdQ	*C-12-HSL*, 3-oxo-C12-HSL	*Pseudomonas aeruginosa* (Jimenez et al. 2010)
AHL acylases Oxidoreductase	Ntn hydrolases	AiiD	C8-12-HSL	*Ralstonia* sp. (Lin et al. 2003)
		QuiP	C-12-HSL	*Pseudomonas aeruginosa* (Wahjudi et al. 2011)

(continued)

Table 3.1 (continued)

Type of enzyme	Enzyme family	Enzyme	Substrate	Source (references)
		AhlM	C8-HSL	*Streptomyces* sp. (Park et al. 2005)
		AiiC	C10-HSL	*Anabaena* sp. (Romero et al. 2008)
		AibP	C12-HSL	*Brucella melitensis* (Terwagne et al. 2013)
		AAC	C-10-HSL	*Shewanella* sp. (Morohoshi et al. 2008)
		AiiO	3-Oxo-C8-HSL	*Ochrobactrum* sp. (Xia et al. 2020)
Oxidoreductase	Cytochrome P450	CYP102A1	C12-HSL	*Bacillus megaterium* (Kang et al. 2011)

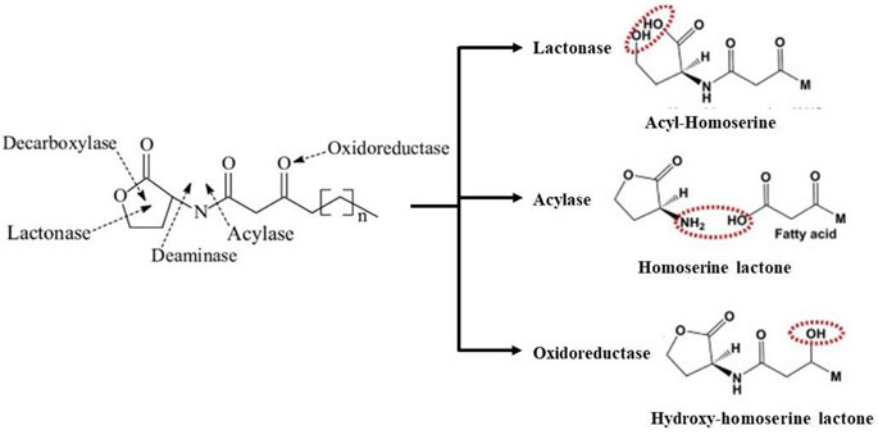

Fig. 3.2 Degradation or modification of N-acyl homoserine lactone signalling molecule by different quorum quenching enzymes: lactonase, acylase, and oxidoreductases

ester bond of the lactone ring of the signalling molecule, thereby forming an open ring structure. These enzymes are found to be highly specific toward acylated lactones with very less to no activity against non-acylated HLs (Amara et al. 2011), although the specificity varies from one lactone to other. In the study carried out by Rémy et al. (2020) with synthetic AHLs of *P. aeruginosa*, it was observed that GcL efficiently hydrolyzed both C4 and 3-oxo-C12 HSL, whereas SsoPox W263I efficiently degraded 3-oxo-C12 HSL but exhibited poor activity toward short-length HSL (C4 HSL). Lactonases can be characterized mainly into three protein families such as metallo-β-lactamase-like (MLLs) or autoinducer inactivator

A (AiiA)-like lactonases, phosphotriesterase-like lactonases (PLLs), and paraoxonases (PONs) (Bergonzi et al. 2018).

3.4.1.1 Metallo-β-Lactamase-Like (MLLs) or AiiA-Like Lactonases

MLLs or AiiA-like lactonases are one among the major classes of lactonases. Autoinducer inactivator A (AiiA) is the most characterized enzyme from this family. AiiA was first discovered in *Bacillus thuringiens*is, having the ability to hydrolyze AHLs (Amara et al. 2011). The main structural highlight of this enzyme family is the presence of a conserved metal-binding motif "HXHXDH" (Bergonzi et al. 2018). HXHXDH motif exhibits a characteristic αβ/βα fold bonded with two metal cations (zinc cations), coordinated by five histidines and two aspartates. Lactone binds the bimetallic center via the two oxygen atoms of its ester group. However, the two metals are bridged by a water molecule. Then, the two metals make a nucleophilic attack on the sp2 carbonyl carbon of the lactone, in turn, forming a tetrahedral intermediate. This activated intermediate breaks down the lactone ring and yields the open ring form of carboxylate and alcohol (Bergonzi et al. 2018).

These lactonases are very proficient enzymes and are found to exhibit a broad AHL substrate preference with respect to the acyl chain length of AHLs (Bergonzi et al. 2018). The mostly characterized enzymes from this family, the autoinducer inactivator A (AiiA), AiiB, AidC, MomL, or GcL, have shown great efficiency to degrade the quorum sensing signals (Rutherford and Bassler 2012). Numerous metallo-β-lactamase-like lactonases have been found among the wide range of microorganisms, AttM, AiiB in *Agrobacterium tumefaciens* (Haudecoeur et al. 2009), AhlK, AhlD in *Klebsiella pneumoniae*, etc. (Chan 2013).

3.4.1.2 Phosphoesterase-Like Lactonases (PLLs)

PLLs are natural AHL lactonases having a promiscuous activity toward organophosphate pesticide (Afriat-Jurnou et al. 2012). PLLs are categorized under amidohydrolase superfamily. Unlike the metallo-β-lactamases, amidohydrolases do not possess a unique fold but instead exhibits a (α/β)8 fold (TIM barrel) (Elias and Tawfik 2012). PLLs can be found in both bacteria and archaea. SsoPox, a phosphoesterase-like lactonase isolated from *Sulfolobus solfataricus*, have shown scavenging activity against a broad range of organophosphate molecules (Hiblot et al. 2012). PLLs are also reported in several extremophiles (Manco et al. 2018). Interestingly, they exhibit remarkable thermal stability (up to 128 °C). Furthermore, this major class of lactones can be classified into two subclasses, phosphoesterase-like lactones A (PLL-A) and phosphoesterase-like lactones B (PLL-B), where PLL-As can hydrolyze δ-lactones, γ-lactones, and AHLs and PLL-Bs are found to be more specific toward δ-lactones and γ-lactones (Bergonzi et al. 2018).

3.4.1.3 Paraoxonases

Unlike PLL and MLL families, paraoxonases were first identified in mammals instead of bacteria. The name paraoxonases is derived from their ability to hydrolyze paraoxons, a parathion derivative (Furlong et al. 2016). Paraoxonases are known to have the same function of lactone hydrolysis as its counterparts only differ in their substrate specificity (Ma et al. 2009). Substrate specificity varies from narrow to a wide range of lactones and lactone derivatives.

Derivatives from mammalian liver isolates are characterized under three families such as PON1, PON2, and PON3 (Shamir et al. 2005). PONs are composed of a six-bladed propeller fold and a central tunnel with two calcium cations. The propeller fold plays a great role in structural integrity, whereas the central tunnels stand for catalytic activity (Khalil et al. 2021). Although initially characterized as mammalian isolates, later on, paraoxonases were also found in bacteria (Harel et al. 2004). However, the isolates from both mammal and the bacteria were found to efficiently hydrolyze N-acyl homoserine lactones (Rehman and Leiknes 2018). Among the three main classes of PONs, PON2 has more specificity toward long acyl chain containing HSLs (Murugayah and Gerth 2019), whereas PON1 and PON3 have shown less activity against HSLs (Elias and Tawfik 2012). Enzymatic activity of PONs has been observed against lactone like and lactones with lipophilic side chains (Elias and Tawfik 2012).

3.4.2 Role of Acylases in Quorum Quenching

Acylases were first discovered in a soil isolate, *Variovorax paradoxus* (Leadbetter and Greenberg 2000). They are one among the three major kinds of AHL inhibitors specifically known for their ability to hydrolyze the amide bond present between HLs, and the acyl chain binds with it (Romero et al. 2015). The first gene encoding for an AHL acylase, called *AiiD*, was cloned from an isolate of *Ralstonia* sp. XJ12B, a biofilm producer (Lin et al. 2003). A study carried out by Lin et al. illuminates the structural characteristics of AiiD. They have suggested that the acylase is composed of well-conserved domains made up of signal peptide followed by an α-subunit, an spacer sequence, and a β-subunit and residues with AHLs degrading activities (Lin et al. 2003). Their study also suggested that AiiD has similarity with N-terminal nucleophile (Ntn) hydrolase–like cephalosporin and penicillin acylases. However, the incapability of this quorum quenchers to degrade penicillin G or ampicillin suggests that AiiD has probably evolved to serve as a dedicated AHL acylase (Lin et al. 2003). Another AHL acylase AibP discovered in the intracellular pathogen *Brucella melitensis* was found to have the ability to degrade endogenous AHL during macrophage infection. Acylase AhlM, an isolate from *Streptomyces* sp., was found to degrade a wide range of AHL (Terwagne et al. 2013). The penicillin G acylase KcPGA from *Kluyvera citrophila* was reported to cleave AHLs with acyl

chains between 6 and 8 carbons in size without oxo substitutions (Mukherji et al. 2014). A well-characterized acylase, PvdQ from *P. aeruginosa*, has shown quorum quenching activities against AHLs with acyl chain lengths exceeding 10 carbons. AHL acylase activities were also found in the *Anabaena* sp. PCC7120 (AiiC; Romero et al. 2008), *Shewanella* sp. (Aac; Morohoshi et al. 2008), *Ochrobactrum* sp. (AiiO; Czajkowski et al. 2011), *Comamonas* sp., and *Rhodococcus erythropolis* (Uroz et al. 2007).

3.4.3 Role of Oxidoreductases in Quorum Quenching

Along with its two counterparts, oxidoreductase plays a great role as a quorum quencher in a wide variety of microorganisms. In contrast, oxidoreductase does not degrade the AHL signalling molecules but acts on by affecting the specificity of HSL receptor binding. Oxidoreductase P450BM3, an isolate from *Bacillus megaterium*, has shown activity against a wide range of substrates such as C12-18-HSL, 3-oxo-C12-HSL, 3-oxo-C14-HSL, C18-HSL, and C20-HSL (Lade et al. 2014). The activity of quorum quenching studied in *Rhodococcus erythropolis* W2 (Uroz et al. 2007) suggests for substitution activity in 3-oxo position of HL signalling molecule with acyl side chains ranging from C8 to C14. This substitution results in the corresponding 3-hydroxy derivatives (Romero et al. 2015). Further, CYP102A1, a cytochrome P450 isolated from *Bacillus megaterium*, has shown the ability to oxidize AHLs and their lactonolysis products at the ω-1, ω-2, and ω-3 carbons of the acyl chain (Chowdhary et al. 2007; Romero et al. 2015). It is also reported that AHL oxidation can make the acyl-homoserines more water soluble and membrane permeable (Chowdhary et al. 2007). NADPH oxidases from phagocytes can able to inactivate *Staphylococcus aureus* autoinducing peptides by oxidizing the C-terminal methionine (Rothfork et al. 2004). A novel oxidoreductase BpiB09 derived from a metagenomic library has shown significant activity against 3-oxo-C12-HSL. Its expression in *P. aeruginosa* PAO1 resulted in significantly reduced pyocyanin production, decreased motility, and poor biofilm formation, although AHLs are likely not the native substrate of this metagenome-derived enzyme (Table 3.1) (Chen et al. 2013).

3.4.4 Quorum Quenching Enzymes Against Other Signalling Molecule

Besides mostly studied AHL signal quenchers, some enzymes have also shown potent inhibiting or quenching activity against other signalling molecules such as the cytochrome P450 of *Bacillus megaterium* (Grandclément et al. 2016), which has been found to be capable of oxidizing the QS signal of the yeast *Candida albicans*

in vitro (Ramage 2002). In *E. coli* and *Salmonella typhimurium*, AI-2 is phosphorylated (P-DPD) by the kinase LsrK. Phosphorylation of AI-2 destabilizes the signalling molecule, thereby preventing intracellular uptake by its cognate receptor and inhibiting the signalling. The quinolone-based QS signals identified in *Burkholderia* sp. (AQ) and *P. aeruginosa* (PQS) found to be degraded by a class of enzymes known as quinolone dioxygenases (Müller et al. 2014). These enzymes inhibit the signalling by opening the PQS ring to form N-octanylanthranilic acid and carbon monoxide. QS signals such as 3-OH PAME can be degraded by recently identified microbial esterase (Sikdar and Elias 2020).

3.5 Advancement of Quorum Quenchers Leads to Higher Effectivity

Rather than being a potential candidate against quorum signalling, commercial application of quorum quenching enzymes suffers from several limitations, such as high cost of production, lack of efficient recovery, and unavailability to reuse. To overcome these limitations and increase the efficiency, the researchers from all around the globe are working continuously to find out a widely acceptable way out. Different nanobiotechnological and protein engineering tools are currently under screening. These tools have been applied to enhance the efficacy and catalytic activities of QQ enzymes (Krzyżek 2019). Among the multiple great advancements in this field of research, some are mentioned below:

3.5.1 Nanotechnological Approaches

In the current scenario, the application of various nanoparticles (NPs) has become widespread to control bacterial infections as an alternative to antibiotics. Generally, NPs are known to follow the novel mechanism of action to target the pathogenic bacteria (Wang et al. 2017). Currently, the technological development in the field of nanoparticles (NPs) such as microbiologically synthesized NPs holds considerable promise in healthcare sectors and development of a better possibility for antibacterial therapies (Capeness et al. 2019). The combination of enzymes and nanomaterials for the enzyme immobilization is used for the effective development of synergetic interactions between enzyme immobilization methods with various nanomaterials and thus increases stabilization of enzyme activities compared to conventional enzyme immobilization approaches (Meena et al. 2021).

Quorum quencher enzymes have the potential to inhibit quorum sensing and thus to control the bacterial infection. For example, AiiA, an acyl homoserine lactonase synthesized by *Bacillus* sp. 240B1 (Bai and Rai 2018), is a strong candidate that can inhibit AHL-mediated QS in industrial and environmental samples. However,

commercial application of AiiA suffers from several limitations, which includes high cost of production of enzyme and lack of efficient recovery means for its reuse. To overcome this limitation, AiiA (r-AiiA) was cloned, expressed, and purified as recombinant enzyme and that covalently immobilized onto magnetic nanoparticles (MNPs). Immobilization of the enzyme makes handling and separation from the reaction mixture very comfortable and in some cases increases thermal and pH stability of enzyme (Beladiya et al. 2015). Gold nanoparticles coated with AHL lactonase proteins (AiiA AuNPs) obtained from *Bacillus licheniformis* were found to have a potent antibiofilm activity against multidrug-resistant (MDR) *Proteus* species. At 2–8 μM concentrations, it can cause reduction in exopolysaccharide production, metabolic activities, and cell surface hydrophobicity, without affecting macrophages. This study suggests that AuNPs coated with AiiA can attenuate pathogens without affecting host cells (Vinoj et al. 2015).

Immobilization of acylase on carboxylated polyaniline nanofibers (cPANFs) results in high enzyme loading and stability. Enzyme loading and stability increase 75 and 300 times, respectively, when immobilized on cPANFs by magnetically separable enzyme precipitate coating (Mag-EPC) (Lee et al. 2017). Kim et al. reported that immobilization of acylase directly onto a polyamide nanofiltration membrane results in a significant decrease in *Pseudomonas aeruginosa* PAO1 biofilm in a nanofiltration process (Kim et al. 2011). It was so effective that even after more than 20 iterative cycles of reaction and washing procedure, the acylase-immobilized membrane was found to retain more than 90% of its initial enzyme activity (Kang et al. 2011). In another study, immobilizing of acylase on intact carbon nanotubes found to retain 66% of its initial enzyme activity for 200 days under rigorous shaking condition. Further, anchoring this highly EAPC on the polyvinylidene fluoride (PVDF) microfilter using polydopamine coatings (EAPC membrane) increases the water permeability and effectively inhibited the biofilm formation by *P. aeruginosa* (Kim et al. 2018).

A study carried out by Zhu et al. stated that combining porcine kidney acylase-I (AC-I) with graphene oxide (GO) and polyvinylpyrrolidone (PVP), adhered to polyvinylidene fluoride (PVDF) casting solutions, may result in longer retention of biological activity of the acylase. It has showed the biological activity of PVDF/GO@AC/PVP membrane could be maintained for about 28–30 days (Zhu et al. 2018).

Silver nanoparticles (AgNPs) are one of the mostly used nanobactericides, used in wound dressing materials and medical devices/implants having a great potency against susceptible and drug-resistant bacteria. They are promising agent against undesirable biofilms and nosocomial infections (Paladini and Pollini 2019).

Amino-bearing (AM) biopolymers are one of a kind of highly efficient membrane-damaging bactericides against both Gram-positive and Gram-negative bacteria. Ivanova et al. in their study suggested that layer-by-layer coating of QQ enzyme acylase and amino-bearing biopolymers with silver nanoparticles (AgNPs) enhanced the antibacterial and antibiofilm activities fourfold against the Gram-negative *Pseudomonas aeruginosa* (Ivanova et al. 2020). They have suggested that the coating of antibacterial AgNPs with membrane-disrupting biopolymer

creates high local positive charge density, which enhances the activity of the nanosized template, improves the interaction of novel hybrid NPs with bacterial membranes, and potentiates the bactericidal activity of AgNPs at lower dosage. Their study also suggests that inclusion of acylase in the nanohybrids inhibits the establishment of *P. aeruginosa* biofilm, thereby making the pathogen susceptible to lower concentration of AgNPs, which is safe to human cells (Ivanova et al. 2020). In a study, the synthesized AuNPs coated with AiiA protein (AiiA AuNPs) were tested for antibiofilm activity against *Proteus*. It was demonstrated that the maximum QQ activities of 2 μM AiiA AuNPs are by degrading N-hexanoyl-L-homoserine lactone (C6-HSL). Moreover, AiiA AuNPs also inhibited the production of exopolysaccharide production, hydrophobicity, metabolic activity, and biofilm formation of the isolated *Proteus* strains DPr1, DPr2, and DPr3 and *P. vulgaris* ATCC 49565 (Vinoj et al. 2015).

The easy production of nanoparticles by chemical reduction method and reduced toxicity when compared with various other nanomaterials have drawn intense scientific and technological interest for potential applications in biomedical field (Devi et al. 2013). These biocomposite materials exhibit unique thermal, mechanical, and biological properties (Balazs et al. 2006; Tang et al. 2006) compared to other free enzymes and proteins.

3.5.2 Protein Engineering Approaches

The protein engineering approaches are applied to increase the stability, modulate substrate specificity, and increase catalytic activity of QQ enzymes. Generally, two kinds of protein engineering approaches are accessible for engineering. The first one is rational designing and the other one is random designing. For rational designing, prior knowledge of protein sequence and their structure and function are required to carry out specific mutations (Bornscheuer and Kazlauskas 2011), whereas random designing is more a kind of blind approach and does not ask for prior knowledge of protein sequence and structure (Murugayah and Gerth 2019). For both approaches (individual or in combination), an enzyme with a decent yield, promiscuous activities, and higher temperature tolerance is used as a "starting template" (Murugayah and Gerth 2019).

3.5.2.1 Random Design

A quorum quenching phosphotriesterase-like lactonase (PLL) isolated from *Mycobacterium avium* was engineered by random mutagenesis coupled with a bioluminescence-based screen led to identification of a single point mutation at N266Y, which is found to enhance its catalytic activity and substrate specificity up to 32-fold. But unfortunately, this variant was identified very unstable for use in potential downstream applications (Chow et al. 2010). The random approach was

also carried out for a thermostable PLL from *Geobacillus kaustophilus* revealing that random mutagenesis led to a double mutant (Glu101Asn/Arg230Ile) with increased AHL reactivity and increased substrate specificity. Another study, AiiA from *Bacillus sp*., was modulated by the same approach that causes double and triple mutant with 7- and 6.1-fold improvement in catalytic activity for C6-HSL (Chow et al. 2010).

A study carried out with the crystal structure of PvdQ, an AHL acylase isolated from *P. aeruginosa*, suggested that a rational engineering approach can also alter the substrate range of PvdQ (from long-chain acyl HSLs toward shorter AHLs) (Sio et al. 2006).

3.5.2.2 Rational Design

Rational design is a powerful approach to directly target enzyme hotspot to improve the catalytic activity. SsoPox, a PLL isolated from the *Sulfolobus solfataricus*, has been chosen as template due to its hyperthermobility for engineering purposes. A rational mutagenesis approach identified a single point mutation W263V that increased the substrate specificity up to 54-fold and mutation W263I that increased the substrate specificity up to 45-fold (Billot et al. 2020). The PTE7-2/254R mutant has improved the catalytic activity of PTE lactonase isolated from *Brevundimonas diminuta* more than 2000-fold (Afriat-Jurnou et al. 2012).

In addition to enhancing activities, this approach is also used to increase the protein solubility or expression in a heterogenous host. The human paraoxonase huPON2 can hydrolyze various lactones, but unfortunately due to self-aggregation property, it cannot be expressed in soluble form. So by using rational approach, three highly hydrophobic helixes have been replaced with hydrophilic polypeptide linkers, which produced two mutants (D2 and E3) with higher protein yield. Further, fusion to maltose-binding protein (MBP) increases soluble expressions up to 50-fold (Liu et al. 2016).

Both random mutagenesis and rational design approach resulted in a new enzyme variant with strongly enhanced activities, involving both the rational design and random design approaches with a thermophilic lactonase from *Geobacillus kaustophilus* (Murugayah and Gerth 2019). Comparatively, the study carried out by Liu et al. (2016) with thermostable metallo-β-lactamase-like lactonase Est816 suggests that protein engineering by using a combined method of directed evolution and rational design enhanced AHL-degrading activity. This tailoring leads to formation of a variant with two-point mutations (Ala216Val/Lys238Asn). These mutants are found having a threefold higher catalytic efficiency toward C8-HSL (Liu et al. 2016). Another study suggests that a site-saturation mutagenesis at Trp residue (Trp263) found in the active site loop of SsoPox (a hyper thermostable PLL isolates from the archaeon *Sulfolobus solfataricus*) gives rise to a variant Trp263Ile. The resulting Trp263ile variant is 45-fold more active against 3-oxo-C12-HSL. A study reported that in *Burkholderia* sp. (found in the lungs of cystic fibrosis [CF] patients), a computational study combined with in vitro testing of site-directed

mutants revealed a double mutant (α-Leu146Trp, β-Phe24Tyr) for C8-HSL. These mutations resulted in an increased hydrolytic activity toward C8-HSL, whereas these lower the activity toward C12-HSL (Koch et al. 2014).

So far, the modulation in quorum quenching enzyme by biotechnological approaches are mainly focused on enhancing the stability and activity of a quorum quenchers. Besides structural and kinetic studies, numerous studies were done on "downstream" methodologies that mainly focus on enzyme formulation and delivery system. QQ enzymes are continuously under screening for their potential application in several fields including human health. For example, formulated dry powders of acylases and lactonases have been identified suitable for pulmonary delivery (Murugayah and Gerth 2019). As QQ disrupts bacterial communication (QS), which regulates various phenotypes associated to virulence including biofilm formation, plays an important role in the medical field to control bacterial infections.

3.6 Medical Applications of Quorum Quenching Enzymes

The rapid emergence of antibiotic-resistant bacteria is a major threat to the field of public healthcare. It has become an urgent research topic to find new methods to inhibit bacterial infection and solve the problem of bacterial drug resistance (Saurav et al. 2016). QQ enzymes have been reported as potent antibacterial agents in vitro. However, several studies have demonstrated the efficacy of QQ enzymes against pathogenic bacteria and their clinical prospects.

Quorum quenchers such as acylases and paroxonases have been identified from humans, rice, mouse, and zebrafish (Teame et al. 2020). Acylase I from porcine kidney has been used in aquaculture and in the healthcare sector to inhibit AHL-mediated biofilm formation by *Aeromonas hydrophila* and *Pseudomonas putida* (Dong and Zhang 2005; Paul et al. 2009). Paraoxonases from human epithelial cells and from the serum of mammals such as rats, goats, bovines, and horses are found to have the ability to inhibit AHL-mediated QS in *Pseudomonas aeruginosa* (Stoltz et al. 2007). AHL lactonase could hydrolyze quorum sensing signalling molecules of the bacteria, thereby reducing its virulence (Rémy et al. 2020). Moreover, the effectiveness of quenching enzymes such as AHL lactonases B565, AIO6, AI-96, and QsdA against *A. hydrophila* was confirmed (Cao et al. 2012) in zebrafish in vivo (AI-96) and (Cao et al. 2012) in vitro. AIO6 QQ enzyme was also effective against *A. hydrophila* when tilapia was fed diets containing this enzyme (Teame et al. 2020).

Both acylases and lactonases have been successfully formulated as dry powders suitable for pulmonary delivery coating medical implants such as urinary catheters with the AHL-degrading *Aspergillus melleus* aminoacylase inhibiting *P. aeruginosa* PAO1 biofilm formation both in vitro and in vivo (Ivanova et al. 2015). Transgenic *Drosophila* harboring human paraoxonases 1 (PON1) were found to be resistant to infections caused by *P. aeruginosa* and *Serratia marcescens*. PON1 acts by degrading AHL signals of the pathogenic bacteria, which thus prevents pathogenesis

(Kalia and Kumar 2015). So, these QQ mechanisms can be used as therapeutic strategy against infections and inflammations.

It is studied that more than 80% of the bacteria can form biofilms and identified as the source of 65–80% of infections. Bacteria living within the biofilm have a higher resistance to antimicrobial agents as compared to free cells. Biofilm in bacteria can infect directly to the tissues or by the contaminated devices such as catheter (Lebeaux et al. 2013). Health-acquired infections associated with contamination of bacteria in biofilm affect 10% of patients in developed countries, and the most frequent type is urinary tract infections (Lobdell et al. 2012). It is also reported that lung infection in the case of cystic fibrosis is very frequent. *Pseudomonas aeruginosa* is a major opportunistic bacterial pathogen in patients suffering from cystic fibrosis (CF) and a frequent cause of nosocomial infections (Moradali et al. 2017). In a study, AqdC and QsdA significantly reduced the mortality of *P. aeruginosa*–infected *Caenorhabditis elegans.* AqdC, QsdA, or both enzymes treated with supernatants of *P. aeruginosa* showed less cytotoxicity to human epithelial lung cells in compassion to supernatants of untreated cultures (Birmes et al. 2019).

It is also reported that treatment of *P. aeruginosa*–infected lungs requires higher doses of antibiotics than in in vitro experiments with bacterial pathogens. In this regard, inhibiting biofilms in healthcare devices such as catheter and from the environment has arisen as challenge. AHL acylase (PvdQ) powder is used for potential application in the treatment of pulmonary *P. aeruginosa* infection (Li et al. 2019). The formulation of both quorum quenching enzymes, acylases and lactonases, as dry powders made them more suitable for pulmonary delivery coating medical implants such as urinary catheters with the AHL-degrading *Aspergillus melleus* aminoacylase. This system significantly inhibited formation of biofilm both in vitro and in vivo in *P. aeruginosa* PAO1 (Ivanova et al. 2015).

The acylase-containing coatings were found to retained 90% activity even when stored dry for 7 days at 37 °C. The coating also enhances stability than the free enzyme in artificial urine and other physiological conditions. The enzyme-containing coatings pondered the future strategy for clinical management of catheter-related infections. It is also promising for prevention of infections in orthopedic applications (i.e., on hip and knee prostheses) and on contact lenses (Grover et al. 2016).

Recently, the acylase and α-amylase coating on silicone urinary catheters degraded the QS molecules and polysaccharides, respectively. Further, hybrid nanocoating of both enzymes was found to inhibit formation of biofilm efficiently as a function of acylase or amylase position in the layers. The biofilm formation of single-species (*P. aeruginosa*) and mixed-species (*P. aeruginosa* and *Escherichia coli*) on silicone catheters under both static and dynamic conditions was significantly reduced by this nanocoating (Ivanova et al. 2015). In a study, the recombinant Ahl-1 lactonase formulated as a hydrogel was tested to control the infection of multidrug-resistant (MDR) *P. aeruginosa*–infected burn using a murine model. After treatment, the systemic spread of the infection and mortality rate were significantly reduced as compared to untreated model. *P. aeruginosa* strain when transformed with

lactonase-producing gene showed less pathogenicity in mouse pneumonia model (Migiyama et al. 2013).

In a combination therapy of lactonase and ciprofloxacin, topical application of both alone controls the systemic spread of *P. aeruginosa* through burned skin and reduced the mortality. But when applied with combination of lactonase (topical) and ciprofloxacin (I/P), significant reduction in systemic dissemination and severity of histopathologic lesions were observed (Gupta et al. 2015). Additionally, skin regeneration was also observed in this study. This indicate that a combination of lactonase and ciprofloxacin has potential to control the virulence of *P. aeruginosa*. This study suggests that future combination therapies would be a promising approach to reduce bacterial virulence and also to restore antibiotic susceptibility by declining biofilm formation (Gupta et al. 2015).

3.7 Future Prospects

Many alternative approaches to traditional antibiotics have been developed to combat antimicrobial resistance and control bacterial infections. Till date some of the strategies have progressed for the clinical trial, while others are still at the laboratory level. Among them, the blocking of QS signalling by quorum quenching enzymes appears to be the promising next- generation antibacterials. In recent studies, the crystal structure analysis of several types of quorum quenching enzymes has provided valuable information on the catalytic mechanisms and mode of actions. Such information is important for understanding the substrate specificity and its catalysis. Moreover, protein engineering can be used to further improve the stability, specificity, and activities of quorum quenching enzymes. However, enzyme activity in vitro does not always translate to efficacy in vivo. More research will be needed to establish quorum quenching enzymes as potential therapeutics. Also, more researches on emergence of resistance to quorum quenching enzymes are highly anticipated. Future studies must investigate the efficacy of this alternative therapy to fully assess their potential. In closing, the field is in its nascent stage, and further research must be invested for the development of quorum quenching enzymes as next-generation antibacterial.

3.8 Conclusion

The current antibiotic resistance dilemma, as well as the inefficacy of existing antibiotics, poses a global threat to humanity. In the past decades, the multidrug-resistant bacteria have emerged frighteningly, while there is a sluggish development of new novel antibiotics. Because of these two circumstances, there are fewer options for treating bacterial infections. As a result, developing alternative approaches is crucial at this time. In this regard, antivirulence approaches have

shown their potential to control bacterial infections by not selective survival pressure on bacteria and thus curb the possibility of a rapid selection and dissemination of resistant bacteria by diverse resistance mechanisms. QQ enzymes, such as AHL lactonase, AHL acylase, oxidoreductase, and paraoxonase, have been identified in microorganisms and are extensively dispersed in a variety of bacterial species with varying substrate specificity. To reduce bacterial pathogenesis, QQ enzymes interfere with QS signalling and restrict superfluous gene expression of harmful phenotypes. Furthermore, QQ enzymes' capacity to reduce the pathogenicity of QS microorganisms without causing antibiotic resistance qualifies them as viable antibiotic replacements. The customization of QQ enzymes could be a unique antibacterial therapy, and it could increase the utility of QQ enzymes in controlling bacterial infections.

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Conflict of Interest The authors declare that there is no conflict of interest.

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Chapter 4

Antibodies as Antibacterial Molecules: The New Era of Antibody-Mediated Immunity

Fahim Rejanur Tasin, Nazmul Haque, and Chanchal Mandal

Abstract The issue of antimicrobial resistance gained current attention to scientists. The biological approaches would be the game changer to beat pathogenic microorganisms. The successful use of antibodies in different diseases raises the hope of using antibodies as antimicrobial agents. However, several obstacles—antibody production cost, allergic reaction to the recipients, and availability of cheap antibiotics—are raised to combat the overall success of antibody-mediated therapies. The new advancement of biotechnology will overcome the problems to enable the use of it. It is a basic need to protect the antibiotics we already have, to develop new antibacterial drugs, and to generate novel therapies against bacterial pathogens for future use. In this chapter, the antibacterial mechanisms of serum antibodies and monoclonal antibodies (mAbs) are summarized. The potential uses of these antibodies as drugs and their advancement in production are the main focal points of this chapter. Additionally, the challenges behind the successful production of antibacterial antibodies are also discussed.

Keywords AAC · Antibacterial · Antibiotic-resistant bacteria · Antibiotics · Antimicrobial molecules · Augmentation · Bacterial infections · Cell lysis · Conjugates · ESKAPE pathogens · Exotoxin · Host immunity · Ig molecules · Ig–antibiotic conjugate · Immunotherapy · Microbiome · Monoclonal antibodies · Neutralization of toxins · Opsonophagocytosis · Passive immunity · Therapeutics · Tissue diffusion

F. R. Tasin · C. Mandal (✉)
Biotechnology and Genetic Engineering Discipline, Khulna University, Khulna, Bangladesh

N. Haque
Department of Oral Biology and Biomedical Sciences, MAHSA University, Selangor, Malaysia

TotiCell Limited, Dhaka, Bangladesh

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Abbreviations

213Bi	Bismuth213
AAC	Antibody–antibiotic conjugate EF Edema factor
FcγR	Fc-γ receptor Ig Immunoglobulins LD Lethal doses
LF	Lethal factor
mAbs	Monoclonal antibodies PA Protective antigen
TT	Tetanus toxin
γDPGA	Poly-γ-D-glutamic acid

4.1 Introduction

Throughout centuries, infection by microbial pathogens by either viruses or bacteria remained as one of the leading causes of disabilities and deaths worldwide. The first disease causing bacterial agent identified was *Bacillus anthracis* by Pollender and Davaine, before bacteriology became a well-established branch of science (Berghman et al. 2005). The most common infectious diseases that led to the death of millions in the last century are respiratory diseases caused by influenza-type viruses, acquired immune deficiency syndrome (AIDS) by human immunodeficiency virus (HIV), diarrhea, tuberculosis, hepatitis B and C, meningitis, malaria, sexually transmitted diseases, etc. (Morens et al. 2004). Some deadly bacterial and bacteria-associated pandemics recorded in recent history were the seventh pandemic of cholera in the 1960s (Cvjetanovic and Barua 1972), co-infection with influenza A in 1918–1919 (Gupta et al. 2008), co-infection in influenza epidemics during 1957 (Martin-Loeches et al. 2017), co-infection with H1N1 in 2009 (Rice et al. 2012), and most recently the role bacterial co-infection with SARS-CoV-2 leading to COVID-19 pandemic (Chen et al. 2020) in humans, killing millions across the past century. The concept of providing greater immunity to the infected individuals by transferring serum from non-lethal-dosed toxin-exposed animals was established by Behring and Kitasato who successfully developed and marketized the first ever antimicrobial serum (in 1893), active against diphtheria toxin (Berghman et al. 2005). Though the properties were unknown at that age, now it's clear that serum from immunized individuals contains antibodies that provide robust immunity in people with suppressed immune function by a vast range of mechanisms (Oral et al. 2002). Before the advent of chemotherapy in the 1930s, antibody treatment was proven to be functional against infectious pathogens with their use for the prophylaxis of measles and for pneumonia, meningococcal meningitis, diphtheria, etc. (Casadevall and Scharff 1995). But the later use of serum for other infectious diseases was ineffective for a particular sort of disease, like antimeningococcal serum (first developed in 1906), which was found to be incompetent due to the lack of activity identified in the 1920s and 1930s against type C meningitis (Krause et al. 1997). In the following years, serum therapy was thrown over due to some side effects and

limitations, such as posttreatment nausea, high chances of disease transmission, batch-to-batch variations, higher cost of animal husbandry, purification of antibodies, storage, and most importantly the introduction of target-specific antibiotics in the 1930s (Berghman et al. 2005; Casadevall and Scharff 1995). The discovery of the first ever chemical antibiotic 'sulfonamide' in 1935 was an epoch- making event in the world of medicine, which was found to be superior over serum therapy due to less side effect and robust action. The mortality rate from pneumococcal pneumonia declined from 30–40% to 10–20% after type-specific serum treatment, whereas sulfonamide reduced the rate to 7% (Casadevall and Scharff 1995). The need of trivalent-inactivated and live-attenuated vaccine in the 1950s and 1960s further obliterated the possible use of antibodies as a therapeutic (Fauci and Morens 2012). Following the apparent success, then-US Surgeon General proclaimed the triumph over infectious diseases in 1967 (Fauci 2001), which later was proven to be an overestimated statement because of the arising of antibiotic-resistant bacteria in later decades (Spellberg et al. 2008). The continuous evolving and faster replication process of bacteria leads to the generation of persistent pathogens such as multidrug-resistant pneumococcus and vancomycin-resistant *Staphylococcus aureus* (Spellberg et al. 2008; Fauci 2001). With the reemergence of pre-existent pathogenic microbes like *Mycobacterium tuberculosis* and *Treponema pallidum* and an uprise in the number of immunocompromised individuals worldwide, the exploitation of chemical antibiotics has become quite inconvenient (Casadevall 1996; Casadevall and Scharff 1995). Furthermore, genetic analysis of microbial metabolism suggested that some microbes had evolutionarily gained resistance to antibiotics like β-lactam more than 2 billion years ago (Spellberg et al. 2008).

Antibodies are glycoproteins existing in the plasma and extracellular fluids of host body that serve their functions in both of the innate and adaptive immunity. Antibodies are generated by specialized B lymphocytes called plasma cells. Their specificity and higher affinity to bind to target antigen resulted in their use as a significant diagnostic and therapeutic tool. The specificity of a particular antibody indicates the unique ability of it to recognize a specific epitope robustly in the presence of others. The term affinity, on the other hand, denotes the binding strength of an antibody to a monovalent epitope (Lipman et al. 2005). The functions served by antibodies are either directly, like bacterial cell lysis, neutralizing toxin and virus, or indirectly, like complement activation and opsonophagocytosis (Wang-Lin and Balthasar 2018; Casadevall and Pirofski 2004). Lysis of bacterial cells can occur after targeting of bacterial epitope by specific antibodies (Sadziene et al. 1994), whereas neutralization of bacterial toxin occurs via binding of released soluble exotoxins with antibodies, which is cleared by mononuclear phagocytic system (Wang-Lin and Balthasar 2018). On the other hand, an important indirect way of antibacterial activity is complement fixation, which occurs by binding of soluble complement proteins, most predominantly C1q, to the antibody after formation of antibody–bacteria complex (Wang-Lin and Balthasar 2018). The complement proteins are a part of host innate immunity that normally stay inactive in the bloodstream but can actively bind to bacterial cell membrane after infection (Mariathasan and Tan 2017). Complement protein C8 had shown to be essential for bactericidal activity of

host serum against *Neisseria meningitidis* strain C-11 (Nicholson and Lepow 1979). The following event is opsonophagocytosis, which is characterized by the marking of antigens by opsonins (antibodies and/or complement proteins) and subsequent internalization of antigen–opsonin complexes by phagocytic cells (Mariathasan and Tan 2017).

In this modern age of emerging infectious diseases, immunoprophylaxis and immunotherapy have gained much attention due to the lack of preventive and treatment measures in a particular form of diseases (Krause et al. 1997). To successfully combat these diseases, recent studies proposed that anti-infective immunotherapy with high specificity can be brought into play (Casadevall 1996). The arrival and expansion of hybridoma technology in the 1970s, generating homogenous, humanized antibodies in different expression systems, making antibody therapy more appealing for modern clinical use (Casadevall and Scharff 1995). The innovation is considered as one of the groundbreaking events in biological science, which came off by the fusion of myeloma cells with splenic B-cells by Köhler and Milstein in 1975, leading to the generation of monoclonal antibodies (mAbs) (Köhler and Milstein 1975). mAbs are homogeneous antibodies produced by clones of a single B lymphocyte showing a highly specific action toward a single antigen, whereas polyclonal antibodies are heterogeneous, generated from different lineages of B-cells with the ability to recognize epitopes (Lipman et al. 2005).

The suitability of antibodies to act on different pathogens like viruses, bacteria, and fungi has provided much attention to mAbs for its therapeutic uses. Neutralization of viruses like IgA-mediated intracellular neutralization of Sendai virus (Mazanec et al. 1992), protection of mice against fungus *Pneumocystis carinii* (Gigliotti et al. 2002), and notably immunomodulatory properties regulating autoimmune and inflammatory diseases (Bayry et al. 2003) lead to the in-depth investigation of mAb activity against pathogenic resistant type of bacteria. Moreover, some of the proven advantages of mAbs include their inherent specificity, minimal chances of bioconversion into toxins, potential to function as conjugate with drugs, and reduced chance of causing resistance, and they are reconsidered as a potential alternative of antibiotics for both therapeutic and prophylactic purposes. Here, in this chapter, we summarize the mechanisms by how serum antibodies and mAbs act against pathogenic bacteria and the associated factors that influence the selection and production of specific antibodies.

4.2 Structure, Classes, and Functions of Antibodies in Host Immunity

Antibodies are consisted of antigen-binding variable region and isotope-determining constant (C) region within its two identical light (approximately 25 kDa) and heavy chain (approximately 55 kDa) basic units (Casadevall and Pirofski 2004). Two distinct types of light chains (kappa and lambda) are found in humans (Wang et al.

2008). The disulfide linkage between one light chain with one heavy chain on two sides leads to their recognizable Y-shape (approximately 150 kDa) (Casadevall and Pirofski 2004). Due to the presence of common structural domain like in many other proteins, antibodies are also referred to as immunoglobulins (Igs). Depending on the variability of C region, mammal Igs are categorized into five isotypes, IgA, IgD, IgG, IgE, and IgM, and each plays an independent role in adaptive immunity of an individual (Chiu et al. 2019; Lipman et al. 2005). Among them, the predominant antibody isotype in human serum is IgG (approximately 80%), and majority of the therapeutic mAbs that gained therapeutic approval are also IgGs (Wang et al. 2008). Because of the polymorphisms in the heavy-chain-conserved regions, two Ig isotypes IgG and IgA have subclasses, like four IgG subclasses: IgG1, IgG2, IgG3, and IgG4, each having specific target antigens (Wang et al. 2008; Lipman et al. 2005). Pentameric IgM activates complement factors most efficiently leading to membrane attack complex–mediated bactericidal activity (Wang-Lin and Balthasar 2018). Their activities are regulated by their antigen-binding 'Fab' region and crystallizable effector 'Fc' region along with flexibility-related hinge region (Fig. 4.1). The proline- and threonine-rich hinge regions enable antibody in lateral and rotational movement for better fitness toward the targeted antigen domain (Chiu et al. 2019). The Fab region of an antibody targets the specific antigen on the surface of a pathogen and neutralizes the toxin or prevents further infection of cells by inhibiting its binding to cognate ligands (Mariathasan and Tan 2017). For instance, human serum antibodies specifically bind to 29/28 kDa protein(s) of the bacteria *Ehrlichia chaffeensis* after infection (Chen et al. 1997). In opsonization, Fab fragment binds to the epitope of an antigen of bacterial surface, and the Fc part becomes tied up with Fc-γ receptor (FcγR) of phagocytic leukocytes such as dendritic cells, macrophages, and neutrophils initiating endocytosis of bacteria–antibody complexes (Wang-Lin and Balthasar 2018; Mariathasan and Tan 2017). Crosslinking of antigen–antibody to FcγR can change the production of inflammatory mediators such as cytokines and chemokines and facilitate antigen presentation (Casadevall and Pirofski 2004). With a few exceptions, naturally occurring antibodies are mostly glycosylated, but the glycosylation is not crucial to determine their antigen-binding ability. Rather the effector functions of IgG, such as Fc binding, complement activation had been affected by alteration of glycosylation level (Plückthun 1991). As antibodies are highly specific for an antigen, any minor alteration in antigen shape or structure can decide the intensity of their interaction. That's why the binding of an antibody to an antigen is regarded as a reversible relationship (Lipman et al. 2005).

4.3 Functions of Antibodies as Antimicrobial Molecules

Antibodies can exert antimicrobial activity by a number of processes and mechanisms. The functions served by antibodies may vary from extreme bactericidal action to nullification of bacterial toxicity.

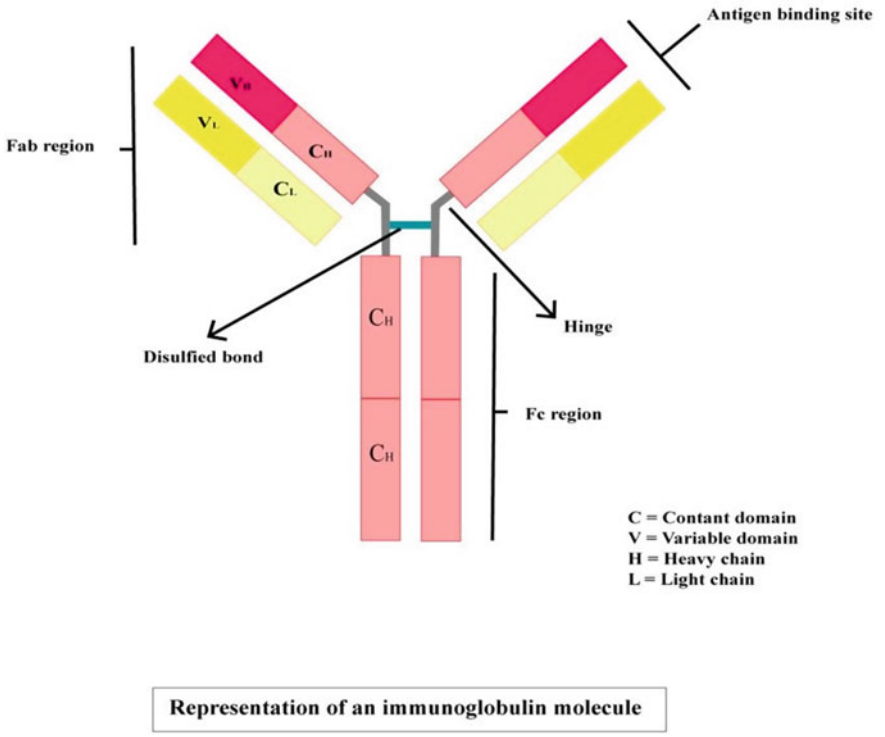

Fig. 4.1 Overview of a fully developed antibody (immunoglobulin [Ig]) molecule. An immunoglobulin (Ig) molecule having a light and heavy chain attached by disulfide bond. Antigen-binding Fab part and cell-binding Fc part contribute to the major functions on antibacterial activities. C_H stands for domains of heavy chain constant region and C_L for domains of light chain constant region. On the other hand, V_H denotes variable domain of the heavy chain, while V_L denotes variable domain of the light chain

4.3.1 Antibodies Functioning Directly as Antibacterial Molecules

By direct action, antibodies put their effects in a relatively vigorous manner by either killing or blocking the bacterial metabolism. Iron is carried throughout our body in stable complexes with transferrin (in serum) and lactoferrin (in mucosal surfaces and milk). The uptake of iron from host body is essential for bacterial survival and growth leading to pathogenesis of diseases. Low-molecular-weighted protein siderophores are ferric ion chelating agent located in the outer membrane of bacteria that help to uptake iron by bacteria growing under low iron stress (Neilands 1995). The bacterial siderophore protein enterochelin help bacteria to transport iron from iron–transferrin complex into their cells (Fitzgerald and Rogers 1980). The compound is a cyclic trimer of 2:3-dihydroxy-*N*-benzoyl-L-serine, which is most likely to emerge after the cleavage of the ester bonds connecting the serine residues. Another

virulence factor is iron-binding catechols that promote bacterial growth both in vivo and in vitro. The suitability of utilizing iron–transferrin complex to uptake iron for their growth is considered the virulency for the bacteria as it enables them to grow in serum antibodies (Rogers 1973). A study published back in 1973 using *Escherichia coli* strain 0111–infected mice model showed inhibition of catechol production after serum treatment. The *E. coli* 0111–immunized horse serum exhibited a 500-fold increase in O antigen agglutinating titer along with a 100-fold higher bacteriostatic titer in the presence of transferrin (Rogers 1973) by the identified antibodies of IgG isotype (Rogers 1976). A concurrent study published in 1978 showed that IgA extracted from human milk sample caused a potent bacteriostatic effect on *E. coli* in the presence of lactoferrin by interacting with bacterial wall lipopolysaccharide, though addition of enterochelin resulted in elimination of this effect (Rogers and Synge 1978). A subsequent study published in 1980 reported that serum antibody exposure lowered bacterial enterochelin production interacting with *E. coli* 0111 lipopolysaccharide component colitose, which is the terminal monosaccharide of the O-specific side chain (Fitzgerald and Rogers 1980). The higher presence of colitose boosts the virulence of *E. coli* 0111 redetermining the interaction between antibodies and bacterial lipopolysaccharide (Medearis et al. 1968). They posited that the reaction between antibodies and colitose blocked the release of enterochelin from the cell, and consequently, in the presence of iron–transferrin complex, bacterial growth is impeded due to lack of iron uptake system (Fig. 4.2a) (Fitzgerald and Rogers 1980). A relatively recent study published in 2001 showed bactericidal effect of IgM mAb by killing the bacteria *Acinetobacter baumannii* (ATCC 19606) up to 80–90% in vitro and also by blocking the siderophore-mediated iron uptake by bacteria (Goel and Kapil 2001).

Inhibitory along with bactericidal activities was displayed by two specific intact mAbs IgG1 and IgG3 (both with kappa light chains) and their isolated Fab fragments' dose dependently against bacteria *Borrelia burgdorferi* (in vitro), indicating complement-independent antimicrobial function. The bacteria is the etiologic agent of Lyme disease, expressing two unique outer surface proteins, OspA and OspB. The study from 1992 concluded that the inhibitory and bactericidal effects of those IgG antibodies were due to their action on an epitope of the OspB disrupting the outer envelope (Coleman et al. 1992). The later study published in 1994 stated that >99.9% cells of *Bo. burgdorferi* strain B31 died after 30 min of treatment to Fab fragments (minimum bacterial concentration of 10 μg/mL) of antibody H6831 of IgG2a isotype, reducing the count by 10^3–10^4-fold. The direct bactericidal effect of the antibody caused lysis of cells targeting OspB epitope at or near residue 253. Another closely related but less intensive agent named *Borrelia hermsii* strain HS1, causing relapsing fever, was tested against antibody H4825 Fab fragment, which generated small blebs in bacterial membrane (Sadziene et al. 1994). Potent bactericidal activity was documented for IgM against *Bo. burgdorferi* in complement-deficient mice model. Mice deficient in late complement protein C5 and early complement protein C3 rapidly cleared the infection, whereas B-cell-deficient mice couldn't (Connolly and Benach 2001). Murine IgM mAb (from polyclonal antibody pool) mediated a rapid damage of *Bo. burgdorferi* outer membrane in both

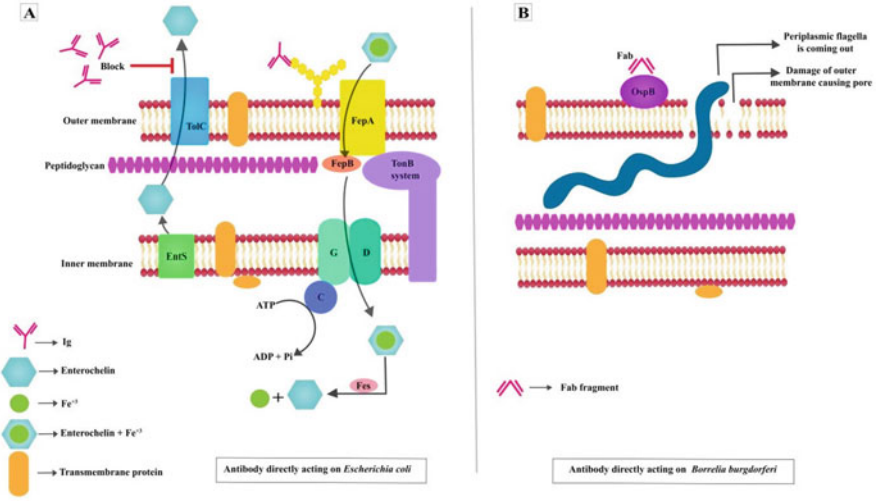

Fig. 4.2 Illustration of Ig molecules acting directly upon bacterial species. (**a**) In Gram-negative bacteria *Escherichia coli*, FepA, FepB, and TonB system and G, D, and C proteins are involved in internalization of enterochelin–iron complex, which is broken down by intracellular FeS protein. Facilitator protein EntS and tunnel protein TolC located in membranes are involved in biosynthesis and cellular release of enterochelin, respectively. Antibody (Ig) primarily binds to the lipopolysaccharide chain on the bacterial outer membrane and inhibits the release of enterochelin. Therefore, the lack of enterochelin turns into lack of iron uptake by the bacteria resulting in death by metabolic dysfunction. (**b**) In Gram-negative *Borrelia burgdorferi*, Fab fragment of antibody targets the outer surface protein OspB and disrupts the membrane. Consequently, the periplasmic flagella come out the cell and that causes loss of structural integrity

B-cell- and complement-C5-deficient mice, revealing a high antimicrobial activity of IgM. The antibody reportedly induced structural damages targeting 20 kDa outer membrane protein. As a consequence, a crucial part of *Bo. burgdorferi* cytoskeleton, periplasmic flagella, was readily exposed (Fig. 4.2b) (Connolly et al. 2004). One of the pivotal roles of the periplasmic flagella besides motility is the maintenance of cellular integrity in the presence of external stress (Kumar et al. 2017). Several other studies published in 1993 and 1994 documented the abilities of IgG and IgM antibodies to detect *Bo. burgdorferi* surface proteins OspA, OspB, and p39 and elicit bactericidal activity in a complement-independent manner (Ma et al. 1995; Aydintug et al. 1994; Scriba et al. 1993). A comparatively neoteric study from 2009 introduced a mAb of IgG1 subclass, which targeted the OspB of bacteria *Bo. burgdorferi*. The activity was specific to *Bo. burgdorferi* only as recombinant *E. coli* expressing OspB protein didn't undergo any bactericidal effect. This complement-independent bactericidal effect created a pore of 2.8–4.4 nm triggering osmotic lysis of bacterial cell (LaRocca et al. 2009). Precipitation of porin compound p66 of outer membrane wasn't found to be responsible (LaRocca et al. 2009), rather a strong relationship was observed between cell lysis and OspB containing temperature-sensitive raft-like glycolipids on the outer surface (LaRocca et al.

2010). Antibody-mediated protection was documented against *Ehrlichia chaffeensis* when transfer of immune serum from immunocompetent C57BL/6 mice to C57BL/6 SCID mice gave protection from infection. The bacteria are an obligate intracellular pathogen that are responsible for human monocytic ehrlichiosis. The targeted antigens were 22, 27, 28, 54, 73, and 88 kDa proteins (Winslow et al. 2000).

4.3.2 Antibodies Functioning Indirectly as Antibacterial Molecules

By indirect operation, antibodies work on bacteria by a number of mechanisms such as opsonization-mediated phagocytosis, complement activation, and inducing cell-mediated immunity. One of the early reports on these types of immunity from a study in 1969 that stated a significant complement-dependent bacteriostatic effect of 15% horse *Pasteurella septica* antiserum in mice *P. septica* infection model. The activity of complement system had been proven as no bacteriostatic effect was shown when the antiserum had been heated by 56 °C for 30 min before its treatment on mice. The effect was also associated with transferrin as reduced bacteriostatic activity was reported when transferrin had been saturated by incorporation of ferric iron (Bullen and Rogers 1969). Non-immunized normal human serum exhibited a well-marked bactericidal effect against two colony types of bacteria *Neisseria gonorrhoeae* GC9 in vitro. The study displayed complement-mediated killing of bacteria as treatment of the same serum after heated by 56 °C for 30 min that showed no antagonistic effect. The association of iron was also evident as addition of ferric ammonium citrate reduced the bactericidal effect, possibly by triggering bacterial siderophore expression on the outer surface (Norrod and Williams 1978). A similar study on *N. gonorrhoeae* demonstrated a complement-mediated lysis of bacteria. The study concluded that complement protein C8 had the key role in human serum antibacterial activity through classical and alternative pathways, as C8-deficient serum of a patient failed to exhibit anti-gonococcal activity (Fig. 4.3a). The serum also opsonized the bacteria *Staphylococcus aureus* 502A and subsequently induced phagocytosis by polymorphonuclear leukocytes (Petersen et al. 1976). A primate model study in 1994 documented antibody-dependent complement-mediated antibacterial activity of immunized serum against *Bo. burgdorferi*. The serum antibody introduced in rhesus monkey (*Macaca mulatta*) killed the bacteria in association with complement proteins, targeting OspA and OspB surface proteins (Aydintug et al. 1994).

Alterations in bacterial metabolism are one of the most vital mechanisms how antibodies can create morphological disabilities and cause death of certain bacterial species. Antiserum and complement system–mediated disturbance of bacterial metabolism was early reported by Melching and Vas in 1971 on *E. coli* strain 0111, where they showed hyperimmune rabbit serum affecting bacterial RNA, DNA, and lipid synthesis. The bacterial RNA accumulation began to decrease

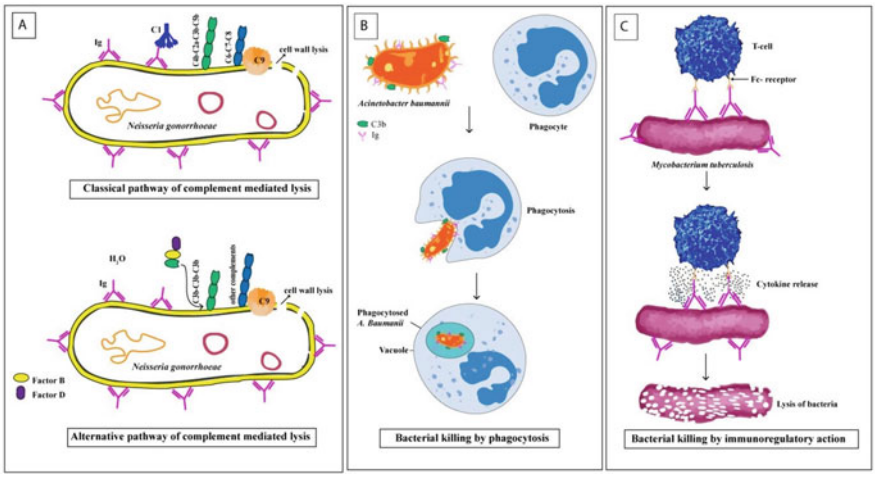

Fig. 4.3 Schematic representation of an Ig molecule acting directly upon bacteria. (**a**) In the classical pathway of complement-mediated activity, antibody (Ig) opsonizes the bacteria *Neisseria gonorrhoeae* and activates the complement system. Complement protein C1 attaches to the Fc region of Ig and later leads to the binding of complement C4b, C2a, C3b, C5b, C6, C7, and C8. Later on, the activation of C9 and membrane attack complex causes the lysis of bacterial wall exposing the intracellular materials. In the alternative pathway, Ig opsonization is followed by activation of C3b by factor B and factor D. Subsequently, activation of other complements including C9 forms membrane attack complex–mediated bacterial lysis. (**b**) *Acinetobacter baumannii* is opsonized by Ig and C3b that is later phagocytosed and degraded intracellularly. (**c**) First, *Mycobacterium tuberculosis* becomes opsonized by Ig, and then, crosslinking with host cytokine causes bactericidal action. Crosslinking occurs by binding of Ig Fc domain with Fc receptor of T-cell and the release of cytokine such as interferon, and interleukin causes damage to the bacterial cell resulting in bacterial lysis

after 15 min and completely ceased after 30 min of serum exposure, and severe impairment of protein synthesis was noticeable. The lipid synthesis had also been halted at 25–30 min that turned into physical defect in bacterial membrane altering the permeability and respiratory function (Melching and Vas 1971). A similar study published in the same year showed that *P. septica*–immunized horse antiserum blocked the net RNA synthesis of *P. septica* in a complement-dependent way and consequently inhibited the synthesis of macromolecules (Griffiths 1971).

Opsonization followed by phagocytosis by the innate immune system is a key factor in antibacterial activity. Fusion of phagosomes and lysosomes creates phagolysosomes, which can efficiently catabolize the infected bacteria (Wang-Lin and Balthasar 2018). According to an estimated calculation, antibody opsonization–mediated phagocytosis can be triggered by a density of only 5.33–26.7 antibodies/μ m^2 on the surface of the targeted bacteria (Lewis et al. 1980). A study by Russo et al. showed mAb 13D6–mediated phagocytosis as a bactericidal mechanism against *Acinetobacter baumannii* in rat soft tissue. The bacterial K1 capsular polysaccharide was detected as the principal target that resulted in the neutrophil-mediated killing of bacteria in vitro (Fig. 4.3b) (Russo et al. 2013). Induced T-cell activity after antibody

opsonization of specific bacteria had shown to be protective for the host organism. A study by Markham et al. reported an antibody-dependent host cellular protection against infection by *Pseudomonas aeruginosa* in mice model. They showed that pathogen-specific IgG3-FcR of T-cell exhibited toxicity against *Ps. aeruginosa* residing in the extracellular environment. The bacteria are the main causing agent of child lethality in cystic fibrosis and the people with immunosuppression. The crosslinking of cytophilic antibody on surface of T-cells induced the release of an antibacterial lymphokine inhibiting bacterial growth (Markham et al. 1991). In another study, for complete clearance of pathogenic bacteria *Ehrlichia chaffeensis*, αβ-T-cells were found to be required where persistent infection was reported in αβ-T-cell-deficient mice as long as 24 days of postinfection (Culkin et al. 1997). B-cell-dependent protective mechanism was shown by Culkin et al. against *Francisella tularensis* strain LVS. The bacterial growth was reportedly restricted rapidly after infection involving interferon-gamma (Culkin et al. 1997). Certain mAb had been shown to have immunoregulatory roles by repairing the enzymatic function of lymphocytes. Introduction of anti-programmed cell-death protein 1 mAb had restored the cytokine secretion and responsiveness of T-cells in the presence of antigen of *Mycobacterium tuberculosis*. The mAb enhanced interleukin-23R and interleukin-17 production along with phosphorylated STAT3 expression in *M. tuberculosis*–cultured $CD4^+$ T-cells ex vivo (Fig. 4.3c) (Bandaru et al. 2014).

4.3.3 Antibodies Functioning in a Conjugate

Antibody–drug conjugates are gradually becoming a significant class of passive-immunity-based therapeutics. The application of mAbs with antimicrobial drug increases the on-target specificity of the conjugate keeping the host normal microflora undisturbed. Two antibody–drug conjugates have already gained approval by the US Food and Drug Administration and the European Medicine Agency to cure patients with cancer (Beck and Reichert 2014). For antibacterial action of antibodies with other substances, a study published in 1974 showed the effect of beta-lysin, lysozyme, and antibody-complement system at the rabbit serum, which led to the destruction of *E. coli* B in vitro. They concluded that the action of beta-lysin caused the cytoplasmic contents to come out the cells after the breaking down the cell wall. The inner peptidoglycan layer of the cell wall was disfigured after lysosomal action, while antibody-complement system destroyed both the middle lipopolysaccharide layer of the cell wall and the cytoplasmic membrane. The mechanism was complement dependent as inactivation of the complement proteins as heating the serum at 56 °C for 30 min knocked out the bactericidal activity (Donaldson et al. 1974). Antibody–enzyme conjugate displayed a robust bactericidal activity in a number of test pathogens. According to the study by Knowles et al. in 1973, glucose oxidase conjugated to human serum IgG acted on *S. aureus*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *E. coli* in the presence of co-factors potassium iodide, lactoperoxidase, and glucose. The conjugate generated hydrogen peroxide, which

is an active bactericidal. The enzyme lactoperoxidase catalyzed the reduction of hydrogen peroxide and simultaneously oxidation of iodine ion. The events led to the halogenation of the bacterium and subsequently killing it. The study suggested that even a lower concentration of 0.01 mg/mL could bring about severe bactericidal activity (Knowles 2nd et al. 1973). Dadachova et al. established radioimmunotherapy-based treatment against bacterial infection in mice model where they used antibody with a radionuclide. The human mAb D11 of IgM isotype was conjugated to alpha-particle emitter Bismuth213 (213Bi) as the radionuclide. The mAb targets capsular polysaccharide 8 of *Streptococcus pneumoniae*, and in vitro incubation of 213Bi-D11 with *St. pneumoniae* serotype 8 resulted in killing of the bacterium in a dose-dependent manner. The conjugate proved to be non-toxic and effective as a treatment for *St. pneumoniae*–infected mice at a dose of 80 μCi, and the survival rate was 87–100%, which was way better than the control ones (Dadachova et al. 2004).

As the groups of antibiotic-resistant bacteria are increasing day by day and the cost of antibiotic development has skyrocketed for particular diseases, the urgency of new therapeutic is quite evident. In addition, the low specificity of antibiotic to affect beneficial microbiota in the gut of healthy is being leaded to facilitate the survival and colonization of pathogenic bacteria. The growth of antibiotic-resistant bacteria *Clostridium difficile*, vancomycin-resistant enterococci, methicillin-resistant *S. aureus*, and multidrug-resistant Enterobacteriaceae can cause inflammatory bowel disease, obesity, and atherosclerosis. That's why targeted therapies for antibacterials became a high-priority call for modern therapeutic platform. Antibody–antibiotic conjugate (AAC) is one of the novel therapeutic approaches for the cure of infectious diseases and has shown outstanding efficacy and specificity. The combined effect of AAC can surpass these obstacles as AAC has antibody of high specificity but low bactericidal activity, and antibiotic with a broad-ranged bactericidal activity can affect target and act and kill certain bacteria type (Mariathasan and Tan 2017). Apart from this, AAC has been shown to be effective against bacteria with intracellular life cycle (Mariathasan and Tan 2017), like *S. aureus* surviving inside of polymorphonuclear neutrophils exploiting inflammatory response of the host (Gresham et al. 2000). The majority of existing antibiotics displayed poor results in killing intracellular *S. aureus* in in vivo mice model (Sandberg et al. 2009). In 2015, Lehar and his associates successfully developed a novel anti–*S. aureus* AAC, THIOMAB™ (Genentech, South San Francisco, CA, USA), which targets and later kills intracellular pathogenic *S. aureus*. The AAC consists of *S. aureus*–targeting antibody of IgG1 isotype, which is covalently linked to a powerful antibiotic dimethylamino piperidino-hydroxybenzoxazinorifamycin (referred to as 'dmDNA31') by a cleavable valine–citrulline linker. This ACC doesn't kill the bacteria in a direct manner, rather the antibody part of AAC opsonizes the bacteria by specifically binding to β-N-acetylglucosamine residues on the ribitol phosphate units of cell wall teichoic acid, followed by cellular uptake. The released intracellular proteases of the host cells remove the linker and bring the antibiotic out in its active form that exhibited potent bactericidal activity against both persistent and stationary-phase *S. aureus* (Fig. 4.4). They reported that one single

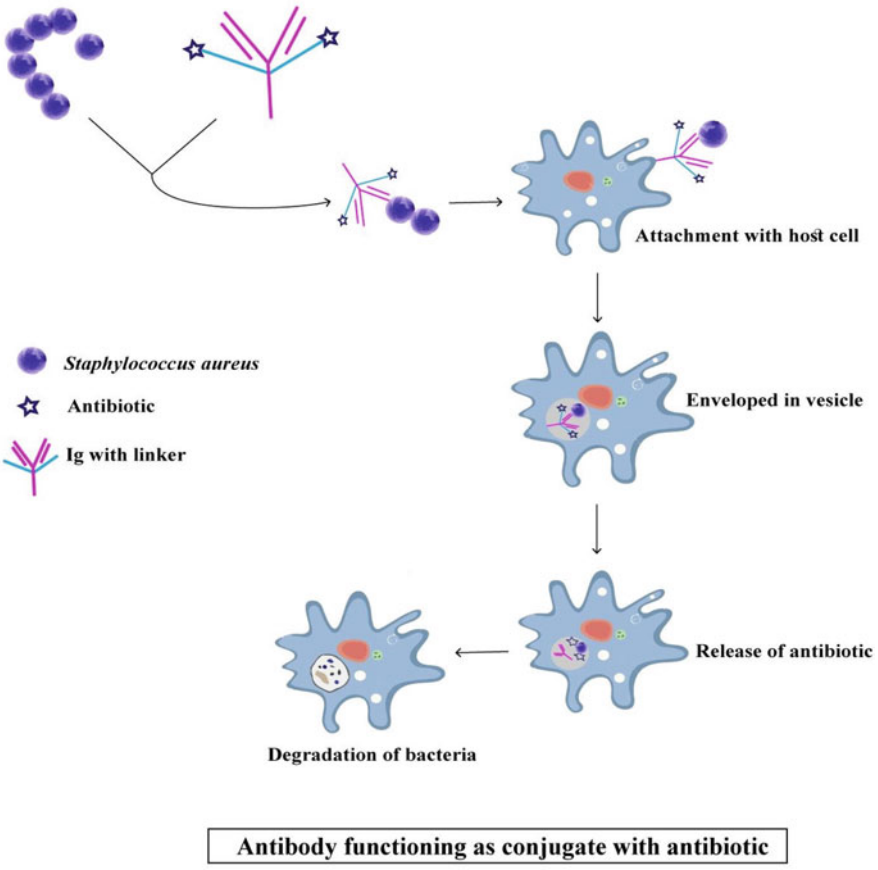

Fig. 4.4 Illustration of an Ig–antibiotic conjugate killing a bacterial species. Primarily, antibody (Ig) is conjugated to antibiotic by a linker that makes the antibody–antibiotic complex (AAC). The Ig part of AAC specifically targets the bacteria *Staphylococcus aureus*, which is followed by attachment with the host cell. *S. aureus*—bound ACC is enveloped by intracellular vacuoles and the protease ligase cleaves the antibiotic from the ACC. Bacteria are later degraded and killed by the released antibiotic, intracellularly

bacterium could be opsonized by thousands of AACs and release free antibiotic within the phagosome to execute bactericidal activity. The efficacy of this AAC was found to be much higher than vancomycin in in vivo infected mice model (Mariathasan and Tan 2017; Lehar et al. 2015). Other AACs like obiltoxaximab-levofloxacin or obiltoxaximab-ciprofloxacin also exhibited higher protective function than treatment of antibiotic only for infection by *B. anthracis* (Greig 2016).

4.3.4 Antibodies Functioning by Neutralizing Toxins

Bacteria can act on infected body by emitting various toxin components, which mainly target cell surface receptors and disrupt cellular equilibrium by interfering with intracellular reactions. As a causative agent of anthrax, *B. anthracis* poses a substantial risk including death for humans when taken by inhalation. The active *B. anthracis* generates three toxins: protective antigen (PA), lethal factor (LF), and edema factor (EF), by which it attacks the hosts. PA is a common cell-binding protein that translocates LF or EF into the cytoplasm (Chen et al. 2011a). LF is a Zn^{2+}-endopeptidase that catalyzes the cleavage of mitogen-activated protein kinase kinases such as MEK1, MEK2, and MKK3 (Vitale et al. 2000), whereas EF is a calmodulin-dependent adenylate cyclase that is involved in the conversion of adenosine monophosphate into 3′–5′-cyclic adenosine monophosphate, leading to the establishment of pathophysiology of anthrax (Leysath et al. 2013). Raxibacumab is an approved drug for therapeutic and prophylactic purpose against anthrax by neutralizing toxin PA. It is a human IgG1 (with lambda light chains) mAb that blocked the attachment of PA to its cellular receptor by binding to the domain IV of PA (Fig. 4.5a). The survival rate of in vivo models was reportedly satisfactory for New Zealand white rabbits and cynomolgus macaques with a respective survival rate of 44% and 64% after 40 mg/kg treatment of raxibacumab. Intravenous administration of raxibacumab in humans at 40 mg/kg dose reportedly had a half-life of

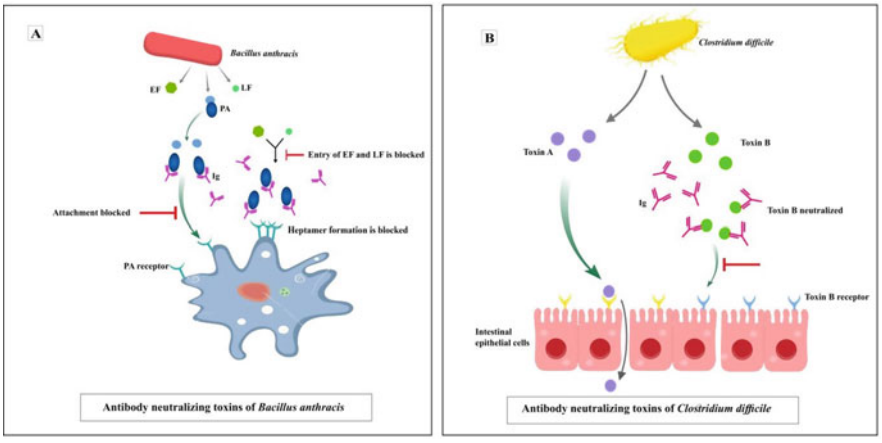

Fig. 4.5 Illustration of Ig molecules neutralizing bacterial toxin inside the host body. (**a**) The three toxins protective antigen (PA), lethal factor (LF), and edema factor (EF) are released by *Bacillus anthracis*. Generally, LF and EF are internalized by PA heptamer on host cell surface causing pathogenesis. Ig blocks the attachment of PA to its receptor by binding to the domain IV of PA and also inhibits the heptamer formation by targeting the PA domain III. As a result, the other toxins EF and LF can't enter the host cell. Events lead to the neutralization of anthrax toxins and antibacterial action. (**b**) Toxin A and toxin B released by bacteria *Clostridium difficile* are targeted to the host epithelial cells. Antibody (Ig) binds to the toxin B and inhibits its binding to the receptor. Therefore, toxin B becomes neutralized, though toxin A enters the host cell

20–22 days with potential clinical benefit (Migone et al. 2009). Another mAb obiltoxaximab of IgG1 isotype (with kappa light chains), also named as ETI-204, co-produced by Elusys Therapeutics, inhibited the binding of PA to its receptor targeting domain IV of PA and prevented the entrance of LF and EF of *B. anthracis* into the host cell providing protective and therapeutic effect against anthrax (Fig. 4.5a). In New Zealand white rabbits, intramuscular injection of the mAb at 20 mg dose displayed total protection from death with 100% survival (Mohamed et al. 2005). Obiltoxaximab received approval on 18 March 2016 for therapeutic and prophylactic use to measure the inhalational anthrax by *B. anthracis* in the USA (Greig 2016). A similar mechanistic effect of human mAb IQNPA was reported and developed as hybridomas from peripheral lymphocytes of anthrax vaccine–immunized individuals. The mAb imparted prophylactic function targeting domain IV of PA and probably inhibited the binding of PA to its cellular receptor (Albrecht et al. 2007). A human mAb named AVP-21D9, co-produced by Avanir Pharmaceuticals, inhibited the heptamer formation of PA by targeting domain III of PA (Fig. 4.5a) and thus reduced *B. anthracis* dissemination from the lungs to the organs. They concluded that the mAb showed protective outcome for Dutch-belted dwarf and New Zealand white rabbit models against *B. anthracis* Ames spores (Peterson et al. 2007). mAb Valortin from PharmAthene/Medarex is a human antibody that reportedly disfigured the previously formed PA-heptamer PA63h by targeting domain III of PA. The mAb formed a PA supercomplex showing a similar ability to that of mouse mAb 1G3 (Radjainia et al. 2010). Anti-LF mAb IQNLF recognized PA-binding domain I of LF and later inhibited the binding of LF to PA.

A single 180 μg dose of IQNLF provided 100% protection to A/J mice that had been intraperitoneally challenged with 24 50% lethal doses (LD_{50}) of *B. anthracis* Sterne spores (Albrecht et al. 2007). For 200 μg use of anti-LF mAb LF10E, 100% protection of A/J mice was also reported after challenged with 1000 LD_{50} of *B. anthracis* Sterne spores (Chen et al. 2009a). Anti-EF mAb EF13D reportedly neutralized EF by preventing edema formation in a subnanomolar range in vitro. The chimpanzee/human mAb EF13D bound to domain IV of EF and later inhibited the attachment process between calmodulin and EF. The mAb also could separate calmodulin from EF–calmodulin complex and reduce the toxic effect of EF on the host (Chen et al. 2009b). Another important virulence factor of *B. anthracis* is poly-γ-D-glutamic acid (γDPGA) capsule, which are targeted by immunized chimpanzee Fab 11D and 4C fragments. Chen and his colleagues constructed IgG mAbs with 11D and 4C Fab fragments that specifically bound and neutralized the γDPGA of *B. anthracis* with an LD_{50} of ≈10 ng/mL in an opsonophagocytic bactericidal assay. The treatment of 30 μg of either of the mAbs provided protection against virulent *B. anthracis* Ames strain in mice model in both pre-exposure and post-exposure conditions (Chen et al. 2011b).

Gram-positive bacteria *Clostridium difficile* infects the colon of susceptible individuals causing rupture, sepsis, and possibly death by the action of their two exotoxins, toxin A and toxin B. Human IgG1 mAb bezlotoxumab shows protective effect against *C. difficile* infection by inhibiting the binding of toxin B to the host cells (Fig. 4.5b). A crucial component for the maintenance of morphology and

homeostasis of the host cell is Rho GTPases, which becomes inactivated by toxin A and toxin B after infection. Bezlotoxumab halts the inactivation of Rho GTPases and its intracellular downstream signaling pathways (Orth et al. 2014). Frank et al. (in 2002) generated a murine antibody mAb166 that showed the ability to neutralize toxin from *Ps. aeruginosa* when coinstilled with the inoculum or introduced into mice by intraperitoneal injection. Opportunistic pathogen *Ps. aeruginosa* causes acute infections in immunocompromised patients and chronic infection in cystic fibrosis (Frank et al. 2002). The mAb targeted the protective antigen of *Ps. aeruginosa* named 'PcrV' (Frank et al. 2002), which is a key component of type III secretion system that enables the bacteria to inject the effector toxin molecules into the host eukaryotic cells (Gébus et al. 2008). The anti-PcrV antibody mAb166 acts by inhibiting the transfer of type III toxin of *Ps. aeruginosa* to the host cell by binding the epitope mapped to the carboxyl-terminus of PcrV (Frank et al. 2002). The protection provided by mAb166 was not Fc mediated, as a single dose of 10 μg of Fab fragments was able to present excellent protection where no detectable *Ps. aeruginosa* cells had been identified in the lungs of 80% of the infected mice after 48 h (Baer et al. 2009).

The combined action of two antibodies had noteworthy antibacterial effects documented in a number of studies. *Clostridium tetani* is one of soil bacteria that is responsible for tetanus by producing exotoxins. The human spleen cells were precultured with pokeweed mitogen along with tetanus toxoid to produce mAbs specific to tetanus toxin (TT). Two mAbs named anti-TT1 and anti-TT2 of IgG1 isotype specifically targeted two separate regions of TT; the anti-TT1 mAb binded the heavy chain portion of the B fragment and the anti-TT2 mAb binded the C fragment of TT. The joint effect of these two mAbs offered a better neutralizing effect on the TT, whereas mice treated with one mAb alone after TT exposure resulted in death within 20 days (Ziegler-Heitbrock et al. 1986). The combination of two other mAbs, namely, cαtx1 and cαtx2, in together called Shigamab, has proven to be protective against Shiga toxins of *Shigella dysenteriae* and *E. coli* (Melton-Celsa et al. 2015). Stx1 and Stx2 are the two chief bacterial toxins and the virulence factors that interfere with the host protein synthesis process and cause bloody diarrhea and hemolytic uremic syndrome in humans (Melton-Celsa et al. 2005). The protective efficacy of Shigamab was reported in mice via neutralization of Stx1 and Stx2, with confirmed safety in human introduction (Melton-Celsa et al. 2015).

Alpha-toxin of *S. aureus* is a compound that causes pore formation in host cell membrane leading to lysis of host cells. The gene coding for this toxin is conserved among numerous isolates all over the world and is considered as a critical content for *S. aureus*–mediated pathogenesis causing skin infection, sepsis, and pneumonia. mAb MEDI4893 of IgG1 isotype specifically targets alpha-toxin of *S. aureus* and prevents its cytotoxic effects. Though the mAb is yet to come in the market till this date, alpha-toxin neutralization ability of this mAb has proven to be effective in protection against ventilator-associated pneumonia, damaging lung epithelium and innate immune cells (Tabor et al. 2016). Other cytotoxins of *S. aureus* important for its pathogenesis, alpha-hemolysin and leukocidins, have shown to be neutralized by

synergistic action of human IgG1 mAb with antibiotic linezolid offering protection in murine pneumonia model (Rouha et al. 2015).

4.4 Challenges and Factors to Consider for the Production of Antibacterial Antibodies

To fight against persistent, multidrug-resistant bacteria, repeated doses of antibody are a requirement for successful therapy. The production of mAbs in large quantities over a long time with desired efficacy remains a major challenge for biopharmaceuticals. Though mammalian cell lines such as human PER.C6 cells, Chinese hamster ovary cells, and murine NS0 cells are the predominant expression systems to commercially generate mAbs (Li et al. 2010), transgenic plants (Hiatt et al. 1989), egg yolk (Carlander et al. 2000), and bacterial expression systems (Plückthun 1991) have shown to be promising. Some of the obstacles for antibacterial antibodies are briefly discussed here.

4.4.1 Augmentation of Bacterial Infection by Antibodies

Quite a surprising event in immunological discovery is the promotion of microbial infection by host's antibodies. An early example of antibody-mediated enhancement of infection is shown by Weiser et al. in 2003 where they suggested a particular antibody-mediated tissue-specific escalation of bacterial infection in humans. Generally, IgA blocks the bacterial attachment on host cells, but the study stated that human mucosal IgA1, which normally targets capsular polysaccharide of *St. pneumoniae*, augments the tissue infection by facilitating *St. pneumoniae* adherence to the Detroit 562 pharyngeal epithelial cells, when cleaved by bacterial IgA1 protease. IgA1 protease splits IgA1 alone, as it specifically targets prolyl-threonyl or prolyl-seryl bonds in the hinge region of IgA1, and the bonds aren't present on protease-insensitive IgA2 and IgG. The negative charges of exopolysaccharide are neutralized by the positive charges of Fab (of IgA1) fragments, which also play a role in *St. pneumoniae* attachment on the epithelial cells (Weiser et al. 2003).

In a very recent time, antibody-mediated augmentation of infection by *A. baumannii* is reported in a published study in 2019, where mAb 8E3 of IgG3 subtype specifically targeted to K2 capsular polysaccharide of the bacteria raised the mortality level in mice with elevated bacterial population in the blood, lung, and splenic samples. One of the hypotheses that the study made was the 8E3–*A. baumannii* complexes that enhanced the invasion of macrophages and human lung epithelial cells NCIH-292 by involving a distinctive FcγR activity (Wang-Lin et al. 2019). A review published in 2010 by Halstead et al. suggested that an idiosyncratic FcγR signaling of macrophages after tethering with IgG–pathogen

complexes led to the rise of intracellular infection. This exceptional FcγR signaling repressed the host immunity rather than boosting it, by provoking interleukin-10 production and altering passage of T-helper-1 responses to T-helper-2 responses (Halstead et al. 2010). Another recently published report from 2016 identified antibody-mediated promotion of *M. tuberculosis* infection when the antibodies are collected from actively infected individuals (Lu et al. 2016).

4.4.2 Target Site Accessibility for the Antibodies

For passive immunity, antibodies are targeted to a surface antigen or the exotoxin of bacterial pathogen. Produced mAbs are tailored to bind evolutionarily conserved proteins on the outer membrane or the exopolysaccharides of the bacteria for elicitation of bacteriostatic or bactericidal effect. For instance, 'outer membrane protein A' of *A. baumannii* was identified as a primary immune target for vaccination that showed >99% conservation from the clinical isolates obtained from the year of 1951 to 2009 (Luo et al. 2012). However, a matter of complication in this event is evident in some bacteria strains where the capsular polysaccharides cover up the evolutionarily conserved antigens, which are to be targeted by the mAbs, resulting in a serious disturbance in opsonization. An earlier study from 1986 substantiated that O-antigenic side chain hinders antibody binding to cell surface of the outer membrane pore protein PhoE of various Enterobacteriaceae (van der Ley et al. 1986). Overproduction of biofilm-forming exopolysaccharide compound poly-N-acetylglucosamine of bacteria *Staphylococcus epidermidis* and *S. aureus* averted the phagocytic killing by not letting enough antibody deposition on the target site. *Staphylococcus epidermidis* along with other staphylococci frequently causes the nosocomial infection, and their exopolysaccharide adds to their virulence in the presence of immune action (Cerca et al. 2006). A study on extraintestinal pathogenic *E. coli* concluded that binding of polyclonal antibodies directed against non-capsular and O-antigenic epitopes becomes obstructed by the presence of barrier components capsule and O-antigen (Russo et al. 2009). Moreover, with the increase of doses of high affinity mAb, the increase in mAb–antigen complexes led to the saturation of target sites, consequently a scarcity of antibody binding sites (Wang et al. 2008). In the study on *A. baumannii*, Wang-Lin et al. posited that mAb 8E3–bacterial capsule immune complexes impaired the function of phagocytic cells, leading to progression of infection (Wang-Lin et al. 2019).

4.4.3 Bacterial Defense Mechanisms

Certain bacteria possess the ability to defend themselves against the host immune response by either directly cleaving the antibodies or neutralizing the antibodies through their enzymatic activity. A well-known human pathogen *Streptococcus*

pyogenes can give rise to some life-threatening diseases such as necrotizing fasciitis and septicemia (Cunningham 2000). The bacteria release IgG-specific enzyme EndoS, which hydrolyzes the glycan moiety of intact IgG. Another protease named streptococcal erythrogenic toxin B (SpeB), secreted by *St. pyogenes*, cleaves the γ-chain of IgG specifically in the hinge region leaving stable Fab and Fc fragments. Heavy chains of other antibody components such as IgA, IgM, and IgD had been cleaved into fragments, whereas complete degradation of IgE was documented by SpeB action (Collin and Olsén 2001). Another IgG-cleaving enzyme secreted by *St. pyogenes* is immunoglobulin G-degrading enzyme of *St. pyogenes* (IdeS), which degrades the IgG molecules that are bound to their cell surface, and thus prevents the phagocytic action (von Pawel-Rammingen et al. 2002). Another enzyme IgA protease produced by clinical isolates of *St. pneumoniae* and *Haemophilus influenzae*, causing respiratory tract infection, specifically cleaved the human serum secretory IgA1 protein, further promoting the infection (Mulks et al. 1980). Neutralization of antibodies was described in the studies on bacterial surface proteins, where protein G of *Streptococcus* sp. and (Björck and Kronvall 1984) protein A of *S. aureus* (Falugi et al. 2013) bind Fc region of IgG resulting in disturbances of opsonophagocytosis and subsequent killing of bacteria.

4.4.4 Tissue Diffusion of Antibodies after Bacterial Infection

The diffusion of polar mAb molecules across the tissues is conducted by extravasation of mAbs from circulation to interstitial fluids and later to the antigens within the tissues. Though diffusion and convection are the two typical mechanisms of mAb transfer (Wang et al. 2008), antibody distribution in mice model suggested a transfer percentage of 98.4% of IgG by convection through the large pore system (Baxter et al. 1994). Attachment of IgG with the cell membrane is facilitated by its binding with Brambell receptor, FcRn, which forms IgG–FcRn complexes and protects the IgG from lysosome-mediated degradation (Wang et al. 2008). The secretion of cytokines and chemokines along with the activation of lymphocytes after bacterial infection results in antibody recruitment on the target tissues. But the elevation of fluid pressure within the tissues reduces the antibody absorption via convection (Wang-Lin and Balthasar 2018). Even after diffusion into the target tissues, the antibody-dependent opsonophagocytosis can be barred by the biofilms made by certain bacteria, like *S. epidermidis* (Cerca et al. 2006).

4.5 Future Prospects

As a portion of traditional antibiotics have continuously failed to be efficient against the resistant types of pathogens, antibodies come as alternatives with their diverse modes of action on pathogenic bacteria. However, there are still some obstacles with

the success of therapeutic antibody-mediated antibacterial. As AAC represents a novel approach against bacteria, choosing a cleavable linker and ratio of antibiotic: MAb are major factors producing this sort of drug. Success has been reported for linkers of cathepsin-cleavable type in AAC targeted to *Ps. Aeruginosa*, and the concentration of 6:1 of antibiotic and MAb in a specific AAC has shown to be been efficient for bacterial clearance. A higher antibiotic level in an AAC has proven to be more antagonistic to a bacterial species after macrophage-mediated bacterial phagocytosis. The interplay of mAbs with complement factors and other inflammatory molecules plays a critical role and needs to be assessed in developing an effective therapeutic mAb model. A recent study of mAb treatment on *A. baumannii*–infected mice revealed a reduction of proinflammatory IL-10 and TNF-α level that became a determinant of host survival. Therefore, a deeper understanding of the action of mAb in the host body environment and the relation of mAbs with effector molecules would provide new opportunities to design an effective antibacterial therapy. We hope that the new sophisticated formats will cope the future challenges to fight against pathogenic diseases.

4.6 Conclusion

Antibodies are selective, specific, robust, functionally recognizable, and exploitable to be introduced with other drugs for the treatment of bacteria-mediated infection. Furthermore, their resemblance with host immunity promotes their use for therapeutic and prophylactic application. The antibacterial use of antibodies can act via a number of different mechanisms, such as cell lysis, opsonophagocytosis, and inactivation or neutralization of bacterial exotoxins. Passive immunization by specific mAbs has asserted their well-grounded credibility, instead of the use of chemical antibiotics. One of the recent advancements of antibody-based therapy is conjugation with other drugs, like antibiotic, targeted to a specific bacterial population. Though antibody is capable of performing as potent antibacterial, there are some obstacles that need to be assessed for proper application. Bacterial defense mechanism and the target site availability are two of the important factors for clinical mAb development. Lastly, the antibodies are the promising agent from a very primitive era to this modern age, and the use of antibacterial antibodies would be the possible solution for drug-resistant bacteria.

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Conflict of Interest None to declare.

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Chapter 5
Phage Therapy: Genomics to Applications and Future Prospects

Abhishek Jaiswal

Abstract Bacteriophages are unique organisms with diverse genomic orientation and enriched with beneficial characteristics. Phage therapy was initially employed nearly a century ago, though it was undermined and neglected after the efficacious insertion of antibiotics. The therapeutic use of bacteriophages is undeniably the utmost explored tool and best suited to be part of the multidimensional strategies to fight against antibiotic resistance. Now, the pipeline for new antibiotics is almost dry with the upsurge of antibiotic resistance. So the forgotten cure is resurfacing with a well-deserved honor. Phage therapy primarily designates obligately lytic phages to kill their respective bacterial hosts, while parting the human cells unharmed and diminishing the shock on commensal bacteria that frequently result from antibiotic use.

This chapter depicts the prime points of bacteriophage genomic diversities, the evolutionary pattern, the calamity of antibiotic resistance that validates the phage use, their interface with the human immune system, and an evaluation within different antibiotic therapies. By going through peer reports of human clinical trials, this chapter will project the versatility of phage therapy. This informative writing will also cover orthodox methods and novel tactics, such as the use of phage-antibiotic permutations, phage-encoded enzymes, utilization of phage endurance mechanisms, and phage ergonomics. Potential and promising recent studies from all over the world define that the time has come to look more minutely at the prospective of phage therapy for future practice, both by strongly supporting new research and by scrutinizing the research already available, raising the optimism that this century-old magical cure will be an answer to multiple problems by varied invasive approaches.

Keywords Anti-phage antibodies · Experimental therapy · Immunomodulation · Lysin · Phage therapy · Phage cocktails · Prophage · Synergy · Macrophage · Alternate medicine · Dendritic cell · Innate immunity · Pathogen

A. Jaiswal (✉)
Department of Microbiology, Viral Research and Diagnostic Laboratory, Midnapore Medical College and Hospital, Midnapore, West Bengal, India

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Abbreviations

CRISPR	Clustered regularly interspaced short palindromic repeat
DS RNA	Double-stranded RNA
FDC	Follicular dendritic cells
GP	Glycoprotein
ICTV	International Committee on Taxonomy of Viruses
MHC	Major histocompatibility complex
PRMC	Phage resisting microbial cells
SS RNA	Single-stranded RNA
STIV	*Sulfolobus* turreted icosahedral virus

5.1 Introduction

Bacteriophages are unique organisms with diverse elements in the biosphere. Since centuries, bacteriophages are used to treat humans. Antibiotic defiance is the prime threat to majority worldwide health issues in this current era. In each decade, a rising number of contagions carrying high fatality rate are a matter of concern and becoming harsher to treat in a cost-effective way. It is clearly evident from the past that phage treatment was scientifically employed approximately an epoch ago, but it was fetched to a cessation after the impressive application of antibiotics. Now, in the shadow of drug resistance, bacteriophage therapy is gaining a well-deserved resurgence. The enormous article lately published worldwide supports the concept and pragmatism of bacteriophage healing approach. This chapter concentrates to deliver a progressive view on bacteriophage cure, encompassing its exceptional potential. Bacteriophages are present not only in almost all the known biomes to human, from the gastrointestinal tract to the global ocean, but also in notable dwellings like oceanic basement (Nigro et al. 2017) and petrified stool specimen (Appelt et al. 2014). Phages exist as lysogens in the human gut and act as magnificent modulators in the gastrointestinal environment. This behavior of phages largely influences the physiology and metabolism of respective hosts (Kim and Bae 2018). In this chapter, strategic facts of the biotic and evolutionary doctrines of phages, genomic diversification in the light of metagenomics, their chemistry with the immune architecture, orthodox methodologies, novel stratagems (including the insertion of phage-antibiotic amalgamations), bacteriophage bioengineering, exploitation of bacteriophage resistance, phage-derived enzymes, and other emerging approaches are detailed.

5.1.1 Ancestry of Bacteriophages

Regardless of the extensive presence on earth, phage ancestry remains a myth. Swap of genetic elements is among the crucial mechanisms utilized by phages in the process of evolution. Irrespective of the genetic exchanges, there is huge scarcity of homology at the genome and amino acid level. It should be noted for most of the bacteriophages, a finite number of virion varieties occur in nature. So a definite question arises; whether the structural resemblances are the outcome of divergent or convergent evolution. A divergent evolution would signify a common ancestor with divergence beyond the noticeable homology in sequence, although they have maintained basic confirmation of their capsid proteins. On the other hand, a convergent evolution ensures about no common ancestor but rather has converged in the separate direction to form an optimum virion structure. Although both the evolution pathways can point to a single and very common pathway but with the accretion of equivalent knowledge about the capsid conformational characteristics, they clearly indicate toward divergent evolution hypothesis and, of course, from a common ancestor. The study findings that backed the divergent evolution are as follows: First, evidence fetched from a relation between double-stranded tailed phages, archaeal virus HSTV-1 (Pietilä et al. 2013), and herpes viruses (Baker et al. 2005). All these virion capsids contain a common fold called HK97. These virions also display similarity in their capsid assembly pathways (Rixon and Schmid 2014).

Tectiviridae family gives us the second evidence in the form of PRD1 phage, where the capsid protein is structurally equivalent to the archaeal virus *Sulfolobus* turreted icosahedral virus (STIV) (Khayat et al. 2005) and mammalian adenoviruses (Benson et al. 1999). Capsid protein architecture was made up of β-barrels. There are four different ways to fold β-barrels, but these viruses display only one common way of folding (Benson et al. 2004). Besides, adenoviruses and PRD1 embrace a linear double-stranded DNA (dsDNA) genome with inverted terminal repeats. Other viruses like phage PRD1 (infects gram-negative host) also display structure such as Tectiviridae, Corticoviridae, archeal, and other eukaryotic viruses (Krupovic et al. 2011).

The flag bearer for the third evidence is Cystoviridae phage phi6, which has a resemblance with the eukaryotic Reoviridae like blue tongue virus (BTV) and Totiviridae (El Omari et al. 2013). It was also evidenced that the inner coat protein of double-stranded viruses is similar in confirmation (Huiskonen et al. 2006). Besides, they also contain segmented genome enclosed within a double-layered capsid structure.

5.1.2 Resurrection of Phage Therapy

Continuous acquirement of insolence in pathogen toward antibiotics and the unstoppable rise in mortality are the prime reason for the resurrection of phage cure

approach. Resistance in pathogens become evident in the form of multidrug-resistant (MDR), expansively drug-resistant, and certainly pan-drug-resistant (Magiorakos et al. 2012) bacteria. The flag bearer pathogens for drug resistance are *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Enterococcus faecium*, and *Staphylococcus aureus*. Though the pathogens drew enough attention of health researchers all over the world, the ultimate result is disappointing as there is no answer toward pathogen. The pipeline of a newer drug production is almost dry, resulting in the reemergence of the alternative treatment in the form of bacteriophage treatment. Immunocompromised and patients hospitalized with surgical, burn, or intensive care necessity are at an extremely high risk of catching multidrug-resistant infections. So if you consider post-antibiotic epoch, even simple infections can result in a reason of fatality (WHO 2014).

A recent study on the effect of multidrug-resistant bacteria on world economy and human civilization reveals ten million lives will cease annually, with a flashing US $100 trillion equivalent cost on world economy (O'Neill 2014). It is crystal clear that the global health is in grave crisis, regardless of age or socioeconomic status in the country (WHO 2015).

5.2 Genomic Diversity in Bacteriophages

Phages display an array of structural morphologies in their genomic versions like double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), single-stranded RNA (ssRNA), and double-stranded RNA (dsRNA) genome phages (Table 5.1).

Despite their tiny genomic organization, bacteriophages exhibit plethora of genomic abilities as found by viral metagenomics. The latest progresses in this field discovered several new phages belonging to non-tailed dsDNA (Brum et al. 2013; Kauffman et al. 2018) and ssDNA (Lim et al. 2015; Roux et al. 2019) family, and some of these bacteriophages are abundantly present in biomes.

In detail, metagenomic study additionally disclosed that genetic archeology of these phages. Remarkably, the sequencing data showed no such homology with reference to phage genomes, which proved the genome-level variety of phages (Paez-Espino et al. 2016; Gregory et al. 2019). Among the discovered phages, most of them contain dsDNA and a prominent tail (Ackermann 2007). The most common structural versions of bacteriophage are tailed phages rather than non-tailed phages. Bacteriophage classification was signified by viral archeology, target choice, and genome type (ssDNA, ssRNA, dsDNA, or dsRNA). In 2018, International Committee on Taxonomy of Viruses (ICTV) diversified bacteriophages into 5 families, 26 subfamilies, 363 genera, and 1320 species (https://talk.ictvonline.org/taxonomy/p/taxonomy_releases). Considering the evolution of genome-based techniques, it is expected that the list of bacteriophages will significantly increase in the future (Adriaenssens and Brister 2017).

Table 5.1 Genome- and morphology-based classification of bacteriophages

Genome	Morphological characteristics	Family	Example
ssDNA	•Non-tailed •Icosahedral capsids •Small circular genome	Microviridae	PhiX174
	•Non-tailed •Filamentous capsid •Circular supercoiled	Inoviridae	M13
dsDNA	•Long contractile tail •Icosahedral capsid	Myoviridae	T4
	•Long noncontractile tail •Icosahedral capsid	Siphoviridae	Lambda
	•Short noncontractile tail •Icosahedral capsid	Podoviridae	T7
	•Long contractile tail •Icosahedral capsid	Ackermannviridae	AG3
	•Long contractile tail •Icosahedral capsid	Herelleviridae	SPO1
	•Non-tailed •Circular genome •Capsid with internal lipidic membrane	Corticoviridae	PM2
	•Non-tailed •Linear dsDNA genome •Capsid with internal lipidic membrane	Tectiviridae	AP50
	•Non-tailed •Circular dsDNA genome •Lipidic envelope with no capsid	Plasmaviridae	MVL2
ssRNA	•Non-tailed •Linear genome •Icosahedral and geometrical capsids	Leviviridae	MS2
dsRNA	•Non-tailed •Tri-segmented genome •Spherical capsid	Cystoviridae	Phi6

5.2.1 Genome Size

Bacteriophage exhibits a varied range of genome lengths. Recently, megaphages were reported with the largest genome size of >540 kb. These phages were demonstrated to be resident of the human and animal gut and projected to infect *Prevotella* species as explored in a viral metagenomic study (Devoto et al. 2019). These megaphages are widespread as evident in baboons, pigs, and humans. Large genome size definitely helps these organisms in carrying genes accountable for replication and nucleotide metabolism, which are absent in small genome phages. Till date, the lowest phage genome to be known is Leuconostoc phage L5 (2435 bp). Certainly, L5 phages do have evolutionary advantage and disadvantages over the large-sized megaphages.

5.2.2 Role of Metagenomics in Bacteriophage

Genomic diversity of bacteriophages is hard to grasp, as phages do not contain a similar genetic indicator. Here comes the rescuer, viral metagenomics, which talks beyond the necessity of culture-based techniques and the dependence on marker genes. Metagenomics deals with the whole viral nucleic acid isolated from various environments. Metagenomic study helped to get the first human gut virome from a hale and hearty individual in 2003 (Breitbart et al. 2003). Phage infectivity toward a wide array of host cell makes the situation more complex as they bear minute or no sequence resemblance. Even a single host infecting distinct phages can exhibit significant sequence differences (Hatfull 2008; Krupovic et al. 2011). The use of statistical method like pairwise evaluation for genetic distance and gene content against 2333 phages displayed no homology for almost 97% cases. Besides, until now, metagenomics gave us 90 viromes from aquatic environments, 8 from soil, and 38 from the human gut. Furthermore, several studies that dealt with ulcerative colitis patient (Zuo et al. 2019), twins (Reyes et al. 2010), and healthy adults (Minot et al. 2011; Manrique et al. 2016) displayed interpersonal viral scenario in between health and ailments. The utmost modern discovery by metagenomics is the supreme abundance and widely distributed phage in the human gastrointestinal loci as crAssphage (Dutilh et al. 2014).

5.2.3 Single-Stranded DNA Phages

The most prominent family of these phages are Microviridae and the best discovered member is phiX174 (5386 bp) (McKenna et al. 1992). Microviridae family was widely ignored, until 258 new ssDNA phages were revealed in the gut environment of *Ciona robusta* (marine invertebrate). These phage groups contain small icosahedral capsid (26 nm) with β-barrel structure. Two subfamilies are enlisted under this group, named as Bullavirinae and Gokushovirinae. These two families are structurally quite different as Bullavirinae have pentameric major spike protein complexes at the end of each capsid apogee. In this occasion, Gokushovirinae "mushroom-like" protrusions can be observed that extend along the icosahedral axes of the capsid (Chipman et al. 1998).

Now, Inoviridae family also represents ssDNA genome. The virions in this family contain circular supercoiled ssDNA packed within a long filament, comprised of major capsid protein (MCP) (Ackermann 2012). The MCPs in the filamentous structure are structured uniquely along the genome of the bacteriophages. The MCPs constitute of α-helix with N-terminal signal peptide. This signal peptide helps this virus to translocate the membrane barrier (Xu et al. 2019). Recently, mining of Inoviridae phage family genome explored 10,295 *Inovirus*-like sequences. Among the vast diversified species network, only 5964 distinct species found were

identified. This study strongly defines a hundredfold allocation of variety than previously described (57 genomes) within the Inoviridae family (Roux et al. 2019).

5.2.4 Double-Stranded DNA Phages

The most abundant and widely characterized phages contain double-stranded DNA (dsDNA) genome. These phages belong to Caudovirales order. This order can be further divided into five families: Myoviridae, Siphoviridae, Podoviridae, Ackermannviridae, and Herelleviridae. Together, an extensive variation in the capsid dimension can be witnessed among the members in this order. Capsid diameter arrays from 45 to 185 nm and perfectly corroborates with the genome size (Hua et al. 2017). The capsid proteins in these phages contain HK97 fold (Duda and Teschke 2019). The HK97 fold was first identified from phage HK97 through X-ray crystallography. The tail of the virions linked to the capsid by a connector complex comprised of portal protein coupled to connector proteins. Several structural studies have discovered that the portal complex is a dodecameric ring with an equivalent overall structure, though they have a low sequence resemblance (Lebedev et al. 2007). The glycoprotein 4 (gp4) (head to tail connector) protein of Podoviridae P22 has an equivalent structure to those present in siphophages SPP1 and HK97.

Myoviridae and Podoviridae exemplify 17% and 12% of the entire bacteriophages, respectively. Metagenomic studies from the Antarctic soil explored that the dsDNA tailed phages were dominant, and the occurrence in Myoviridae and Siphoviridae was inversely correlated (Adriaenssens and Brister 2017). Herelleviridae and Ackermannviridae are the furthermost fresh addition to Caudovirales order. It is evident that >85% of the bacteriophage genomes belong to Caudovirales order in the genome database. It has been perceived these two families are the utmost homogeneous family in view of genome analysis, which characterizes as Ackermannviridae (Myoviridae morphology with tail spikes at the base of the tail; e.g., AG3) and Siphoviridae (long noncontractile tail; e.g., phage lambda). Though Herelleviridae is an officially defined family, it reflects similar morphological pattern as Myoviridae.

In the case of non-tailed phages, we describe three families: First, Corticoviridae (circular dsDNA genome) family members enclosed within the capsid constituted of lipidic membrane (internal) enclosed by MCPs (e.g., PM2). Second, Tectiviridae (linear dsDNA genome) has an internal lipidic membrane inside an icosahedral capsid (e.g., PRD1). A signature feature of these two-phage family members is their specialized trimeric form of major capsid protein (MCP), comprised of a double β-barrel sheet structure. Structural analysis of PRD1 phage and their main capsid protein displays N-terminal α-helix formation. It can interact directly with the inner phage membrane and shares exclusive confirmational homology with adenovirus MCP (Benson et al. 1999). Besides, icosahedral apices of PRD1 phage and PM2 exceedingly corroborate structurally to N-terminal domain adenoviruses (that infect all human) (Abrescia et al. 2008). In this context, it should be noted that phage PRD1

do not contain a tail to transport its genome into its gram-negative host, but its membrane can perform as a proteo-lipidic tube, which can also pierce host envelopes (Peralta et al. 2013). Most pleomorphic phages belong to Plasmaviridae family, and it contains double-stranded circular DNA genome, which is surrounded by an envelope but without any capsid structure (e.g., MVL2).

5.2.5 Single-Stranded RNA Phages

Single-stranded RNA phages represent the group of Leviviridae. They are known to be the simplest known positive (+) sense single-stranded RNA. These genomes signify a size of 3.5–4.4 kilobases. Leviviridae, a ssRNA genome (e.g., MS2, R17) bearing phage have capsids with icosahedral and spherical geometry, with a span of 30 nm. The virions of these family encode mostly four proteins such as MCP, replicase, maturation, and lysis proteins. Curiously, MS2 was the first ssRNA virion whose whole genome sequence was decoded. These virions are made of MCP and an RNA-interacting protein of the replicase-encoding gene (Valegard et al. 1990). The most auspicious use of ssRNA phages is in vaccine development, which is a boon for human civilization.

5.2.6 Double-Stranded RNA Phages

Cystoviridae is the family that represents the dsRNA bacteriophages. These phages display an exclusive confirmational upgradation in the form of tri-segmented genome enclosed within a spherical capsid (e.g., phi6). These phages armored with three-layered structural integrity, two-layered inner capsid, and an exterior lipid membrane (Vidaver et al. 1973). The genome of Cystoviridae members transcribed into three segments: large (L), medium (M), and small (S). These three segments behave as an independent polycistronic mRNA and believed to be translated into 12 proteins with the help of host machinery.

5.3 Bacteriophages as a Therapeutic Agent

Some stringent features of bacteriophages like lytic in nature, target pathogen specific, cidal activity, and removal of bacterial endotoxins enhance the refinement of bacteriophages as a therapeutic agent. Besides, knowledge of phage-specific receptors on the host cells is technically essential for resisting phage resisting microbial cells (PRMCs). This knowledge will also guide the future strategy on using combination therapies.

As we know, bacteriophages signify two modes: lytic and lysogenic. From the point of treatment, lytic phages are the prime choice over the lysogenic phages as they can hamper the bactericidal activity, by inducing a prompt rise in homoimmunity and also by lysogenic conversion effect. Phage receptors present on the cell walls, capsules, pilli, and flagella (Bertozzi Silva et al. 2016) invite gram-positive (species or strain specific) or gram-negative (species or strain specific) phages. Attachment of bacteriophages to its highly specific receptors acts as lock and key mechanism. The receptor conformation can guide the phages against a widespread range of potential host. A growing knowledge of host receptors stimulates the research in host–phage interactions.

5.3.1 Why Bacteriophage Remedy Is Superior than Antibiotics

Phage therapy is undoubtedly a multidimensional, dynamic, and multidisciplinary option against rising resistance (Table 5.2). Whereas antibiotics are chemical compounds targeted against particular physiological process in bacterial cell, collateral damage is the common consequence of antibiotic treatment. Antibiotic treatment generally interrupts the microbiome of the host, in the form of antibiotic-associated diarrhea, metabolic or immunologic disorders, etc. (Langdon et al. 2016; Perepanova et al. 2020).

Bacteriophages are extremely precise to target, and this feature can sometimes act as a disadvantage (Table 5.3), as phages need precise etiological agent, occasionally

Table 5.2 Pros of phage therapy compared to antibiotics

Sl. No.	Pros
1	Ubiquity: Naturally copious
2	Specificity of action: It does not affect the normal microflora
3	Higher safety: No effect in normal mammalian cells.
4	Reproduction in exponential rate: Helps to formulate lower dosage
5	Self-limiting: Once the host is lysed, phages will automatically cease to work
6	Evolution: If resistance against phages develop, phages adapt alongside bacteria to counter the resistance
7	Higher tolerability: The immune system tolerates the phages in a better way than the antibiotics
8	Administration does not always require neutralized pH environment
9	Site-directed effect: Fire and forget approach as it is limited to the site of infection
10	Biofilm lysis: Very effective against biofilm inactivation
11	Less expensive: Simple purification and abundance of technology for low-cost formulation of phage therapy
12	Remarkable benefits after genetic engineering

Table 5.3 Cons of phage therapy compared to antibiotics

Sl. No.	Cons
1	Narrow spectrum action: Causative organism must be identified sometimes to the strain level
2	Stimulation of phage-neutralizing antibodies inside the humans is an issue to be resolved in the clinical level
3	The number of successful and scientifically designed clinical studies on humans is very small
4	Unlike antibiotics, there is a lack of explicit regulatory and legal authorities regarding intellectual property rights

to the strain level, and this methodology is resource and time consuming (Caliendo et al. 2013).

Target specificity also gives phages upper hand over the antibiotics as they don't disturb the microbiome. It has been explored that early commencement of phage therapy can significantly reduce pathogen count (Jaiswal et al. 2013; Chhibber et al. 2008). Altogether, it is evident that collection of new phages should be in pipeline in a constant manner. Not only isolation but well-defined strain-level portrayal of bacteriophages should be refined to use it as potential phage candidate. These potential phage candidates should have short latency period, large burst size, and expansive host range (Nilsson 2014; Whittard et al. 2021). A current study in Australia validated safety and efficacy of a good manufacturing procedure where a three-phage cocktail inoculated intravenously to 13 patients with hostile *S. aureus* infections (Petrovic Fabijan et al. 2020).

The role of the immune system is crucial against these dual therapeutic techniques. The most lethal side effects of antibiotics are stimulation of hypersensitivity and mostly IgE-mediated anaphylaxis (Legendre et al. 2014). Relatively, there is no such documented occurrence of anaphylaxis with phage cure in humans (Sarker et al. 2012; Speck and Smithyman 2016). It should be noted that the human evidence-based study with bacteriophage remedy is small. It is to be noted that the immune system counterparts both the healing approaches.

Lytic phages are killers of their target, and still, they are not able to eradicate all the pathogen on their own. A well-known argument for the above statement is that eradication of host will result in cessation of the virus as well. That is why bacteriophages activate dynamic strategy as "kill the survivor," (Campbell 1961; Maslov and Sneppen 2017) where host abundance is rapidly reduced to an assured extent that the immune system can take over the clearance protocol. The immune system assists the phages in clearing the pathogen, which can be an immunophage synergy (Roach et al. 2017). So these findings establish the theory that bacteriophages elicit both innate and acquired immune system. In view of all the valid points between phage therapy and antibiotics, there are some similarities as well (Table 5.4).

Table 5.4 Similarities between phage and antibiotic therapy compared to antibiotics

Sl. No.	Shared characteristics
1	Effectivity very much influenced by the immune system
2	The development of resistance is a common phenomenon in both the cases
3	Initiation of treatment and the time interval highly regulate both the therapies
4	Therapeutic approaches can be utilized by several routes of administration

5.3.2 Antiphage Mechanism of Bacterial Host

Bacteriophages are not immune against resistance like antibiotics. Lytic phages employ an intense antimicrobial selective pressure, which generates rapid phage-resistant bacterial mutant. One of the first mechanisms employed by pathogens are (a) mutation in the phage receptors in the host so that phages are inhibited against and adsorption and superinfection step; (b) restriction modification system that is another strategy where host genome is kept protected, but the phage genome remains vulnerable by the target restriction enzymes; and (c) besides, clustered regularly interspaced short palindromic repeat (CRISPR)–Cas (Labrie et al. 2010) tactic where host cell keeps memory of the previously confronted foreign DNA and degrade again if it enters. Altogether, these mechanisms can be termed as prokaryotic immune system. In 2018, Doron et al. explored in research that beyond 45,000 bacterial and archaeal genomes have new antiphage mechanisms that are yet to be established.

5.3.3 Phages and the Human System

It is proved that phages in the human gut encode a population of hypervariable (large sequence variation) proteins (Minot et al. 2012). Most of these hypervariable proteins possess C-type lectin fold, and some of them displayed immunoglobulin (Ig)-like domains (Medhekar and Miller 2007). Interestingly, this fold was previously reported from Bordetella phage BPP-1 tail fibers. Ig-like domains are displayed in many structural proteins of phages (Fraser et al. 2006, 2007). In vitro growth conditions recommended that these Ig-like proteins aid in the attachment of their target prey under environmental conditions (McMahon et al. 2005; Fraser et al. 2007).

Phages are capable of crossing the mucosal barrier at a concentration where it can bypass and intermingle with the cellular epithelium. In vitro research findings using cell lines support the theory that phages can enter and cross epithelial cell layers by non-specific transcytosis mechanism, from apical to basal direction (Nguyen et al. 2017). Bacteriophage transcytosis phenomenon occurs across different types of epithelial cell (e.g., gut, lung, brain, liver, and kidney cells). This phenomenon works for diverse phage types and morphologies (e.g., Myoviridae, Siphoviridae, and Podoviridae). Critical experiments on transcytosis explored that merely 10% of

the entire phages endocytosed through the epithelial cells, which found to be localized within the membrane-bound vesicles. These phage particles travel through the Golgi apparatus, before being exocytosed as functional units in the basal layer. This transcytosis process across epithelial layers certifies the manifestation of phages in the human system even in the nonappearance of disease or pathogen (Nguyen et al. 2017).

Phages are qualified of directly interacting with mammalian cells, as discovered by Bloch in 1940. This study validates the attachment of phages to cancer tissue, which primes the tumor inhibition. Later, it was reestablished that phage can not only bind to cancer cells in vitro and in vivo but also are quite capable of attaching lymphocyte plasma membrane (Wenger et al. 1978; Dąbrowska et al. 2014). Several studies started to understand the molecular basis phage–mammalian interaction. In 2003, Gorski et al. hypothesized that cross talk happens due to the existence of a tripeptide motif Lys-Gly-Asp (KGD). This motif found to be present on T4 capsid protein gp24 and verified to be acting as ligand for the β3 integrins on cells. In this framework, a significant study has to mentioned where phage capsid protein motif (WDC-2, containing a TRTKLPRLHLQS peptide motif) was modified. This amendment resulted in potential tumor-specific phages with 93% success rate (Eriksson et al. 2009).

5.3.4 Bacteriophage Interaction with the Immune System

The human body interacts with several viruses each second. A majority of the microorganisms settle first at body surfaces (intestine, skin, and respiratory tract) that are open to welcome microbes. Studies from the last decade explored that microflora of human beings also inducts viruses (White et al. 2012), especially bacteriophages. Metagenomic studies have discovered the hidden world of phages inside the human system (Minot et al. 2011).

It will be fascinating to discuss whether phage–host dynamic relation of the human intestine could significantly modify the composition of the normal flora and influence on the spread of pathogenic viruses (Duerkop and Hooper 2013). Niche occupation by phage and limiting pathogen colonization and resource use is the signature effect of host immunity. Few viruses target mammalian cells, but phages donate to the mainstream of the viral community (Cadwell 2015).

As we know, phages are daily commuters of the human body, but questions persist over the safety and immunogenicity regarding phage therapy (Cooper et al. 2016). It is evident now that bacteriophages have not only antibacterial properties but also a part of mucus layers and even migrate through the cell layers (Nguyen et al. 2017). When these phages interact with individual immune system, they induce both innate and adaptive immune responses (Van Belleghem et al. 2017; Majewska et al. 2015; Miernikiewicz et al. 2013; Hodyra-Stefaniak et al. 2015). No doubt bacteriophages adhere or attack nontarget tissues or cells to some point (Table 5.3). During portal entry, some phages are taken up by the gastrointestinal tract into the

blood. This phenomenon ascertains phage to epithelium transition and reticuloendothelial system (Merril 2008; Górski et al. 2006).

In 2012, Gorski et al. discovered that purified phages show anti-inflammatory activities via the suppression of reactive oxygen species (ROS) and also NF-kB inhibition. Phages also affect the macrophage-mediated cytokine production (Van Belleghem et al. 2017). The immunomodulating effect of phages in the blood has been suggested by different studies of Górski et al. (2012, 2017). Besides, inhibition of inflammation caused by bacterial infections was also proved. A recent study explored that the immunomodulating effect of bacteriophages was regulated at the transcription level from monocyte-related immunity genes (Van Belleghem et al. 2017).

5.3.5 How Does the Immune System Work Against Bacteriophages?

5.3.5.1 Phagocytic Response

Mononuclear phagocytic cells were the prime members of innate immune system. Simultaneous inoculation of phage, together with host bacteria, undoubtedly stimulates bacterial phagocytosis, and this effect is ensured by certain opsonization of bacterial cells by phages. Besides, it was observed that phages stay functional and virulent to their host bacteria even after ingestion by granulocytes (Kaur et al. 2014). Therefore, some authors also endorsed that phages can be operative in lysing the phagocytosed bacteria, catalyzing the activity of phagocytic cells (Górski et al. 2012). It was speculated that phages might inhibit the platelet and T cell adhesion to fibrinogen (Kurzepa et al. 2009) and thus strengthens the critical role of bacteriophages in transplant rejection, metastasis, and angiogenesis. These phagocytic cells can effectively remove these viruses from the circulatory systems (Navarro and Muniesa 2017; Górski et al. 2012). Several antigenic combinations can perform as pathogen-associated molecular patterns (PAMPs), and in this case, phage nucleic acid can act as potential PAMP. These PAMPs are recognizable by cognate, toll-like receptors (TLRs) or TLR9 (responsible for recognition of DNA). Inside the cell, phage nucleic acid is unprotected and vulnerable to cellular defenses. Further, experiments on λ phage revealed phagocytic cells rapidly remove λ phage from the circulatory system in humans (Merril et al. 1973), but certain λ phage mutants can prevail for a longer period in the blood system than the wild-type version (Merril et al. 1996).

5.3.5.2 Cytokine Response Against Phage

Bacteriophages certainly stimulate cytokine response as evident by different studies. But most of the studies were performed with phages having bacterial endotoxins or

proteins. A study conducted with 51 patients suffering from suppurative bacterial illnesses (antibiotic-resistant bacterial species) of numerous organs and tissues, who were treated with phages. Afterwards, blood sample was taken and analyzed for IL-6 and TNF-α. The authors witnessed a sharp decline in the illustration of these cytokines after a long-term treatment (i.e., 21 days). In another experiment, mice were remedied intraperitoneally for 5.5 h with phage T4 or capsid proteins (i.e., gp23, gp24, Hoc, Soc) and analyzed for cytokine expression. This experiment displayed no such inflammatory-mediating cytokines (Miernikiewicz et al. 2013).

Another instance, where phages and antibiotics were mutually used as a treatment measure in mice, showed that phage-cocktail-treated group had a gradual decrease in both IL-6 and TNF-α level for 3 days (Jaiswal et al. 2014). Astonishingly, phages also exhibit conserved anti-inflammatory properties. In a designated study, phage specific to *Pseudomonas aeruginosa* and *Staphylococcus aureus* has been shown to stimulate equivalent immune responses (Van Belleghem et al. 2017). Their expression levels regarding inflammatory markers like IL6, suppressor of cytokine signaling 3 (SOCS3), and IL-1 receptor antagonist (IL1RN) are quite similar. The above findings were also endorsed in murine models of xenografts (by Górski et al. 2016) where he described an immunosuppressive effect of phages.

5.3.5.3 Antibody Response Against Phages

It is obvious that phages do stimulate the manufacture of cognate antibodies as observed in humans and animals (Górski et al. 2012; Puig et al. 2001). It is the most potent barrier in using phages as therapeutics. Shortly, after the discovery of phages, antibodies against phages were also encountered (Jerne 1952, 1956).

One important observation regarding phages is that they don't follow a simple rule of stimulation. Rather, the route of inoculation from individual to individual matters. Besides, the dose and application schedule individually also influence the antibody response (Dąbrowska et al. 2014; Łusiak-Szelachowska et al. 2014). The antibody response stimulated against phages can be detrimental (Huff et al. 2010), but as evident by scientific experiments, antiphage antibodies do not rule out a favorable therapeutic practice of phages in humans (Łusiak-Szelachowska et al. 2014). It is clearly known that humoral immunity can be stimulated by inherently occurring phages. So antiphage antibodies can be spotted in the serum of different species (e.g., human), even before any kind of phage treatment.

Since the 1970s, bacteriophages were used comprehensively to detect and track immune deficiencies in humans. One such example is the immunization of human with φX174 to gather detailed information about primary and secondary immunodeficiencies. This study elaborates no such adverse or severe antiphage antibodies in patients where phages were found to be present in the bloodstream for a prolonged period (Ochs et al. 1971; Rubinstein et al. 2000; Shearer et al. 2001). Earlier studies based on T4 phage confirmed that no antibodies were stimulated in volunteers at all (Bruttin and Brüssow 2005). These findings were further supported by Dąbrowska et al. (2014), where 50 healthy (never been subjected toward phage therapy or

phage-related work) individuals were taken to observe phage T4 activity. Surprisingly, they showed 82% decreased activity against phage T4, whereas positive antiserum cases showed increased intensities of IgG antibodies against Hoc, Soc, gp23, and gp24 proteins. The antibody response was generated mainly due to the gp23 protein.

A study where 20 patients infected with *S. aureus* treated with MS1 phage cocktail by either oral route or local inoculation showed an elevated level of IgG, IgM, and IgA (Żaczek et al. 2016). Within them, few patients showed lower response, whereas some showed higher antibody response levels. This can be enlightened by several opinions. But ultimately the good news is that the presence of antiphage antibodies did not undermine the clinical effectivity of bacteriophage therapy.

5.3.6 Bacteriophage Pharmacokinetics

The route of administration of phages crosstalk with phage pharmacokinetics. There are variety of proven administration routes for phages. Several studies regarding topical (wound infection, eye infection, otitis), transectal (prostatitis), intravenous (septicemia), transnasal (lung infection), transurinary (bladder, kidney), or oral use of pages have been documented. It is evident when bacteriophages were directed by topical, transnasal, transurinary, and intravenous routes, phages can candidly interact with target pathogens and eradicate them. In contrast, when oral or transrectal routes are utilized, the phages need to travel from the gut environment to the blood and lymph by invading epithelial cell layers. Here, we will discuss briefly about the recent human experiments done on humans and their consequences from the pharmacokinetics angle.

5.3.6.1 Topical or Intranasal Applications

In Georgia, cystic fibrosis patient was treated for multidrug-resistant *Achromobacter xylosoxidans*. A mixture of two phages was administered orally and via inhalation through a nebulizer at a concentration of ~10^8 (plaque-forming units, pfu). This dose was continued for twice daily for 20 days, and the dose was repeated at 1,3, 6, and 12 months. This study showed a promising result against the infection and improved the patient clinically (Hoyle et al. 2018). In USA, *Staphylococcus aureus*–infected diabetic toe ulcer patients were treated with phage Sb-1. The bacteriophages were given to wound cavity with 0.1–0.5 ml phage suspension-soaked gauze. This treatment dose was given once in a week up to 7 weeks, and the patients recovered (Fish et al. 2016). In 2018, Fish et al. again successfully treated patient with digital staphylococcal osteomyelitis in diabetic foot ulcers infected with *S. aureus*. The soft tissues around the ulcer were injected with 0.7 ml once a week for 7 weeks and found significant recovery among patients. In Georgia, a young patient with Netherton

syndrome (a rare disease) accompanied with severe *S. aureus*–infected skin disorder, treated with Sb-1 phage and other *S. aureus* cocktail phages. Patient limbs were sprayed and cream treated with around 10^7 phages. Patients also medicated with oral application of phages.

5.3.6.2 Intravenous Application of Therapeutic Phages

Here, phage particles were directly introduced into the bloodstream, so it doesn't require any translocation to the host site. The safety of this administration route was experimentally proven by Speck and Smithyman (2016) to some extent.

In 2017, Schooley et al. did an experiment on multidrug-resistant *Acinetobacter baumannii* in diabetic patients with necrotizing tissues by percutaneous drainage catheters with phage φPC (a cocktail of phages AC4, C1P12, C2P21, and C2P24). In addition, the above phage φIV (a cocktail of phages AB-Navy1, AB-Navy4, ABNavy71, and AB-Navy97) was inoculated intravenously. After this treatment, the patient recovered from coma. This treatment was continued for 59 days, and the patients recovered with time. In 2017, patients infected by *P. aeruginosa* with acute kidney damage in Belgium were treated with 50 μl BFC1 cocktail (14/1 and PNM) and one *S. aureus* phage ISP. The dosage was 6 h intravenous infusion for 10 days, and also wound was irrigated with 50 ml of the cocktail every 8 h for 10 days. This treatment protocol resulted in a speedy recovery of the patients.

5.3.6.3 Oral and Intrarectal Administrations of Therapeutic Phages

The most challenging route of inoculation is oral and intrarectal where phages are not capable of interacting directly with the target pathogen, as the bacteriophages must transit from the gastrointestinal tract to the bloodstream. Though there are different successful studies with oral or rectal administration, the exact mechanism of bacteriophage translocation is still under the cloud. Oral administration has been explored to be effective against systemic infections (Alisky et al. 1998; Weber-Dąbrowska et al. 2000; Górski et al. 2006). It is highly essential to neutralize gastric acid before administering bacteriophage through oral route as low pH in the stomach may inactivate the bacteriophages (Alisky et al. 1998; Weber-Dąbrowska et al. 2000). Jaiswal et al. in 2014 showed neutralization of gastric acid prompted the phages to move from the stomach to the intestine and diminish the intestinal colonization of pathogenic bacteria in an animal model. Bacteriophage translocation from the stomach to the blood was experimentally proved in both neutropenic and healthy models (Matsuzaki et al. 2014; Międzybrodzki et al. 2017). Międzybrodzki showed that the transit of bacteriophages might highly influence the phage and animal species or characteristics. This experiment clarifies two animals (rat and mouse) and two bacteriophages (*Escherichia coli* phage T4 and *S. aureus* phage A5/80) were used. The very first observation was for phage T4 and A5/80 where these orally inoculated phages could travel from the gut to the small intestine faster if

gastric acidity is neutralized. Second, it was detected that phage A5/80 effectively translocates from the gut to the blood in mouse, but considering T4 phage, the efficacy of transit was considerably lower. Interestingly, there was no translocation for both the phages in mouse. In this setting, Bari et al. in 2017 enlightened the possible reason behind the low-frequency translocation of T4 phage in mice. As explained by Bari et al. 2017, the reason may be the incidence of T4 head of immunoglobulin-like non-essential Hoc protein. In the intestine, T4 head attaches to the mucus layer via Hoc and leaves the tail tip free. So this adherence connection may be due to the probable missing link between lower or non-translocation of the bacteriophage from the stomach to the bloodstream.

Administration of phages through the intrarectal pathway against human enterococcal prostatitis has demonstrated to be efficient (Letkiewicz et al. 2009, 2010; Górski et al. 2018). The transit of phages in this situation was believed to be similar to that of the previous cases. Therapeutic phages have been used significantly in Georgia, Russia, and Poland for many years. Phages were applied through versatile administration routes like intravenous, oral, and transrectal routes. We have discussed successful human clinical trials around the world that emphasizes the effective and useful nature of phages irrespective of their method of administration, though it should be documented that oral path of administration is specially preferred due to its versatility. The precise mechanisms dealing with phage translocation into the blood are still under the lens of molecular biology and cytology experiments. Regrettably, however, there is very little evidence on the bacteriophage dynamics in the human body. So a detailed knowledge of phage pharmacodynamics is highly desirable to improve the suitability of bacteriophages as a perfect therapeutic agent.

5.3.7 Bacteriophage-Derived Lysins

Bacteriophage lysins are highly effective molecules, which facilitate release of its progeny phage during its replication cycle. These enzymes are specific to the five major bonds in peptidoglycan. It must be notable that lysins don't have signal sequences, other than few exceptions. This factor bars the lysins to translocate through the cytoplasmic membrane and interact with their substrate in the peptidoglycan layer. The intermingle of lysins with their cognate substrate is tightly regulated by holing the lytic system (Wang et al. 2000). At a definite time during phage replication inside the target cell, holin molecules are injected into the bacterial cell membrane and create a patch and ultimately induce a localized membrane disruption (Wang et al. 2003). Now, the cytoplasmic lysins that were accumulated in the cytosol are free to interact on the peptidoglycan and cleave specific bonds. This causes the cell to lyse immediately. Unlike large DNA phages, small RNA and DNA phages engage a different strategy like interfering with the enzymes responsible for peptidoglycan synthesis (Bernhardt et al. 2001). It is clear now that phages evolved from several years to bear features like lysin production. Just like naturally

occurring antibiotics in bacteria that help in combatting other organisms around them, bacteriophages do maintain their surrounding with the help of lysins.

It was proven that some lysins (isolated from gram-positive bacteria) can kill their targets within second of contact (Loeffler et al. 2001; Nelson et al. 2001). Such an example is nanogram amounts of lysins that could reduce 10^7 *Streptococcus pyogenes* by >6 log minutes after enzyme addition. This is a remarkable feature of lysin, as no biological compounds are found to kill bacteria at such speed other than chemicals. In 2001, Nelson et al. coined the phrase "enzybiotics" to designate these novels highly destructive antibacterial compound. It should be noted that until now, only two lysin-based ointments (polysporin and mupirocin) are extensively used to control colonization of pathogenic bacteria in mucus.

5.3.8 Lysin Structure and Mechanism of Action

5.3.8.1 Gram-Positive Specific Phage Lysin

Lysins secreted by DNA phage (specific to gram-positive bacteria) ranges between 25 and 40 kDa. Only one exception was discovered in the form of PlyC lysin (114 kDa), isolated from C1 phage of group C streptococci. A common trait of gram-positive host-specific phages is their two-domain structure. The two domains can be divided as catalytic domain or N-terminal domain and cell-binding domain (CBD) or C-terminal domain.

The activity of N-terminal domain can vary widely such as attack on (1) an *N*-acetylmuramoyl-L-alanine amidase (or amidase) that hydrolyzes the amide bond linking the glycan strand and peptide moieties (Loessner 2005), (2) an endo-β-*N*-acetylglucosaminidase or *N*-acetylmuramidase (lysozymes) that equally affects on the glycan moiety of the wall peptidoglycan, (3) an endopeptidase that cleaves the stem peptide moiety, or (4) a phage lysin with γ-D-glutaminyl-L-lysine endopeptidase efficiency that has also been discovered (Pritchard et al. 2007), but these enzymes are rare to find compared to others. It is also evident that in some bacterial phage (some staphylococcal and streptococcal phage), two to three types of catalytic features can be observed in a single domain (Cheng et al. 2005).

On the other hand, C-terminal or cell-binding domain (CBD) mostly targets cellular carbohydrates in the cell wall for attachment. Cell membrane–specific choline-binding lysins were discovered in pneumococcal phage lysin Cpl-1 (Hermoso et al. 2003). Later PlyL lysin and Ply21 lysins were discovered in *Bacillus anthracis* and *B. cereus* phage TP21, respectively. Interestingly, these phage lysins contain hairpin confirmation. This hairpin confirmation ensures that the catalytic domain and CBD interact with each other. This confirmation also defines that catalytic domain remains inactive until cell-binding domain interacts with its target molecule in the cell wall or membrane.

5.3.8.2 Gram-Negative Specific Phage Lysin

It is fascinating that lysin in gram-positive and gram-negative lysin behaves differently toward their target. Their characteristics are also different. Most gram-negative lysins behave like lysozymes. Gram-negative lysins are more complex as explored by Lood et al. (2015). He explored several lytic clones and found four distinct domains, unlike gram-positive bacteria. The four domains are (1) a TIGR02594 domain, (2) a catalytic domain, (3) a binding domain, and a (4) lysozyme domain. In this study, it was exclusively reported that TIGR02594 and lysozyme domain were flanked on a single side or both sides of the lysin with short positively charged amino acids. Thus, it helps the lysin both break the peptidoglycan with functional enzymatic domain and disrupt the outer membrane with charged domain, resulting in highly effective lysis of host cell (Thandar et al. 2016). It is established that gram-negative bacteria have lower internal turgor pressure (~3–5 atm) than their gram-positive (~15–25 atm) siblings. So in the case of gram-negative cells, there may be scarcity of sufficient pressure that supports the above theory.

5.3.9 Synergistic Approaches with Lysin

Several pneumococcal phage lysins were isolated, demonstrated, and categorized into two groups: amidases and lysozymes. Interestingly, both classes of lysins display similar C-terminal choline-binding domain but different N-terminal catalytic domains. Like cocktail phages, in a study, it was evident that a blend of two lysins with diverse peptidoglycan specificities was more applicable in shielding against a disease (Loeffler and Fischetti 2003; Schmelcher et al. 2015). It should be noted that a combination of lysins significantly enhances the effectivity of killing kinetics and also reduces the chance of emergence for enzyme-resistant mutants.

Considering an unusual combination (lysin and antibiotics), pneumococcal lysin Cpl-1 and gentamicin are highly effective in slaying pneumococci, while Cpl-1 and penicillin displayed synergy against an extremely penicillin-resistant pneumococcal variety. In another experiment, staphylococcal phage lysins and antibiotics against methicillin-resistant *Staphylococcus aureus* (MRSA) also found to be effective in in vitro and in vivo condition (Daniel et al. 2010). So it can be concluded that a correct combination of lysin and antibiotic could not only control antibiotic-resistant bacteria but also reestablish the use of specific antibiotics (against which resistance already reported). The good news regarding lysin is that till now no such bacterial resistance is reported. Experiments have been designed to comprehend the resistance pattern of bacterial cells against lysin. A 10 μl drop of dilute lysin was dropped on a bacterial lawn. After incubation, a clear lytic zone was formed and colonies from the periphery of the clear zone were taken to test the resistance. But in >10 cycles of bacterial contact to diluted concentrations of lysin in liquid media, no resistance was found (Loeffler et al. 2001; Schuch et al. 2002). It is obvious that the lack of resistance development is connected with the evolution of binding domain, which may be designed to avert lysin spill during lysis phenomenon.

5.3.10 Clinical Approach of Lysin

In 2016, Czaplewski et al. revealed in a review that diverse strategies has been considered earlier to find best substitute against antibiotics which include bacteriophage lysins as therapeutics, antibodies and vaccines as prophylactics, and probiotics as a treatment. In 2018, a company called ContraFect has used lysin (CF-301) against *S. aureus* bacteremia and endocarditis in phase 2 trial worldwide. Results of that trial against MRSA explored a 42% improvement over antibiotic treatment. Later, follow-up studies also supported the above findings, as humans treated with drug–lysin combination were discharged earlier and had fewer relapses compared to drug-alone-treated patients.

As we are surrounded by multidrug-resistant gram-positive and gram-negative pathogens, phages can be employed in indirect way to isolate and apply lysins against them. The lysins under use or under trial are astonishingly heat stable (up to 60 °C) and can be purified and marketed in large quantities with rapid manner. In the future, protein engineering, domain swapping, and gene shuffling approaches could be handy in coping up against continuously adapting bacterial pathogens.

5.4 Genetic Engineering of Bacteriophages

Bacteriophage diversity and adaptive potentials definitely make them an ideal candidate for genome engineering. Engineered phages can be applied to different sectors like in synthetic biology (Lemire et al. 2018), material science (Cao et al. 2016), and biomedical fields (Kilcher et al. 2018). There are several techniques to engineer phages.

5.4.1 Homologous Recombination–Based Techniques

This phenomenon talks about the swap of sequences in between two DNA strands, which share identical sequences. Phage cross is such a classical strategy to produce a mutant phage with a desired phenotype by combining characteristics from two parents (Karam and Drake 1994). Sometimes, host cells were coinfected with two dissimilar phages, and the homologous recombination inside the host cell generates the desired progeny with the desired phenotype. The progenies were chosen by nominating selective markers.

Homologous recombination among the plasmid and phage genome was also developed where plasmid containing the desired mutation flanked by homologous sequences was commenced into the host cells, which is also coinfected with the desired phage to be engineered (Namura et al. 2008). Though the overall rate of frequency of recombination is very low (Tanji et al. 2004), in some cases, a high

frequency of recombination is also observed (Oda et al. 2004). So this genetic strategy is complicated and time consuming to find the preferred recombinants unless there is a properly designed selection strategy.

5.4.2 *Bacteriophage Recombineering of Electroporated DNA (BRED)*

This is a very popular technique based on the homologous recombination principle. This method exploits a RecE/RecT system of Rac prophage and Red system of lambda phage to enrich the frequency of recombination (Murphy 2012; Nafissi and Slavcev 2014). Red system is a well-coordinated system, composed of exo (α), bet (β), and gam (γ) genes. The target of exo gene is double-stranded DNA (dsDNA) end to transform to single-stranded DNA (ssDNA) substrate, whereas beta performs as a ssDNA-binding protein that anneals ssDNA target and cognate recombination zone in phage genome. Lastly, gamma works as an inhibitor of *E. coli* RecBCD exonuclease complex (designated to degrade linear dsDNA substrate).

BRED needs co-electroporation of the bacteriophage DNA and donor DNA expressing RecE/T-like proteins into recipient cells via either plasmid or chromosomally inserted so that homologous recombination can be promoted in an efficient manner (Marinelli et al. 2008, 2012; Thomason et al. 2009). It was observed earlier that recombination occurs only after starting of bacteriophage genome replication, and further purification of recombined phage is highly essential apart from initial PCR screening (Marinelli et al. 2008). This technique was first employed on mycobacteriophage and later applied to several other phages by constructing replacements, deletions, and insertions of heterologous genes (Marinelli et al. 2012). This technique is not so useful regarding gram-negative bacteria as they have low transformation efficiency, compared to gram-negative bacteria. So in the case of gram-positive bacteria, instead of transforming both bacteriophage DNA and donor DNA, bacteria that enclose phage-defined recombination system can be transformed with donor DNA only (Pan et al. 2017). The phage mutants can then be generated by infecting bacterial cells with wild-type phage. The main issues with high background of wild-type phage are a matter of concern, and also, it needs screening of recombinants.

5.4.3 *CRISPR–Cas System–Based Engineering*

At the dusk of the last century, a novel, highly effective immune system of prokaryotes was discovered. It's a defense mechanism of prokaryotes against foreign invasion, named as clustered regularly interspaced short palindromic repeats (CRISPR)–Cas system. This system has two main components CRISPR RNA

(crRNA) and Cas proteins. CRISPR–Cas system can be classified and can be further diversified into six types. These six types can be brought under two classes (class 1 or 2) based on mechanism and phylogeny (Koonin et al. 2017). It was proved that class 1 systems include types I, III, and IV recruit multiple Cas proteins in association with effector complex, whereas class 2 systems (type II, V, VI) employ single Cas protein in association with effector complexes to nick the target DNAs (Koonin et al. 2017).

The CRISPR–Cas effector complex is target specific and binds their target sequences intervened by the crRNA with a complementary region to the sequence of action. Here Cas proteins cleave the DNA and generate a break into the double-stranded DNA (Shmakov et al. 2017; Knott and Doudna 2018). It is well documented that the CRISPR–Cas system was first applied in genome editing in 2014 to identify a phage mutant with a deletion of a non-essential gene, gene1.7 (Kiro et al. 2014). This study denotes CRISPR–Cas system can be easily used as a screening utility to eradicate wild-type phage from the recombinant population. The plasmid relied type I CRISPR–Cas system was defined to target gene 1.7 and cleave the wild-type T7 phage genome, whereas mutant phages lacking gene 1.7 were protected from Cas9 complex and could propagate normally. Later in 2016, Box et al. used type I CRISPR–Cas system of *Vibrio cholerae* to engineer *V. cholerae* lytic phages. In the above study, both the donor DNA and CRISPR–Cas components were assembled in a single plasmid. Those plasmids were then harbored by *V. cholerae*; as phages attacked the cells, phage genomes were cleaved by CRISPR–Cas but immediately repaired by homologous recombination with the donor DNA and give rise to recombinant phages with deletion or insertion mutation (Box et al. 2016).

Streptococcus pyogenes CRISPR–Cas is the most commonly used type II system, selected for bacteriophage genome engineering (Lemay et al. 2017; Schilling et al. 2018; Shen et al. 2018). It should be noted the first type II CRISPR–Cas system utilized for phage genome modification belonged to *Streptococcus thermophilus* (Martel and Moineau 2014). Recent works on *Listeria monocytogenes* also confirmed the existence of CRISPR–Cas system and are found useful in valuable engineering program for *Listeria bacteriophages* (Hupfeld et al. 2018). Generally, the three components Cas9, crRNA, and transactivating crRNA (tracrRNA) of CRISPR–Cas system were attached or cloned to a single plasmid. It was interesting to observe that crRNA and tracrRNA could be expressed either separately (Lemay et al. 2017) or as a sole fusion RNA (Schilling et al. 2018). Due to a successful transformation into recipient cells, the machineries form CRISPR–Cas9 complex will specifically connect to the phage genome and craft a double-stranded DNA break during phage genome invasion. It was also explored that Cas9 of *S. pyogenes* is highly capable of cleaving T4 phage genome (highly protected from most of the restriction endonuclease enzymes due to high levels of 5-hydroxy methylation and glucosylation to its cytosine molecules (Tao et al. 2017, 2018b). It has to be mentioned that sometimes cleavage potential of CRISPR–Cas9 complex on its target crRNA (protospacer sequence) is very high, and then, only the recombinant phages can survive. Consequently, all resultant progeny phages are recombinant mutants. If

somehow the protospacer sequence is feebly cleaved or an overburden of parental phage was employed that time, it could lead to error-prone repair and also may result in inclusion of random mutations in the protospacer sequence ensuing in dodging from CRISPR–Cas cleavage. This phenomenon is occasionally reported from equally type I and II CRISPR–Cas systems (Barrangou et al. 2007; Fineran et al. 2014).

Next, *Staphylococcus epidermidis* represents the type III CRISPR–Cas system. This system allows engineering of virulent staphylococcal phages. Type III system comprises of native endogenous CRISPR–Cas10 system but is accompanied with the crRNA transcribed from an exogenous plasmid (Bari et al. 2017). Interestingly, CRISPR–Cas10 system has an excellent cleavage frequency against elevated dose of staphylococcal phage infection.

5.5 Future Prospects

Bacteriophage till now, evidenced to be a versatile candidate, can be utilized both as prophylaxis and as therapy against an infection. Therefore, it can be applied equally before and after bacterial infection (Debarbieux et al. 2010; Chanishvili 2016; Tao et al. 2019).

5.5.1 Vaccines

Recent studies ventured the potential of phages in vaccine platform other than prophylactic or therapeutic platform. Bacteriophages are natural virus that display several properties like size, geometry, multivalent display, and ordered and repetitive structure equivalent to a natural mammalian virus (Bachmann and Jennings 2010; Zepp 2010). These characteristics are crucial for stimulating immune response and guidance for vaccine design, and the only difference is that they infect bacteria (Jończyk-Matysiak et al. 2017). In recent past, several efforts have been employed to enhance the use of phage in vaccine platform, such as T4 (Tao et al. 2013), MS2 (Fu and Li 2016), phages λ (Nicastro et al. 2014), and others (Tissot et al. 2010).

The foundation for using phages as antigen carrier vehicles engages assimilation of the viral antigen on phage capsid either in vivo or in vitro to form an original virus-mimicking particle (VMP) through fusion of antigen to a virus capsid protein. This enabled antigen to be displayed by the phages in a repetitive format, which is crucial for innate immune system activation (Shepardson et al. 2017). For small genome phages, fusion of antigen and capsid protein is easy to produce, in comparison to multifaceted phages such as T4. Bacteriophages do contain CpG (a vital ligand for human immune system Toll-like receptor 9), a crucial viral character, so phages can definitely elevate the level of innate immune response (Sartorius et al. 2015). Hence, this nature of phages displaying antigens confirms itself as a highly

effective self-adjuvating vaccine delivery system that is qualified of eliciting a long-lasting immune response without any external adjuvants. Indeed, in 2013, Tao et al. proved that antigens embedded on T4 capsid elicited a much higher immune response associated to their soluble antigens. In another experiment, a phage QB VMP enveloped with CpG sensitizes antigen presenting cells in a faster and better way than a simple mixture of antigen (Gomes et al. 2017). Apart from that, it was also explored that phage QB capsid was able to bind natural IgM due to the display of highly ordered and repetitive format antigen. This capsid can also fix complement component 1q and easily deploy follicular dendritic cells (FDCs). Deploying of FDCs is indispensable or the choice of B cell during germinal center reactions (Link et al. 2012). Another huge advantage of phage VMPs and their display of highly restricted epitope density in a particular zone is the facilitated presentation by both class I and class II major histocompatibility complex (MHC). The feature of phage display sets off both CD4+ and CD8+ T cells, helping to generate long-lasting effective memory immune response (Tao et al. 2013, 2018a). A licensed viral vaccine must contain vastly localized epitope density and that is widely disseminated by bacteriophages (Cheng 2016). A promising strategy to enrich vaccine efficacy is by aiming of antigens to immune cells (Kastenmuller et al. 2014; Macri et al. 2016). Dendritic cells (DCs) are one of the crucial immune cells as they play the connecting link between innate and adaptive immune system (Steinman and Banchereau 2007). Although it is clear that some phage may have mammalian tropism, but most of them are not. So phages can be cleverly engineered to target DCs by displaying DC-specific targeting molecules. Sartorius et al. performed an experiment in 2015, where phage fd was modified to display a single-chain variable fragment of antibody against a DC-specific receptor-205 and separate group of phage also engineered to exhibit only ovalbumin through pVIII and pIII capsid protein. These groups were inoculated into the mice and found phages with DC-specific receptor that generated a higher titer of antibody compared to another group.

It must be mentioned that though phages have exclusive advantages, till now, there is no licensed vaccine based on phage platform that has yet been commercialized. Although several phage carrier–based vaccines are under clinical trial (Low et al. 2014; Huang et al. 2017), but most of these are still constrained to basic biological research. The reason behind this is quite clear: (a) A fair percentage of phages are unable to display the antigen in a highly localized or dense manner as an original mammalian viral vaccine, which is an utmost prerequisite to cultivate high titers of antibody. (b) Several pathogens are quite enabled to mutate specific prime amino acids in the epitopes, making vaccines constructed on one or limited epitopes, less to none effective. (c) Bacteria and bacteriophages don't display posttranslational modification, so phages cannot display antigens that require posttranslational modifications. (d) Like natural protein nanoparticles, bacteriophages are also naturally enabled to elicit immune responses (Dąbrowska et al. 2014); therefore, these significantly decrease their chance of usage when multiple doses of vaccinations are required, although this setback can be fixed, first by epitope engineering. The epitopes that stimulate robust immune response (immunodominant epitope) (Akram and Inman 2012) can be identified, and their expression levels can be

reduced by phage engineering. Second, PEGylation (addition of polyethylene glycol) is a process that enables phages for better solubility and diminution in renal clearance, hence increasing their bioavailability in the circulatory system (Suk et al. 2016).

5.5.2 *Clinical Phage Therapy and Phage-Assisted Approaches*

Phage is the undisputed winner in the list of alternative treatments against bacterial infection. Because phages follow "survival of the fittest policy," they evolve with the selection condition and overcome bacterial resistance mechanism. Restriction modification (R-M) system is employed by bacteria to destroy invading DNA, but phages (not all phages) can integrate base modifications to keep their genome protected from bacterial R-M system (Samson et al. 2013). But phages like T4, restructured their genome cytosines by two alterations, first by 5-hydroxymethylation and second by glucosylation, and this enabled T4 phages to be impervious to majority of the restriction endonucleases of *E. coli* (Bryson et al. 2015). Phages can escape CRISPR–Cas through either expressing anti-CRISPR proteins (Pawluk et al. 2018) mutation through prime nucleotides responsible for CRISPR–Cas complex binding/cleavage (Tao et al. 2018b). Bacteria can make themselves unreachable by modifying their phage receptors, but phages can reclaim the capability of binding to their receptor by modifying the receptor-binding protein to adapt with the evolving bacterial population (Samson et al. 2013). Hence, co-evolution of phages parallel to host bacteria is a never-ending process, and this phenomenon makes bacteria a less protective form of phage therapy than antibiotics. Bacteriophage therapy also faces issue like highly specific and narrow host range. This feature of phages sometimes limits its use against all strain of a particular species. This limitation can be overcome (De Jonge et al. 2019). The host range can be expanded by the help of genetic engineering. Swapping the long tail fiber genes of T2 bacteriophage with those from phage PP01 swung the host of T2 from *E. coli* K12 to *E. coli* O157:H7 (Yoichi et al. 2005). Switching cognate receptor–binding protein genes between more indistinct bacteriophages could even empower an orchestrated *E. coli* phage to attack *Klebsiella* bacteria and vice versa (Ando et al. 2015). Various phages aiming various strains can be sequestered from the natural environment to target multiple strains. An excellent example will be a recent study of the "San Diego patient," who was infected with a multidrug-resistant *A. baumannii* strain and regained their health after multiple intravenous injection of phage blends (Schooley et al. 2017).

Interestingly, phage-associated lysins, depolymerases and endolysin, can be consumed to lyse bacteria (Maciejewska et al. 2018). Here, depolymerases are polysaccharide-degrading enzymes, which are utilized to disintegrate capsular polysaccharides of pathogens, and in that way, phage gets access to cellular receptor on the bacterial cell surface. In preliminary experiments with depolymerase of PHB02

phage, when injected intraperitoneally, a significant surge in the persistence of mice pre-infected with *P. multocida* has been observed (Chen et al. 2018). Even though some of the phage-derived enzymes also have constricted infective capability, and they are competent to lyse a given bacterial species other than a single strain (Maciejewska et al. 2018).

All the above experiments do convey a message that synergistic approach of bacteriophages can be the most promising and also an emerging way to counter bacterial resistance in an efficient manner. As predicted, the most noticeable combination is antibiotics and phages. When utilized simultaneously, phages and antibiotics have displayed synergistic effects and effectiveness against biofilms (Chaudhry et al. 2017; Akturk et al. 2019), where the distinct treatments had limited success. Some experiments showed recurring medication with phages, which augmented the biofilm assembly but the mutual use of phage and antibiotics occasioned in biofilm eradication (Henriksen et al. 2019).

Synergism in between phages and antibiotics does not work for all phage–antibiotic blends, and a slightly increased dosage of antibiotics can effectively antagonize phage propagation (Dickey and Perrot 2019). This is predominantly evident when applying antibiotics that aims cellular protein synthesis (Akturk et al. 2019). But in rare cases, even though no synergism was observed, antimicrobial activity is displayed, and the combined utilization of phages and antibiotics drastically lowers or even limits the creation of antibiotic- and phage-resistant bacteria (Coulter et al. 2014; Dickey and Perrot 2019).

Phages and enzymes can be co-administered for enhanced result against stubborn infections, such as simultaneous use of depolymerases and phages that doesn't naturally express them to get better efficiency against biofilms (Gutiérrez et al. 2015). Phages may be utilized combinedly with DNAse enzymes to diminish the DNA elements of the biofilm matrix and increase effectivity of bacteriophage activity (Hughes et al. 2006). Besides, some distinct productive cases of commingled phages include triclosan, chlorhexidine, chlorine (Zhang and Hu 2013), hydrogen peroxide (Agún et al. 2018), cobalt (II) sulfate (Chhibber et al. 2013), xylitol (Chhibber et al. 2015), probiotics (Woo and Ahn 2014), and honey (Oliveira et al. 2017).

So far, we have observed that majority of the phage-orchestrating technology have concentrated on lytic phages, but some experiments do talk about engineering of temperate bacteriophages for phage remedy purposes. The most confident methodology talks on genetically modifying temperate bacteriophages to become utterly virulent or lytic. This was successfully performed by deletion of the genomic segment accountable for the regulation and instituting lysogeny (Zhang and Hu 2013; Kilcher et al. 2018). The transformation of temperate wild types to otherwise virulent mutant phages can simply explore the miscellany of phages available for therapeutic use. A beautiful example of this methodology can be given; a recent study elaborated utility of cocktail phages constituted of one natural wild-type lytic phage and two engineered temperate phages efficiently that treated a 15-year-old patient with cystic fibrosis with a disseminated *Mycobacterium abscessus* infection (Dedrick et al. 2019).

Therefore, engineering tactics can possibly expand the antimicrobial features of phages and produce pioneering strategies for rebellious bacterial infections. The significances of genetic remodelling of phage genomes must be sensibly countered, but bacteriophage engineering tactics should be sincerely considered as an impending therapeutic alternative. Furthermore, it is noticed that engineered bacteriophages contain more commercial interest, as getting patent for engineered phages is far more easy than natural phages.

5.6 Conclusion

We have already entered into the post-antibiotic era and the imminent threat of antibiotic defiance, which requires instant action in the form of phage therapy or phage-assisted therapy. From this chapter, it is crystal clear that phage cure is well matched to denote itself a part of the multidimensional stratagems with versatile administration and engineering opportunities to fight against hostile, stubborn pathogens. So the theory says that phage remedy needs to be commenced in our stash of treatment approaches against multidrug-defiant pathogens, and certainly the sooner the better. Moreover, there is no such outstanding efficient approach to clinical use of phage remedy, and in fact, its miscellany and flexibility with the changing environment are among its utmost advantages. Although there are knowledge gaps that must be enlightened before we can take up the practice of phage remedy on a regular basis, this arena is like a never-ending gold mine and rapidly advancing. A better perceptive of bacteriophage pharmacology, genetics, and immunological interaction over the years has also signify bacteriophages as critical therapeutic agents. In conclusion, we analyzed that phage remedy is a rapidly evolving dynamic alternative against antibiotics, and extensive use of phage therapy is an extremely difficult task to be undertaken, but considering its medical, technological, societal, and economic prospects, it is the one and only leading alternative in repertoire of treatment strategies.

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Chapter 6
Alternatives to Antibiotics in Animal Farming

Rejuan Islam, Anirban Pandey, and Tilak Saha

Abstract Antibiotics are utilized in animal farming to cure various diseases, avert or regulate infection, and increase animal productivity and growth. To increase animal growth, antibiotics are given to animals which do not even require. This practice is meant for making their feed more efficient. Due to the over and mostly unreasonable application of antibiotics in livestock production, concerns about resistance to antibiotic have emerged as a worsening global threat. Although many developed countries have restricted the utilization of antibiotics in feed of animals for promoting growth, this is far from reaching global implementation. The complete ban of growth promoters has also resulted in poor animal performance and sometimes increased incidence of certain animal diseases. As a consequence, research on true antibiotic-free alternatives for animal farming has been in prime focus to the scientist's world over. There are different alternatives available which are reported to play an important part both in the growth and prevention of diseases. Use of probiotics, prebiotics, feed enzymes, phytochemicals, metallic antimicrobial elements, antimicrobial peptides, organic acids, bacteriophages, endolysins, vaccines, and CRISPR-Cas9-mediated gene editing are potent alternative strategies. These alternative approaches serve important roles in shifting the interest of farmers towards adopting more sustainable and less harmful alternative animal husbandry strategies which can eventually decrease or even stop the widespread usage of antibiotics. The aim of this study is to abridge the positive impacts of currently available alternatives on fitness and growth performance of the animals reared in farms with the vision of attaining antibiotic-free animal raising.

Keywords Antibiotics · Antibiotic alternatives · Antibiotic resistance · Antibiotic resistance gene · Multidrug resistance · Growth promoters · Probiotics · Prebiotics ·

R. Islam · T. Saha (✉)
Immunology and Microbiology Laboratory, Department of Zoology, University of North Bengal, Siliguri, West Bengal, India
e-mail: tilaksaha@nbu.ac.in

A. Pandey
Department of Zoology, A B N Seal College, Cooch Behar, West Bengal, India

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Feed enzymes · Phytochemicals · Metallic elements · Antimicrobial peptides · Organic acids · Bacteriophages · Endolysins · Vaccines · CRISPR-Cas9 · Gut microbiota · Short-chain fatty acids · Immunity · Antibody

Abbreviations

AIV	Avian influenza virus A
Alba	Acetylation lowers binding affinity
AMPs	Antimicrobial peptides
ARGs	Antibiotic resistance genes
DNA	Deoxyribonucleic acid
EOs	Essential oils
FCR	Feed conservation ratio
FOS	Fructo-oligosaccharides
GIT	Gastrointestinal tract
IgA	Immunoglobin A
LCFA	Long-chain fatty acids
LPS	Lipopolysaccharide
MAMPs	Microbe-associated molecular patterns
MAPK	Mitogen-activated protein kinase
MCFA	Medium-chain fatty acids
MDR	Multidrug resistance
NDV	Newcastle disease virus
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PRRs	Patterns recognition receptors
QS	Quorum sensing
RNA	Ribonucleic acid
SCFA	Short-chain fatty acids
TOS	Trans-galacto-oligosaccharides
VSCFA	Volatile short-chain fatty acids

6.1 Introduction

Antibiotics have played a pivotal role in preventing, controlling, and curing vast spectrum of both animal and human diseases which are infectious and hence mass killers. Antibiotic application in animal feeds is a technique to improve feed efficiency, boost animal growth and improve the amount of livestock products. Sub-therapeutic quantities of antibiotics added to animal food were the earliest to report the effect of antibiotics on boosting performance when they discovered that birds fed with streptomycin had higher growth responses (Moore et al. 1946). So, antibiotics have been utilized in animal feeds for decades in many countries to boost

growth effects and protect rearing animals from the harmful effects of pathogenic and non-pathogenic enteric microbes (Lin 2004).

However, antimicrobial resistance (AMR) is increasingly becoming a major global health concern which surfaced due to the over and injudicious application of antibiotics both in human and in animal rearing. The application of antibiotics for metaphylactic, therapeutic, or prophylactic reasons in medicine and their application in the feed of animals to increase growth are significantly connected to the rise of cross-resistance and multiple-antibiotic resistance in harmful bacteria in both humans and farm animals. Multidrug resistance (MDR) is linked to the application of non-therapeutic antimicrobials, including resistance to medicines that were never used in the animals of the pen (Marshall and Levy 2011). Sweden restricted the utilization of several antibiotics in foods for animals in 1986 as an outcome of this concern (Castanon 2007). In the United States, the application of medically significant antibiotics for promoting growth was similarly abolished on January 1, 2017 (AccessScience Editors 2017). However, these prohibitions have resulted in issues with animal performances as well as a growth in the prevalence of certain diseases (Fiorentin et al. 2005; Valdez et al. 2014). As an outcome, developing antibiotic-free alternatives is a promising approach. Non-antibiotic additives have been designed and manufactured for prophylactic usage against diseases or as promoters for growth as an outcome of public health concerns and farmer demands to avoid economic losses. Probiotics, prebiotics, feed enzymes, phytochemicals, metallic elements, antimicrobial peptides, organic acids, bacteriophages, endolysins, vaccines, and CRISPR/Cas9 have been discussed in this chapter as potential alternative strategies for application in animal farming.

6.2 Status of Application of Antibiotics in Livestock Farming

There are diverse mechanisms that explain how antibiotics at less than therapeutic levels better animal growth. Sub-therapeutic antibiotic administration helps animals to spend less energy maintaining their gut commensal bacteria, freeing up more energy for growth (Gaskins et al. 2002). This statement is supported by an experiment on germ-free chickens which when grown in isolation did not exhibit any signs of progress in their growth when fed with antibiotics (Coates et al. 1955). Under normal circumstances, intestinal bacteria reside in a host's gastrointestinal tract and regulate crucial immunological, nutritional, and physiological factors that keep the host healthy (Jandhyala 2015). Animals with these bacteria staying in their intestines have larger guts, broader gut walls, and a greater number of villi present in their intestines than germ-free animals (Coates et al. 1955). However, the bacteria consume foods, expel metabolites, increase the turnover of intestinal epithelial cells, and reduce fat digestion (Dibner and Richards 2005). This can favour bacterial

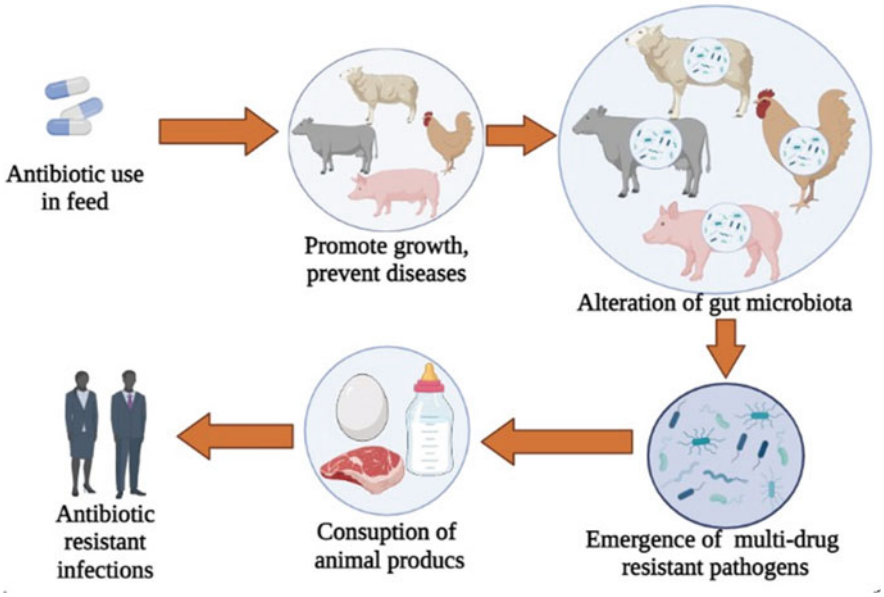

Fig. 6.1 Shifting of antibiotic resistance from animal husbandry to human being

overgrowth in the small intestine, which is connected to weight loss, malabsorption of food, and poor health condition in the animals, and it affects their growth too.

Antibiotics, even at doses less than therapeutic levels, were thought to limit harmful bacterial development during the animal's growth phase, resulting in enhanced comprehensive weight and health increase (Kim et al. 2016b). Antibiotics may function by changing the amount and activities of bile acid-converting enzymes such as cholyltaurine hydrolase found in the stomach, lowering growth-suppressing metabolites like bile breakdown products, resulting in increased weight gain in the animals (Feighner and Dashkevicz 1987). Antibiotics help to strengthen the intestinal barrier by reducing inflammation and increasing nutritional absorption (Oh et al. 2019). Antibiotics have a well-established anti-inflammatory action on cells which are inflammatory, and thus this concept holds up (Bersani et al. 1987; Broek 1989). Antibiotic growth-promoting agents have a mode of action which is complex in nature, and thus it is difficult to pinpoint. Antibiotic growth-promoting agents influence both the constitution of microbiota present in the alimentary canal and physiology of the animals. When antibiotics are added with feed for animals, it helps agricultural animal fitness and welfare, but their continued use at less than therapeutic levels poses major health issues to the wider population due to the enhancement of resistant microbes. Antibiotic residues are also transferred from farmed animals to human beings through the consumption of different products, posing a risk to human health (Fig. 6.1). Application of antibiotics in animal diets has been linked to more risks than benefits (Manyi-Loh et al. 2018; Landers et al. 2012). As a consequence,

antimicrobial resistance has been recognized as one of the most prominent public health challenges of our time.

6.3 Antibiotics and Ecosystems

Antibiotics used indiscriminately and abusively can bring about greater antibiotic concentrations in the surroundings. Prophylactic or therapeutic application of antibiotics pollutes streams of human waste, while antibiotics which are used in animals for promoting growth, prevention, and treatment purposes contaminate animal waste streams as well. As a consequence, these are the primary sources of release of antibiotics into the surroundings (Dolliver and Gupta 2008). Because the antibiotics are not totally degraded and are let out into the surroundings, such as soil, manure, or water, unchanged. The kind of antibiotic applied, the animal's species and age, and also the dose at which the antibiotics are administered play the determining factors behind the quantity and speed at which the antibiotic is let out into the surroundings (Zhao et al. 2010). Nevertheless, both antibiotics and their resistant genes will be present in these streams of waste; both are contaminants for the surroundings, and their providence in the surrounding is variable (Martínez 2009). As an outcome, these let-out antibiotics are usually of different kinds, and due to that, they do not break down at a similar rate in the surroundings during the course of time, i.e., they break down at various speeds in the surroundings over time through the main eradication procedures, like photo degradation, sorption, biodegradation, and oxidation (Li et al. 2014; Liu et al. 2016).

The layout of antibiotics and their resistance in our environment is not restricted to a single ecosystem because soil, plants, and animals are all interconnected. Antibiotics travel along all of these channels (Karesh et al. 2012), forming a network in which ARGs also traverse. Antibiotics are applied in animal feed, agriculture, and aquaculture to aid growth. They also protect to avert and handle diverse types of diseases. However, in the case of animals, complete absorption of these antibiotics does not occur in the stomach, and they are removed from the body in the form of faeces and urine, causing the formation of manure. This manure, which is a rich source of organic substances and nutrients, improves soil fertility, but it also becomes accountable for the transference of antibiotic residues from the animal world to the ecosystem of the soil and, eventually, to plants (Chambers and Deleo 2009). It was seen that resistance to antibiotics among soil bacteria increases because of manure application on agricultural land. It is already manifested that long-term use of fresh and composted swine dung in agricultural soils results in the creation of resistance against tetracycline (Walson et al. 2001). A study has manifested that the use of manure near animal pens has been also linked to a high level of resistance against chlortetracycline as the bacteria isolated from this kind of soil encode tetracycline resistance genes (Ghosh and LaPara 2007). A couple of experiments have also suggested that applying antibiotic-laced manure to crops can produce a rise in antibiotic resistance in various crops. Kumar et al. (2005) found tylosin resistance

in corn, green onion, and cabbage. In aquaculture, antibiotics find their way into streams and pens surrounding the area of the farm. Nevertheless, there are quite a few reasons that affect the break out of resistant genes against antibiotics. For instance, of physical influence, we can find human activity, wind, and watershed that have ensued in a rise in resistance among usual harmful microorganisms. According to research, it was observed that *E. coli* isolates associated with wild animals had a high ubiquity of resistance (Souza et al. 1999). Antibiotic resistance is more prone to developing in the bacteria present in the alimentary canals of animals which are nearby to people, as antibiotics are purposefully supplied to animals for quick development and high productivity to satisfy the daily requirements of the rising population.

At last, we can come to a conclusion on the basis of the above research that excessive application of livestock manure causes resistance genes against antibiotics to continue in soil bacteria, which is then passed on to plants, animals, and people via cycling. Besides, antibiotics, which are utilized for promoting growth, appear to impose a pressure applied selectively on food animals, resulting in enormous stores of interchangeable resistance against antibiotics in different kinds of ecosystems (Witte 2000).

6.4 Mechanisms of Antibiotic Resistance

Antibiotic resistance methods used by various microbes can be divided into two categories: (1) mechanisms with a genetic basis for microbial resistance and (2) mechanisms with a mechanistic basis for antimicrobial resistance. Genetic resistance develops as an outcome of mutations that alter antimicrobial targets, reduce drug absorption, enhance chemical efflux, and alter metabolic pathways (Fig. 6.2). It also transfers genes horizontally via transformation, transduction, and conjugation. Besides, mechanistic resistance, is brought about by antimicrobial molecule alteration, preventing chemicals from reaching antimicrobial targets, bypassing target sites, and resistance caused by global cell adaptation mechanisms (Fig. 6.2) (Munita et al. 2016).

A process known as protein promiscuity has recently been revealed to be accountable for antibiotic resistance, in addition to these fundamental pathways. Proteins attach to their ligands/substrates in a certain way via their preformed binding sites, according to classical biochemistry. However, recently, various sorts of deviations from the norm, known as promiscuous behaviour/protein promiscuity, are already seen (Gupta et al. 2020). An experiment on the antibiotic albicidin found that even structural changes to a drug may not be enough to conquer drug resistance (Rostock et al. 2018). Albicidin is an antibacterial drug that works against Gram-negative and Gram-positive bacteria by blocking DNA gyrase (EC 5.99.1.3). However, in other circumstances, bacteria develop resistance to the emergence of drug-binding proteins, which prevents the molecule from binding to the gyrase. In 2018, Rostock et al. (2018) revealed that the drug-binding domain of

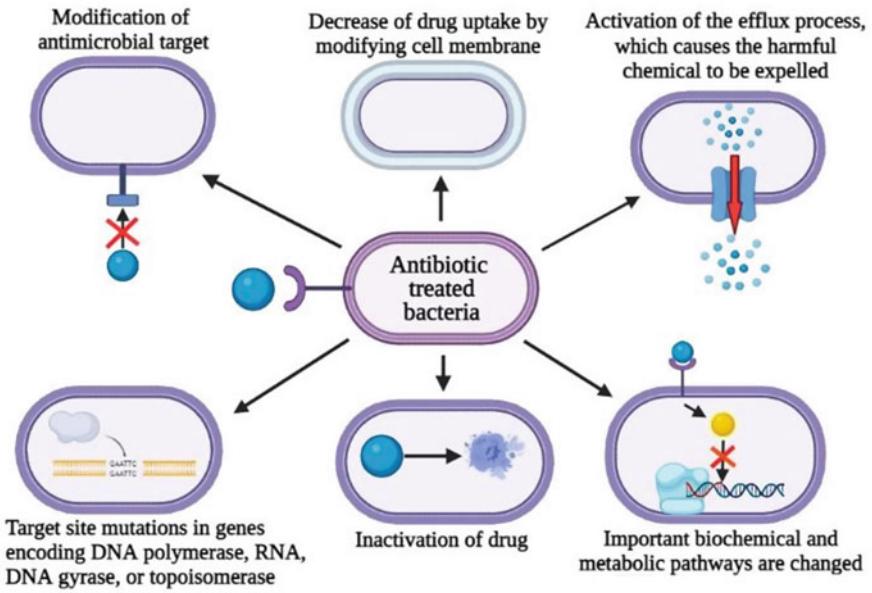

Fig. 6.2 Different antibiotic resistance mechanisms in bacteria

the protein AlbA in *Klebsiella oxytoca* displayed significant promiscuous behaviour by attaching to various albicidin compounds. Because antibiotics have dissociation constants in the nanomolar range, these interactions render them unavailable to their molecular targets, resulting in drug resistance. Another similar study looked at the formation of resistance to the broad-spectrum antibiotic fosfomycin (Brown et al. 2009). The drug's reaction with glutathione, cysteine, and water is catalyzed by the proteins FosA, FosB, and FosX, respectively. In compliance with the findings, a protein called FosXmt may function as a primogenitor of FosX and FosA. This primogenitor protein was discovered to have promiscuous catalytic activity and very poor glutathione transferase and epoxide hydrolase functions. They demonstrated that only a 10% variation in sequence (from wild proteins) resulted in antibiotic resistance activity from the progenitor when compared to mutants. Antibiotic resistance in aminoglycosides (such as gentamycin and amikacin) is produced by enzymatic adenylation, phosphorylation, and acetylation of the medicines. Because it could bind to 10 different aminoglycosides, a phosphorylation kinase was discovered to have a high level of promiscuity (Fong and Berghuis 2002).

Resistance against antibiotic is stimulated by enzymatic adenylation/phosphorylation/acetylation of aminoglycosides (e.g., gentamycin, amikacin). Because it could adhere to 10 different aminoglycosides, a kinase enzyme involved in phosphorylation was discovered to have a high level of promiscuity.

6.5 Alternatives to Lessen the Use of Antibiotics in Animal Farming

There are different alternatives, but they differ in how their uses are to be timed to assure effectiveness. Vaccines, for example, must be administered well in advance of infection since they rely on the animal building a safeguarding immune response, which takes time. In contrast, products like bacteriophages, which are effective because they directly interrelate with and kill disease-causing bacteria, must be given close to the time of infection; they will only do work if bacteria are present in large numbers and causing infections, and they may be quickly inactivated in the absence of bacteria. However, the different available alternatives till date whose uses can reduce antibiotic application in livestock farming are discussed in this chapter (Fig. 6.3).

6.5.1 Probiotics

When live cultures of microorganisms (such as yeast, fungus, and bacteria) are added to the diet to help in maintaining the gastrointestinal tract's microbial ecosystem balance in the rearing animals, they are collectively known as probiotics

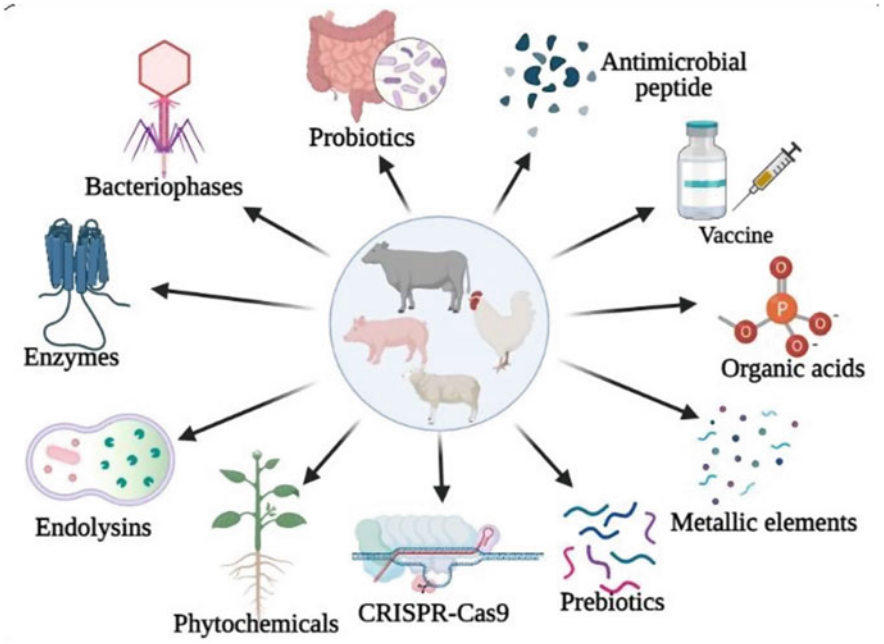

Fig. 6.3 Use of different alternatives to reduce antibiotic resistance in animal farming

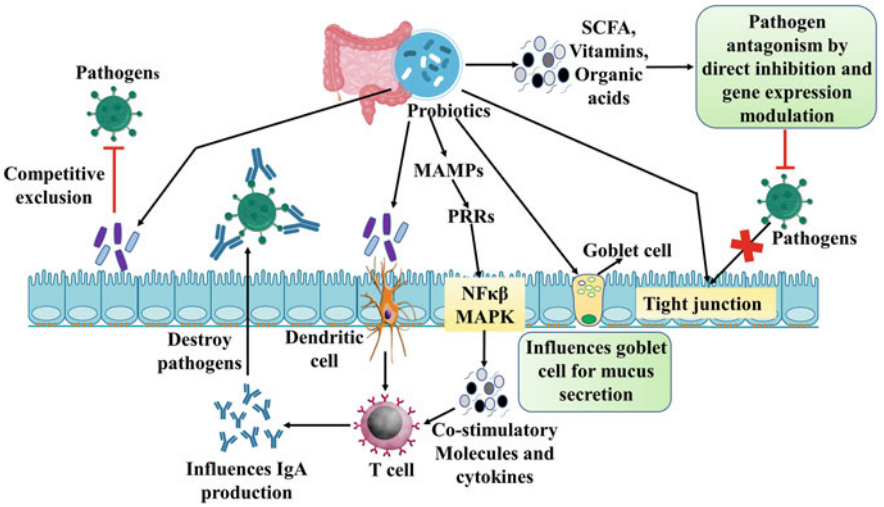

Fig. 6.4 Mechanisms of action of probiotics and their byproducts. *IgA* immunoglobulin A, *MAMPs* microbe associated molecular patterns, *PRRs* patterns recognition receptors, *MAPK* mitogen-activated protein kinase, *SCFAs* short-chain fatty acids

(Chaucheyras-Durand and Durand 2010). Probiotic organisms that adhere to the digestive tract may withstand harsh conditions and contribute stability to the ecosystem of the intestine. They have been found in many studies to lessen the chance of infections and digestive problems in the rearing animals and promote their growth. In egg laying hens, probiotics have resulted in statistically significant increases in productivity, as assessed by egg production (Kurtoglu et al. 2004).

The intestine is such an abundant source of nutrients that microbes are unlikely to go hungry. Probiotics have a great fermentative activity and help with digestion. It is very common for an animal's digestive system to show problems during stress (e.g., weaning). If antibiotics are applied as nutritional modifiers in this situation, both the pathogenic and useful bacteria are affected. So, the application of probiotics in such situation may be a better option than the application of antibiotics. Besides, probiotics are planned in such a manner that they may favour definite bacterial strains at the dropping of unwanted ones (McDonald et al. 2010). Probiotics compete with pathogenic bacteria for adhesion sites and organic substrates (mostly carbon and energy sources) (van Zyl et al. 2020). They are trusted to dissuade pathogens by the process known as "competitive exclusion," which might entail competing for critical nutrients or more antagonistic interactions, including antimicrobial chemical production and contact-dependent inhibition (Fig. 6.4) (Hayes et al. 2014; Chen et al. 2018). Thus, beneficial bacteria are thought to be capable of fully occupying accessible niches and stopping disease formation as an outcome of these coupled activities. This is also accomplished by microbe-microbe interactions (Freilich et al. 2011). Probiotic bacteria can interrelate with epithelial cells for improving mucosal permeability and modulate mucosal defences like antimicrobial peptide expression,

mucosal/secretory immunoglobulin A (IgA) induction, and the mucus layer (Fig. 6.4) (Wlodarska et al. 2014). Secretory IgA can affect microbiota composition by monitoring and reducing commensal microbe overgrowth, and it protects the host too by binding bacteria and toxins and blocking their adhesion to mucosal surfaces and absorption (Boyaka 2017; Macpherson et al. 2018). Antimicrobial compounds produced by several probiotics can have antipathetic effects on other microorganisms. Bacteriocins are antimicrobial peptides produced by specific bacteria and are capable of targeting the cell membrane, DNA, RNA, and protein metabolism of vulnerable bacteria (Cotter et al. 2013). Organic acids, hydrogen peroxide, and a variety of other antimicrobial chemicals are in some instances antimicrobial substances. Organic acids, which lower gut pH and have antibacterial effect against particular microbes, are typically carboxylic acids and include lactic acid and short-chain fatty acids (SCFAs). Some SCFAs are also sources of energy and carbon for microbial community members and the host (Dittoe et al. 2018). Probiotics may also put out quorum sensing (QS) molecules and reduce the expression of virulence genes in various types of pathogens (Fig. 6.4) (Bondue et al. 2016). Lactic acid, for example, has been discovered to suppress the synthesis of QS molecules in several *Pseudomonas aeruginosa* isolates, resulting in downregulation of motility, elastase, protease, pyocyanin, and biofilm development (Kiymaci et al. 2018).

Biosurfactants, which hinder attachment of pathogens to the epithelial cells, and bile salt hydrolases, which deconjugate bile acids and create antimicrobial compounds, are also produced by probiotics. Bile salt hydrolases can also detoxify bile salts, giving *Lactobacillus* spp. a competitive lead that would otherwise be stopped up (Allain et al. 2018). The immune system is induced by probiotics. Probiotics can improve nonspecific cellular immune responses by activating antigen-specific cytotoxic T lymphocytes, natural killer (NK) cells, and macrophages and releasing various cytokines in a strain- and dose-dependent manner (Ashraf and Shah 2014). The mucosal epithelium's multiple cell types work together to balance toleration of the natural microscopic organisms of that particular environment and their products while also defending against dangerous infections and dietary allergies (Fig. 6.4). The efficacy of probiotics in promoting growth and preventing sickness in chickens and turkeys has been quantified in a number of research investigations. For example, one study report showed enhanced productivity and intestinal health in newly hatched birds and over 20% less mortality compared to control flocks. It was also seen that the depletion in the death rate was similar to that obtained with antibiotics (Dersjant-Li et al. 2013).

6.5.2 Prebiotics

In addition to probiotics, prebiotics are also utilized as natural feed additives. Prebiotics are organic food ingredients, such as sugars, that are nondigestible to animals but are broken down by specific beneficial microbes present in their alimentary canal, which selectively enhance the growth of these and other microorganisms

when introduced to the diet (Vyas and Ranganathan 2012). At least three requirements must be met for a dietary substrate to be classified as a prebiotic: (1) The substrate must not be hydrolyzed or absorbed in the stomach or small intestine; (2) it must be selective for beneficial commensal bacteria in the large intestine, such as *Bifidobacteria*; and (3) the substrate's fermentation should result in beneficial luminal/systemic functions in the host (Scantlebury-Manning and Gibson 2004). Thus, prebiotics may help to induce the existence of good bacteria in the intestine.

Carbohydrates and oligosaccharides with various kinds of molecular structures are the highest frequently identified prebiotics; dietary carbohydrates, like fibers, are candidate prebiotics, but nondigestible oligosaccharides are the highest favourable. Some examples of commonly applied prebiotics are fructo-oligosaccharides (FOS), gluco-oligosaccharides, oligofructose, trans-gluco-oligosaccharides (TOS), gluco-oligosaccharides, lactitol, lactulose, stachyose, malto-oligosaccharides, xylo-oligosaccharides, raffinose, etc. (Davani-Davari et al. 2019; Orban et al. 1997; Patterson et al. 1997; Collins and Gibson 1999; Patterson and Burkholder 2003).

Both probiotics and prebiotics aid good bacteria in outcompeting bad bacteria. Besides, they may show other benefits too such as immune system modulation. Prebiotic efficacy appears to be determined by various kinds of parameters, including prebiotic type, animal age and species, animal health state, housing type, and management practices (Davani-Davari et al. 2019); all of those must be considered when deciding whether to utilize these alternatives.

Although there have been few studies testing the efficacy of prebiotics for illness prevention in chickens, notable depletions in pathogen shedding and betterment in gut health have been reported (Gaggìa et al. 2010). Prebiotics have been reported in several trials to promote growth in pigs, with improvements in average daily gains of up to 8% in pigs very soon after weaning (Halas and Nochta 2012).

6.5.3 Phytochemicals

Phytochemicals, also known as phytobiotics or phytogenics, are natural bioactive molecules produced by plants that are added to animal feed to increase production (Gadde et al. 2017). Essential oils (EOs) (volatile lipophilic compounds which are collected by steam/alcohol distillation or cold extraction) and oleoresins (extracted using non-aqueous solvents) are two types of phytochemicals which are employed in dried, solid, and grinding textures or as apozem (concentrated or crude) (Gadde et al. 2017). Polyphenols are considered the primary bioactive molecules in phytochemicals. The structure and density of polyphenols can differ according to the harvesting season, geographical origin, storage conditions, surrounding factors, and techniques of processing (Gadde et al. 2017).

In the ruminant industry, poultry industries and swine phytochemicals are already employed as natural boosters for growth recently. Coriander, cinnamon, thyme, rosemary, oregano, yarrow, ginger, garlic, black cumin, marjoram, and green tea are just a few of the spices and herbs that are already applied to chickens to see

whether they could be utilized as replacements for antibiotics as growth promoters (Gadde et al. 2017). There are numerous other plant extracts available that can be used as growth promoters like grape seed, grape pomace, macleaya cordata, fruit of cranberry, yucca extract, and garlic powder that had no influence on performance metrics (Gadde et al. 2017). Besides herbs and spices, EOs (carvacrol, thymol, cinnamaldehyde, and eugenol, star anise, coriander, garlic, ginger, rosemary, turmeric, caraway, basil, sage, and lemon) have been applied one by one or in mixes to enhance animal performance and health (Gadde et al. 2017). Some EOs, such as cinnamaldehyde and a combination of cinnamaldehyde with thymol boosted the attainment of broiler's body weight. Other EOs such as star anise and thymol enhanced the efficiency of feed, as reported by a lower feed conversion ratio (FCR) (Lee et al. 2010, 2011, 2013). Curcuma alone itself or in addition with capsicum (Lee et al. 2013; Kim et al. 2013a) improved resistance to necrotic enteritis and coccidiosis. The discrepancies in the consequences might be ascribed to variances in the content, source, and type of the required oils utilized, the inclusion level of the trial, and ambient conditions (Gadde et al. 2017). Nonetheless, European countries approved the first plant-based feed for enhancing performance in livestock and broilers. A combination of commercial phytonutrients (containing cinnamaldehyde, capsicum oleoresin, and carvacrol) helps to boost the innate immunity and control the harmful effects caused by intestinal pathogens (Lee et al. 2010, 2011). Several trials using this kind of commercial mixture consistently demonstrated a significant impact on growth enhancing and feed consumption efficiency (Lee et al. 2010, 2011; Bravo et al. 2014). It was found on a meta-analysis on 13 broilers that include this commercial mixture in diets boosted to gain body weight, the ratio of feed conversion and mortality decreased (Bravo and Ionescu 2008).

The way of working of phytochemicals is unknown; however, it is believed to be dependent on some active substances present in the applied product. Because of their antimicrobial and antioxidant properties, phytochemicals have a positive role in host defence mechanisms. Phytochemicals have antimicrobial properties against various pathogenic bacteria; including them in the diet changes and stabilizes the gut microbes. It is known that phytochemicals also reduce toxic metabolic products produced by gut microbes, resulting in relief from intestinal complications and immune stress, thus enhancing performance (Kim et al. 2015). Another key benefit of including phytochemicals in someone's diet is a decrease in oxidative stress in different tissues and an increase in antioxidant activity, which results in good health (Settle et al. 2014). Phytochemicals also have immunomodulatory effects, like increasing immune cell proliferation, modulating cytokines, and increasing antibody titers (Lee et al. 2010, 2011, 2013, 2017; Kim et al. 2010, 2013a, b, c). Furthermore, use of *Allium hookeri* in the diet of young broiler chickens can reduce inflammation, and expression of tight junction proteins is increased in the intestine when LPS is induced in young broiler chickens (Lee et al. 2017).

6.5.4 Feed Enzymes

Enzymes, which may be appended to animal feed, are a promising approach for promoting animal growth. Dietary substances which are either not broken down by digestive enzymes or absorbed so slowly that bacteria remain available in the host alimentary canal go in for them are a vital source of nutrition for bacterial multiplication and growth in the tract of the intestine. Exogenous enzymes are able to impact nutrient absorption, and they also create nutrients for definite bacterial populations (Bedford and Cowieson 2012). As a consequence, their use has an immediate effect on microfloral populations (Apajalahti et al. 2004).

In-feed enzymes assist in the breakdown and digestion of plant components such as cellulose and pectin, which the animals would otherwise be unable to utilize properly (Thacker 2013). Differences in the microbiota make up of the alimentary canal, prevention of damage caused by undigested plant parts rubbing against the inner lining of the intestine, breakdown of larger molecules into compounds with prebiotic activity, or roles in the structure of intestinal content and its digestibility are all possible mechanisms behind the potentiality of infeed enzymes as promoters for growth (Pourabedin et al. 2015). In-feed enzymes could potentially help avoid infections like necrotic enteritis in chickens (Kiarie et al. 2013). When in-feed enzymes were utilized to promote development and enhance food intake in chickens, promising effects were seen (Yang et al. 2009).

In-feed enzymes as growth promoters in pigs have yielded mixed results. Some enzymes, such as phytases, appear to be more successful than others at enhancing performance (Ohh 2011). The most frequently applied feed enzymes are a brew of glycanases, while phytase is the single-use degrading enzyme (Ravindran and Son 2011). Phytase has notable role on calcium, phosphorus, and mineral digestibility and also intestinal mucus synthesis and endogenous losses; all of them influence nutrient supply and the environment of the intestine, changing bacterial species' selection pressures (Bedford and Cowieson 2012). The antibacterial effect of in-feed enzymes is not evident as enzymes do not directly destroy bacteria, but rather limit the substrates for their growth.

6.5.5 Metallic Elements

Copper, zinc, and other heavy metals are naturally occurring and required trace components in the food, although they are frequently supplemented with higher quantities for promoting growth and, on rare occasions, as therapy for gastrointestinal disease (Wales and Davies 2015).

A meta-analysis revealed that copper is effective in stimulating growth in broiler chickens and swine, and zinc oxide boosted piglet growth (Sales 2013). Experiments have shown that when broiler feed was added with a brew of inorganic minerals such as copper, iron, manganese, and zinc, daily gains in chickens were greatly increased;

these inorganic supplements caused a statistically remarkable enhancement in broiler weight gain (Bao et al. 2007). Copper has been proved too to boost laying hen performance in scientific research, and zinc oxide has been reported to lessen the prevalence of diarrhoea in pigs after weaning (Pesti and Bakalli 1998). Other chemical substances like clay minerals (e.g., zeolites, bentonites) and rare earth elements have been taken into account as growth promoters (e.g., lanthanum, scandium) (Thacker 2013). However, worries about possibly dangerous heavy metal residues in the meat must be thoroughly assessed.

6.5.6 Antimicrobial Peptides

Antimicrobial peptides (AMPs) are another potentially promising growth-promoting option that could help with illness prevention and therapy. AMPs are small antimicrobial compounds which are harmful to specific bacteria (Lei et al. 2019). Microorganisms are often the source of these peptides. AMPs also contain host defence peptides produced by other animals like mammals (Moretta et al. 2021). There are so many AMPs described, with significant variances in the kinds of bacteria they are active against and also their modes of action (Brogden 2005), which could indicate disparities in the establishment of resistance (Wang et al. 2016). AMPs produced by the host are intriguing medication alternatives being investigated as natural therapies for animal treatment. As they have broad-spectrum antibacterial action and various activation methods, these are considered natural antimicrobials. The size, sequence, and localization of AMPs vary, and they are classified into four structural groups: amphipathic α-helical, β-sheet, β-hairpin or loop, and extended versions (Huan et al. 2020). Provided their broad-spectrum antibacterial activity and distinct activation methods compared to classical antimicrobials, AMPs are already investigated as possible therapeutic origins of natural antimicrobials (Kumar et al. 2018).

AMPs are intriguing solutions for promoting chicken development and preventing diseases. Even though their efficacy in avoiding specific diseases is inconsistent, such peptides are useful in growth performance and general gut health in yellow-feathered chickens by using plectasin. (Zhang et al. 2021a). Dietary plectasin can boost H9N2 AIV and NDV antibody levels; plectasin can improve gut structure, suppress *E. coli* and proinflammatory cytokines in the gut ileum, and improve blood biochemical indicators in yellow-feathered chickens at 21 and 35 days of age (Zhang et al. 2021b). Antimicrobial peptides used in broiler chickens and pigs enhance the health condition of the intestine and suppress dangerous bacteria by boosting the growth of beneficial microorganisms, according to in vitro studies (Wang et al. 2016). In chickens challenged with *E. coli*, it has been evidenced that an antimicrobial peptide, cLF36, produced from camel milk, can enhance growth performance, mitigate intestinal morphological alterations, and restore gut microbial balance. Furthermore, supplemental cLF36 may improve the immunological action to *E. coli* challenge through its influence on cytokine and mucin expression. In addition, by upregulating the expression of tight junction

proteins, cLF36 can improve the intestinal integrity of *E. coli*-infected chickens (Daneshmand et al. 2019).

AMPs are already reported to have the potential for gaining of weight and disease prevention in pigs in several investigations. WK3, an AMP, enhanced growth performance and decreased diarrhoea like incidence in piglets when challenged with *E. coli* K88 enterotoxigenically. WK3 also have antibacterial function and a fall in immunological response due to its capability to regulate cytokine release and expression. WK3 also improved intestinal probiotics and reduced oxidative damage in piglets (Zhang et al. 2021c).

Some researchers have focused on the potentiality of AMPs in dairy cattle and found promising results for promoting growth and prevention of udder infection and treatment. Buffalo (*Bubalus bubalis*), an essential livestock for milk and meat production, reported a greater disease resistant than cattle. Many AMPs, such as defensins, cathelicidins, and hepcidin, are found in them, and they play a very significant role in neutralizing invading pathogens. Application of AMPs on Buffalo have shown antibacterial action against Gram-positive and Gram-negative bacteria across a broad spectrum. Synthetic analogues like the natural counterparts of AMPs applied on buffalo have shown antimicrobial properties against bacteria and even fungi, making them a promising target for the development of therapeutic antimicrobials (Chanu et al. 2018).

6.5.7 Organic Acids

Organic acid is one more recent choice being utilized in animal rearing as a growth stimulant and for disease prevention. Organic acids have been shown to have antibiotic-like properties, and the European Union has also given them permission to be used in animal feed. (Adil et al. 2010). Organic acids are mostly constitute with organic compounds that have acidic properties (Papatisiros et al. 2013) and also consist of carboxylic acids (-COOH). It is known too that organic acids constitute short-chain fatty acids (SCFA) such as volatile short-chain fatty acids (VSCFA), medium-chain fatty acids (MCFA; C7 to C10), and long-chain fatty acids (LCFA; C11) (Dibner and Buttin 2002; Cherrington et al. 1991).

Organic acids are divided into two categories. The first category (citric, lactic, and fumaric acids) is effective in lowering the pH of the stomach and therefore indirectly reducing the acid-sensitive bacteria present in the stomach. On the other hand, the second category (butyric, formic, propionic, sorbic, and acetic acids) is capable of reducing the pH of the gastrointestinal tract (GIT) by acting on the cell walls of Gram-negative bacteria (Papatisiros et al. 2013; Diener et al. 1993). Organic acids improve GIT conditions by lowering pH, promoting the functions of proteolytic enzymes and the ability to digest the nutrients, enhancing the secretion of the pancreas, encouraging the functions of digestive enzymes, establishing microbial population stability, and increasing the growth of some useful bacteria while preventing the growth of harmful bacteria (Papatisiros et al. 2013).

Organic acids, for example, citric or acetic acids, are also promising options for promoting growth and preventing disease. The power of an organic acid to destroy bacteria contributes to its capability to induce growth. Organic acids may affect gut microbiota by promoting the growth of acid-loving beneficial bacteria, and they also improve stomach physiological processes by raising acidity levels (Huyghebaer et al. 2011). Organic acids have been also reported that it have a good influence on performance and the turning away of some digestive illnesses such rumen acidosis in cattle, although more study is required (Martin et al. 1999). Organic acids have been shown to have direct favourable growth benefits on broiler chickens and grain-fed beef cattle, with weight increase improvements of roughly 17% and more than 8%, respectively, when added to the diet (Samanta et al. 2010). Organic acid supplementation in piglets, for example, was observed to lessen the incidence and severity of post-weaning diarrhoea syndrome in piglets when compared to pigs catered for a diet without organic acid supplementation (Tsiloyiannis et al. 2001).

6.5.8 Bacteriophages

Bacteriophages are a type of viruses that can infect and kill bacteria and have long been thought to be a method which is used to treat infections caused by bacteria (Wittebole et al. 2014). They are got in ample on earth (Weinbauer 2004). Bacteriophage has been reported to regulate host immunity, and it also fights against pathogenic bacterial infection (Górski et al. 2012; Chhibber et al. 2015; Brown-Jaque et al. 2016).

Bacteriophages can influence innate as well as adaptive immune responses by using cytokine responses and phagocytosis activity (Van Belleghem et al. 2019). By affecting the firmness of the microbiota, bacteriophages can control the immunological and metabolic functions of the intestine (Górski et al. 2012). Bacteriophage can be utilized as an alternative to antibiotics for growth enhancement as it is recently investigated in weaned piglets. It is reported that application of bacteriophage can reduce intestinal inflammation, increase gut barrier performance, and maintain gut microbiota composition in weaned piglets. (Zeng et al. 2021).

Most bacteriophages can infect only a few bacterial strains, and in certain situations, they can only infect a single bacterial strain. Bacteriophages can thus be applied in a very targeted manner with little unwanted consequences for other bacteria or the host. Furthermore, the capacity of bacteriophage for bacterium killing is not hampered by antibiotic resistance. So, bacteriophages are now being considered as a therapy of choice for infections due to the emergence of MDR bacteria (Lood et al. 2015).

6.5.9 Endolysins

In the 1950s, endolysins were found for the first time (Ralston et al. 1955). They are synthesized by bacteriophages at precise times in their life cycle, just before the virus kills the bacterium. Bacteriophages produce endolysins which act as antimicrobials (Schmelcher et al. 2012). Glucosidase, endopeptidase, amidase, and trans-glycosylase are endolysins produced late in the lytic cycle of phage. These enzymes digest the peptidoglycan coating of infected bacteria, allowing additional phages to be released. During this stage, freshly produced bacteriophages are released with the help of endolysins (Cheng et al. 2014). Endolysins are peptidoglycan hydrolases that are encoded by phages. These enzymes, along with an associated holin protein, accumulate within the host cell independently of the phage virion. Endolysins have no signal sequences of their own. Holin protein helps endolysin to access the peptidoglycan of bacteria by creating a pore on cytoplasmic membrane. It is a tightly controlled event that occurs serially only when the holin concentration attains a fixed point. In this way, endolysins can access and degrade peptidoglycan, also disrupting the osmotic balance of the cell and ultimately leading to cell death (Kashani et al. 2018). Endolysins along with holins are essential for a successful phage infection process. Having lytic activity, endolysins are now considered as possible antimicrobials because when lysin is practised exogenously, this activity could even occur.

Endolysins have the ability to kill the susceptible strains faster than phases, and the activities are easier to detect. Their ability has been limited through long-term evolution such that they can only target some of the key elements on bacterial cell walls (Loeffler et al. 2003). As the bacteria are quickly lysed by endolysins, they do not have enough time to develop resistance (Fischetti 2005).

6.5.10 Vaccines

Vaccines have been used for years in veterinary science to protect against viral or bacterial infections, and they are proving to be a crucial alternative choice to antibiotics in some situations that may lead to a decrease in antibiotic use (Hoelzer et al. 2018). Vaccinations elicit an immune response for protection that is similar to the consequences of a natural infection, but without the unfavourable consequences resulted by the disease's clinical course, and there is a long history of vaccines for successful application in animals (Postma et al. 2015). It has been demonstrated that using immunizations instead of antibiotics in a quasi-experimental study improved the biosecurity and cost-effectiveness of farrow-to-finish pig farms in Belgium (Rojo-Gimeno et al. 2016). Similarly, in Belgium, the deployment of action plans for herd animals, which included vaccination enhancements, resulted in lesser consumption of antibiotics and an improvement in production indicators such as mortality rates and daily weight gains (Postma et al. 2017).

Most studies have reported that the application of different viral and bacterial vaccinations in animal populations can lead to a large reduction in antibiotic usage (Murphy et al. 2017). For example, in the farmed salmon business, the introduction and extensive use of vaccines against *Aeromonas salmonicida* resulted in a considerable reduction in antibiotic use (Morrison and Saksida 2013). Similarly, an experiment has shown that *Lawsonia intracellularis* vaccination, which can cause ileitis, able to reduce the consumption of oxytetracycline by nearly 80% in pig farms in Denmark; vaccination also resulted in significantly fewer pigs being administered with oxytetracycline and also improved productivity factors like carcass weights and average daily gains (Bak and Rathkjen 2009). After vaccination of *L. intracellularis*, betterment in rates of mortality, pig uniformity, feed conversion ratios, the happenings of clinical diarrhoea, and the requirement of antibiotic therapy have been reported, as the results were sometimes minor and most of the experiments did not measure statistical significance (Bak 2011; Adam 2009; Bak et al. 2009). Notably, research of 64 farms in nine European nations found that after *L. intracellularis* immunization, most of the pig operations reported cost cuttings for antibiotic medicaments; however, not all the farms were capable to minimize their antibiotic utilization (Adam 2009).

Vaccines have a great potentiality for disease prevention, but their implementation is not easy. For example, majority of the vaccines are administered via injection, which increases labour expenses (Meeusen et al. 2007), and excessive handling, which can influence the immunological response of an animal (Marangon and Busani 2007) and may also take part to reduce of weight gain. Furthermore, some vaccinations are only functional against a few numbers of bacterial or viral strains, while others pose the threat of unexpected consequences, such as reversion to a disease-causing virus that might cause disease (Cheng et al. 2014). A large number of these issues are still being investigated, for example, the possibility of mass vaccination delivery or the discovery of the ways for eliciting more protective immune actions. Under the above circumstances it might be thought that vaccination may be a better alternative to antibiotics in near future (Gerdts et al. 2006).

6.5.11 CRISPR/Cas9

CRISPR/Cas9 gene editing technology is one of the most promising modern techniques that have the capability to reverse antibiotic resistance in certain harmful bacteria (Kim et al. 2016a). CRISPR/Cas9 systems have been applied to successfully targeting the virulence components and ARGs of bacteria, making them an interesting option for the development of programmable and sequence specific antimicrobials (Bikard and Barrangou 2017).

CRISPR/Cas9 has a great potential for significant improvement over existing gene-editing methods in respect of ease of use, speed, efficiency, and cost. Genome-editing technology has been widely employed to decipher gene function in disease pathogenesis and immunological responses in the host. The CRISPR/Cas9 approach

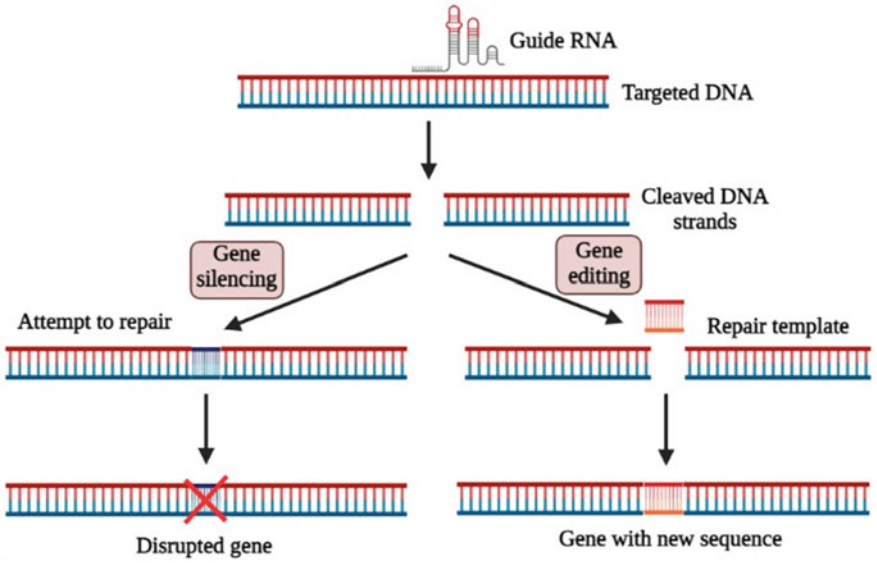

Fig. 6.5 A mechanism of gene editing and silencing with CRISPR. A guided RNA is used which instructs the molecular machinery to cut both strands of the targeted DNA. The DNA is broken, and the gene is inactivated during gene silencing. A repaired template which contains a specified sequence is added and incorporated into the broken DNA for gene editing. The targeted DNA has now been modified to carry this new sequence

also allows for a considerably broader range of alterations, including gene knockout, base-pair replacement, targeted insertion/deletion of larger genomic areas, and gene expression regulation (Petersen 2017). CRISPR-based antimicrobials have a distinct advantage over all other techniques in that they are able to destroy bacteria according to their sequences of genes. Because CRISPR guide RNAs are highly specific, they may be tailored to aim at certain chromosomal and virulence genes, eliminating the requirement for broad-spectrum antibiotics to cure illnesses in animals (Fig. 6.5) (Shabbir et al. 2019).

6.6 Future Prospects

Though there are different types of alternatives to antibiotics that are used in animal farming, the exact one to be selected for use should be perfect. Generally, the alternatives should have at least some common features such as the following: (1) they should not be toxic or have any side effects on the animals, (2) they must not promote bacterial resistance, (3) they must be safe for the animal's gastrointestinal tract, (4) they must not kill the normal intestinal microbiota, (5) they should

increase the feed efficiency, enhance animal development, and prevent their diseases, and (6) they must be eco-friendly.

Probiotics include bacteria, bacteriophages, microalgae, and yeasts (Llewellyn et al. 2014). Despite the fact that many microorganisms have probiotic potential, *Lactobacillus, Streptococcus, Enterococcus, Lactococcus*, and *Bifidobacteria* are the most commonly used probiotic agents in livestock recently (Collado et al. 2005; Llewellyn et al. 2014; Abdelqader et al. 2013). Multistrain probiotics have proven to be a viable alternative to antibiotics in ruminants, poultry, and swine, and their use in animal husbandry is increasing. The effects on and responses of host animals, on the other hand, vary across the literature. As a result, more research is needed to understand the interaction mechanisms between the combined microbes and the host's gut microbiota, as well as the unique role of each microbe.

Foot and mouth disease virus (FMDV), peste des petits ruminants virus (PPRV), bovine viral diarrhoea virus (BVDV), bluetongue virus (BTV), bovine herpesvirus type 1 (BHV-1), capripoxvirus, and other viruses cause fatal diseases in ruminants, wreaking havoc on both social and economic conditions. When no broad-spectrum antiviral pharmaceuticals are available, the only methods for avoiding or managing virus infections are vaccination and hygienic precautions to reduce exposure. Live attenuated vaccines for BTV, BVDV, LSDV, PPRV, and SPV are some of the vaccines that have recently been used against these diseases (Caufour et al. 2014; Chen et al. 2010; Coetzee et al. 2020) and inactivated for FMDV, BVDV, and SPV (Coetzee et al. 2020; Sun et al. 2019; Wolff et al. 2020; Gardos and Cole 1976). Because of the enormous economic losses caused by various ruminant diseases, ruminant vaccine development programmes have received high priority. The advancement of molecular genetics and a better understanding of infectious disease immunobiology have aided in the development of more protective vaccines. Identification of virulence factors and immunogenic antigens has been critical in the development of new vaccine generations, which has been aided by rapid advances in recombinant vector technology.

Plant extracts are generally regarded as safe and effective against a variety of bacteria. They are widely used in feed as growth promoters and health protectants (Hashemi and Davoodi 2011; Abreu et al. 2012), particularly in Asian, African, and South American countries, and have only recently begun to be used in developed countries. In pig rearing, there are several plant products used: oregano, cinnamon, Mexican pepper, thyme, oregano, and Camellia sinensis can decrease pathogenic microbial mass in the intestines (Manzanilla et al. 2004; Namkung et al. 2004; Zanchi et al. 2008); sangrovit, aged garlic extract, and allicin are potential for body weight gain (Borovan 2004; Tatara et al. 2008); and thyme, clove, oregano, eugenol, and carvacrol are potential for improving pig growth (Oetting et al. 2006; Costa et al. 2007). The effects of phytogenic feed additives on poultry production performance are also discussed (Hashemi and Davoodi 2010). Because of their complex composition, it is difficult to conduct systematic and comprehensive toxicology studies and safety assessments on herbs and their extracts. The challenge is to identify and quantify the numerous actions that must be taken in order to claim improved feed utilization, animal physiology, and health status. Despite the fact that

phytobiotics are a class of natural additives, more research on their mechanisms of action, compatibility with diet, toxicity, and safety assessment is required before they can be used more widely in animal feeds.

There have been numerous studies that show AMPs have a protective effect on humans (Guani-Guerra et al. 2010) and animals (Leonard et al. 2012). Numerous bacteriocins have been identified, including nisin, lactacin, lactocin, helveticin, fermenticin, sakacin, lacticin, plantacin, subticin, and others. Bacteriocins have strong killing and suppressive effects on a variety of pathogens, including resistant pathogens, according to in vitro tests (Field et al. 2011). Pediocin PA-1 from *Pediococcus* is now available on the market. Pure bacteriocins, on the other hand, have only a few and limited authorized uses in foods. Although AMPs have good bactericidal effects and are easily digested by bodies without affecting the taste of feed or polluting the environment, a number of constraints have accompanied the advancement of AMP research. Because of their high production costs, AMPs are not widely used as effective antibiotic alternatives for livestock. Bacteriocins are traditionally produced by cultivating wild strains, but the yield is low and the purification process is complex.

Overall, recent alternatives may look to have more potential than the application of antibiotics, but in some instances, their efficacies are assessed only experimentally. Their actual benefits should be tested and verified on a broad scale by applying them to commercial animals in real husbandry conditions. Thorough study and development facilities should be required to establish these alternative approaches as marketable and cost-effective products. In spite of the fact that a number of commercial food animals, producers are already adopting accessible alternatives, such as probiotics and vaccinations, to increase growth and disease prevention. Experimental data from academic research are still insufficient and, hence, should be supplemented with more information on their uses. Such information might be shared through public-private partnerships, and effects could be communicated more widely through extension programmes. A thorough comparative understanding and benefits of applying alternatives to the utilization of antibiotics in animals may be circulated among animal farmers by conducting several training, learning, and educational programmes by both government and private organizations. Focused research will help bringing in the promising technologies to mend veterinary products and their application. Thus, use of antibiotics in animal farming will be decreased without jeopardizing animal health, productivity, or welfare.

6.7 Conclusion

Complete abolition of antibiotic administration in livestock is currently impossible as it may cause severe disruptions in global meat production. A feasible solution to this age-old problem would be to use alternative agents that are both effective and cost-effective for disease prevention and growth promotion, while continuing to use antibiotics on veterinary prescription for treating animal diseases. This is a viable

option because controlled use of antibiotics in livestock has been shown to reduce resistance levels to original parameters in a specific area.

Due to a constant increase in consumer demand for livestock products produced without antibiotics, there is a pressing need for the implementation of alternatives to antibiotics which will not compromise the health conditions of the farm animals and their quality to be used as food. Besides, considering the several drawbacks of using antibiotics in animal farming, the approach of using alternatives is very much significant to ascertaining sustainable development. For sensible application of antibiotics and also for the implementation of regulations or policies restricting their usage, competent authorities must issue clear guidelines for target specific use of antibiotics in animals along with predefined treatment duration and withdrawal period. Simultaneously, we must increase legal oversight and enforcement to keep antibiotic residue in the food chain within acceptable limits. Further, we have to carefully improve animal nutrition and production cleanliness management. Prudent antibiotic use and continual research on alternatives to antibiotics are necessary to assure the long-lasting viability of animal farming.

The search for antibiotic substitutes may be a lengthy process. In addition to research and development of new efficient and safe alternatives, we should strengthen research into the effects of combined antibiotic use and alternatives with the goal of maintaining a healthy agricultural economy and preserving potent antibiotics for efficacious therapy in humans.

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Part III
Chemical Alternative Approaches

Chapter 7
Metal-Catalyzed Synthesis of β-Lactam Antibiotics

Suchandra Bhattacharya, Satadal Adhikary, and Basudeb Basu

Abstract The necessity of antibiotics has increased multifold in recent times in the healthcare system. In this context, the β-lactam unit is the most frequently used scaffold in antibiotics, which include monobactams, penicillins, cephalosporins, and carbapenems. This four-member cyclic amide moiety is being fabricated with the help of a plethora of strategies for years. It is essential to monitor the progression of β-lactam synthesis amidst a fully dedicated domain of synthetic strategies in terms of reaction conditions, selectivity, functional group tolerance, and catalysts from time to time. Herein we present a brief review of critical aspects of metal-catalyzed β-lactam synthetic approaches over the last 5 years.

Keywords β-lactam · Antibiotic · Metal-catalyzed · Relay catalysis · Cycloaddition · Antimicrobial resistance · Interrupted Kinugasa allylic alkylation · Multicomponent reactions · Catalytic pathways · Monobactams · Penicillins · Cephalosporins · Carbapenems · Asymmetric Kinugasa reaction · Carboamination · Cyclic aldimines · Ketimines · Staudinger reactions · Drug

Abbreviations

AMR	Antimicrobial resistance
IKAA	Interrupted Kinugasa allylic alkylation
MCR	Muticomponent reactions
MQ	5-Methoxyquinolin-8-amine

S. Bhattacharya
Department of Chemistry, ABN Seal College, Cooch Behar, West Bengal, India

S. Adhikary
Post Graduate Department of Zoology, ABN Seal College, Cooch Behar, West Bengal, India

B. Basu (✉)
Department of Chemistry, Cotton University, Guwahati, Assam, India

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7.1 Introduction

The trajectory of science in the twenty-first century has undergone a major paradigm shift from the researches of basic science towards an interdisciplinary avenue. The two major facets that coalesce into pharmacology are synthetic organic chemistry and biology, mostly diminishing the gap between these two. This has eventually formed the basis of drug design, discovery, and production—all of which have significant contributions to human civilization amidst a densely populated world, where people are obliged to get exposed to ever-mutating pathogens. In the past few years, researchers have witnessed a notable progression in the synthetic strategies of the β-lactam scaffold (Hosseyni and Jarrahpour 2018; Newman and Cragg 2007). This class of antibiotics has turned out to be indispensable in the cure of bacterial infections and plays a pivotal role in this regard (Zango et al. 2019; Gupta and Halve 2015; Mandal and Basu 2012). With the seminal discovery of penicillins and cephalosporins as quintessential antibiotics, the field of lactam chemistry has flourished throughout the past decades (De Rosa et al. 2021; Troisi et al. 2010; Wilke et al. 2005; Barrett et al. 1985). Moreover, clinical applications as antibacterial agents as well as potential synthons (Bhalla et al. 2016; Alcaide et al. 2007; Ojima and Delaloge 1997; Annunziata et al. 1993; Brieva et al. 1993) towards the development of diverse heterocyclic synthesis (Bhalla et al. 2017; Jarrahpour and Zarei 2006) have inspired both pharmaceutical and synthetic chemists to look for unique possibilities. This includes the practice of conventional methods and also the exploration of novel synthetic strategies to produce functionalized β-lactam antibiotics (Vessally et al. 2018; Brandi et al. 2008; Meloni and Taddei 2001). Keeping in view the diverse applications of the β-lactam moiety, this chapter presents a brief overview of different metal-catalyzed processes for its synthesis over the last 5 years.

Lactams are cyclic amides that are classified into several categories depending upon the ring size which include β-, γ-, δ-lactams, etc. β-Lactam, a four-member ring also known as azetidinone, is classified into different categories depending upon the size of the ring fused with the four-member structure (Fig. 7.1) (De Rosa et al. 2021; Deketelaere et al. 2017; Singh 2004).

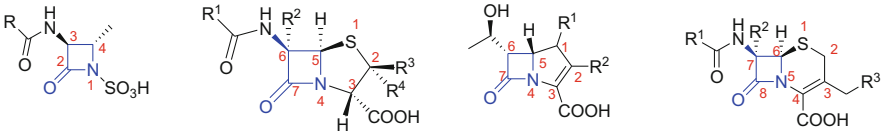


Fig. 7.1 Representative core structures of various β-lactam scaffolds

7.2 Development Throughout the Last Few Years

In consideration of the enormous utility of β-lactam-based antibiotics, it is quite evident from the literature that the synthetic strategies have been evolving day by day (Barrett et al. 1988; Aranda et al. 2013; Mandal and Basu 2013; Singh and Sudheesh 2014; Deketelaere et al. 2017). A summary of two different viewpoints, viz., the types of reactions involved and the bonds formed during the process, is depicted along with the metals used in the form of catalysts (Figs. 7.2, 7.3, and 7.4) (Magriotis 2014; Hosseyni and Jarrahpour 2018). Here, we will be reviewing the metal-catalyzed reactions of β-lactam synthesis, emphasizing the advancements

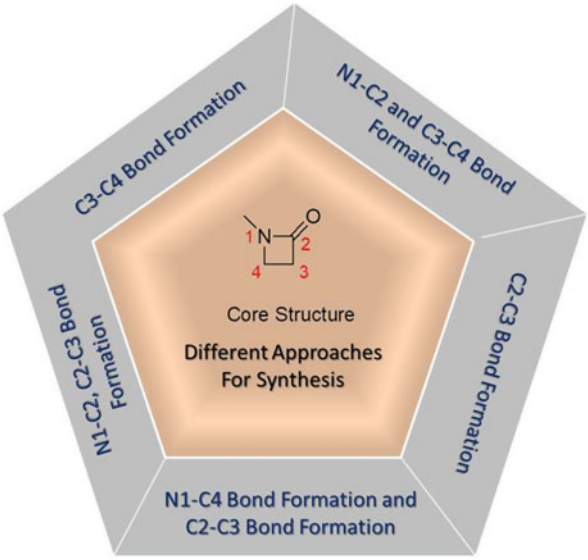


Fig. 7.2 Types of bonds that can be formed during β-lactam synthesis

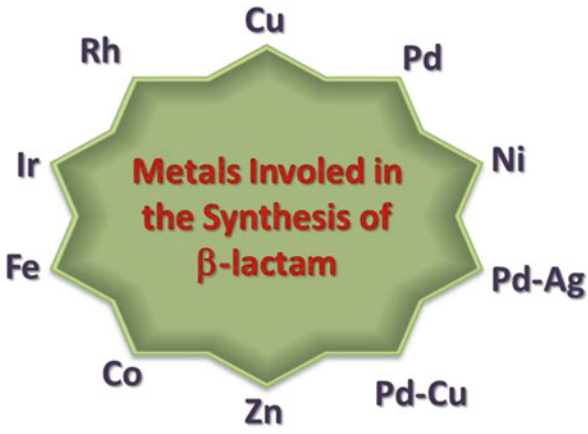


Fig. 7.3 Metals that are involved in catalysis

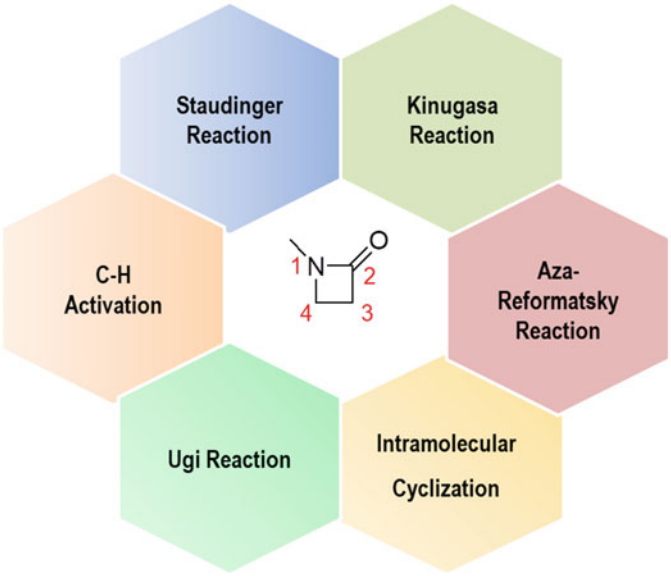


Fig. 7.4 Different approaches to furnish β-lactam skeleton

reported in the last 5 years (Hodous and Fu 2002; Lu and Li 2006; Zhao and Li 2008; Fontana et al. 2010; Xu et al. 2015; Isoda et al. 2015).

7.2.1 Staudinger Reaction

One of the most frequently used and oldest methods of β-lactam ring synthesis is the Staudinger reaction, which is a [2 + 2] cycloaddition reaction between a ketene and an imine (Omidvari and Zarei 2018; Dong et al. 2016; Pitts and Lectka 2014; Tuba 2013; Méndez et al. 2007; Taggi et al. 2002). Tang et al. 2016 established rhodium and iridium catalysts bound with "cod" and Me-pma (*N*-methyl-1-(pyridin-2-yl) methanamine) ligands and employed them in a carbene carbonylation reaction to furnish β-lactam rings (Fig. 7.5) (Tang et al. 2016). Here, an in situ carbene formation and subsequent cycloaddition transpires good yield utilizing the crucial role of metal-bound carbenes.

Sun and coworkers reported an unfamiliar carbene formation from metal-carbene intermediates with *N*-hydroxyanilines by virtue of Wolf rearrangement catalyzed by rhodium catalyst followed by benzoylquinine-mediated Staudinger reaction (Chen et al. 2019). This relay catalysis appeared to be productive in terms of diastereoselectivity in the β-lactam ring synthesis (Fig. 7.6).

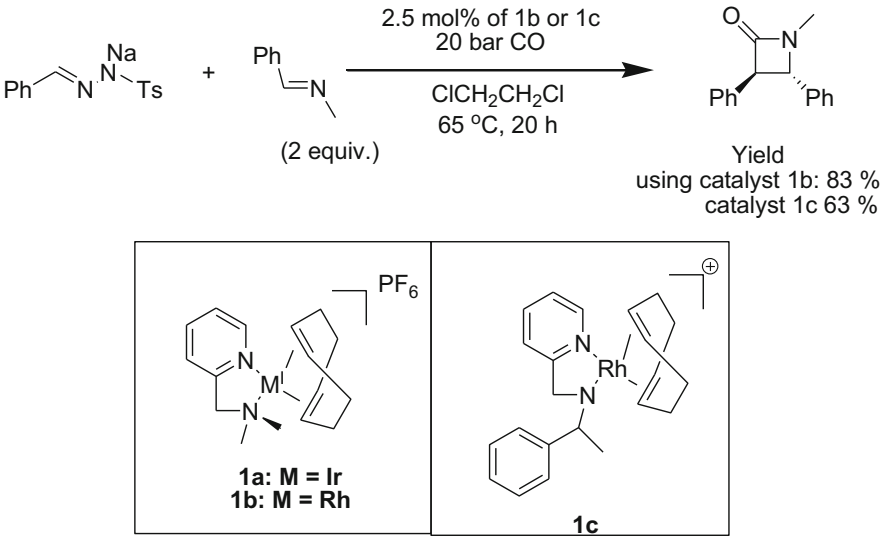


Fig. 7.5 Staudinger reaction via carbene carbonylation pathway catalyzed by Ir(I) and/ Rh (I) catalysts to synthesize β-lactam scaffold

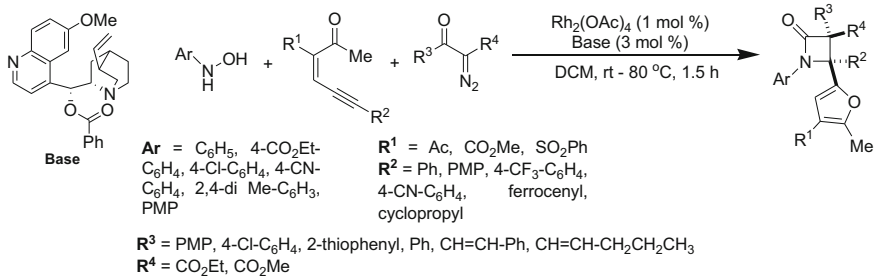


Fig. 7.6 A rhodium catalyst associated with an organic base in relay catalysis to furnish β-lactams

7.2.2 *Kinugasa Reaction*

In search of an alternate way other than the classical methods like the ketene-imine cycloaddition (Mandal and Basu 2013; Marco-Contelles 2004) or condensation of enolate-imine (Tomioka et al. 1999; Fujieda et al. 1997), the Kinugasa reaction came into play to overcome the shortcomings of the earlier methods. This influential reaction offers a cycloaddition between nitrone and acetylene (Ding and Irwin 1976; Kinugasa and Hashimoto 1972) to produce highly functionalized and biologically potent β-lactams. The general mechanistic pathway of a copper- catalyzed Kinugasa reaction has been provided in Fig. 7.7 (Hosseyni and Jarrahpour 2018).

With the increasing requirements of β-lactam antibiotics, many research groups developed new catalytic pathways for the Kinugasa reaction. One such example is

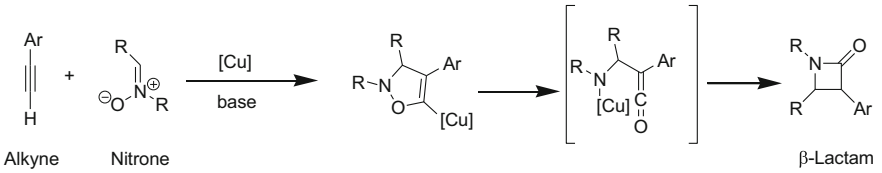


Fig. 7.7 General mechanistic path of the copper-catalyzed Kinugasa reaction

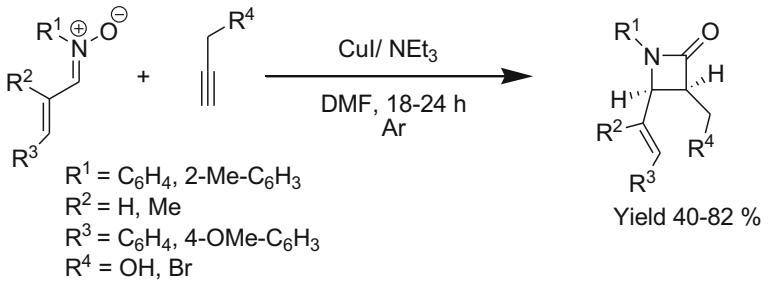


Fig. 7.8 Kinugasa reaction involving an α, β-unsaturated nitrone

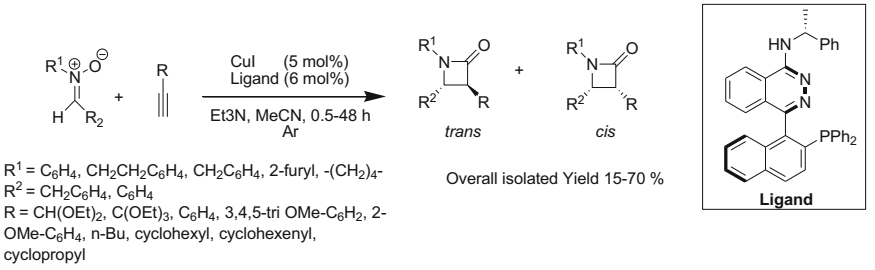


Fig. 7.9 PINAP-CuX system for the fabrication of β-lactam with a special case of cyclic nitrone

found in the work of Bhargava and coworkers, where the use of an α, β-unsaturated nitrone can be seen to give rise to 3-substituted-styryl lactams with diastereoselectivity and regioselectivity (Fig. 7.8) (Kumar et al. 2016).

Wolosewicz et al. (2016) published the synthetic route of β-lactam via Kinugasa reaction with acetylene and nitrones where the use of cyclic nitrone can be found for the first time (Fig. 7.9) (Wolosewicz et al. 2016). The selection of copper salt for PINAP/CuX complexes, generated in situ, was crucial in terms of configuration at the C-4 position. The use of a cyclic nitrone furnished a bicyclic β-lactam (with a commendable cis/trans ratio of 97:3) as anticipated from the mechanistic knowledge.

A report of highly regio-, chemo-, enantio-, and diastereoselective chiral spirocyclic lactam synthesis is provided by Enders' group (Shu et al. 2018). This is the first copper-catalyzed Kinugasa/Michael domino reaction in which alkyne-tethered cyclohexadienones couple with nitrones to construct the chiral lactams with

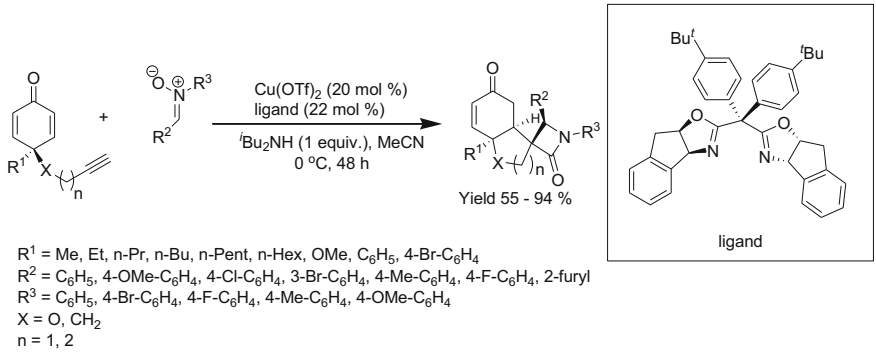


Fig. 7.10 A synthetic path towards spirolactam with a fused bicyclic framework via Kinugasa reaction

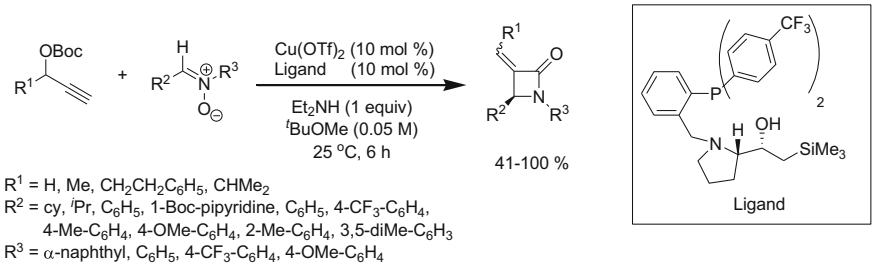


Fig. 7.11 Copper/prolinol-phosphine catalytic system in a Kinugasa reaction towards the alkylidene-β-lactam formation

excellent stereoselectivities (Fig. 7.10). In this context, it is worth mentioning that desymmetrization, i.e., the process of converting a prochiral moiety into an enantioenriched moiety, provides access to unprecedented spirocyclic lactams bearing a fused bicyclic framework with atom economy.

In the following scheme, a recently published work from Imai et al. regarding a copper and prolinol-phosphine-based chiral ligand-mediated asymmetric Kinugasa reaction is depicted (Imai et al. 2019) (Fig. 7.11). In search of the utility of their catalytic system, they have envisaged that this catalyst could be improvised in the development of chiral α-alkylidene-β-lactams. Few interesting aspects of this protocol include a one-step synthesis, high enantioselectivity, use of propargyl alcohol-derived alkyne, etc.

A three-component approach towards a thiol-funcionalized chiral β-lactam unit is depicted by Qi et al. 2021 (Fig. 7.12). This asymmetric synthesis is an interrupted Kinugasa reaction where various benzenesulfonothioates were utilized as the sulfur electrophile. This protocol offers the first synthesis of chiral β-lactam disulfides with a disulfur transfer reagent (TsSS^{t}Bu) using Cu(I) catalyst.

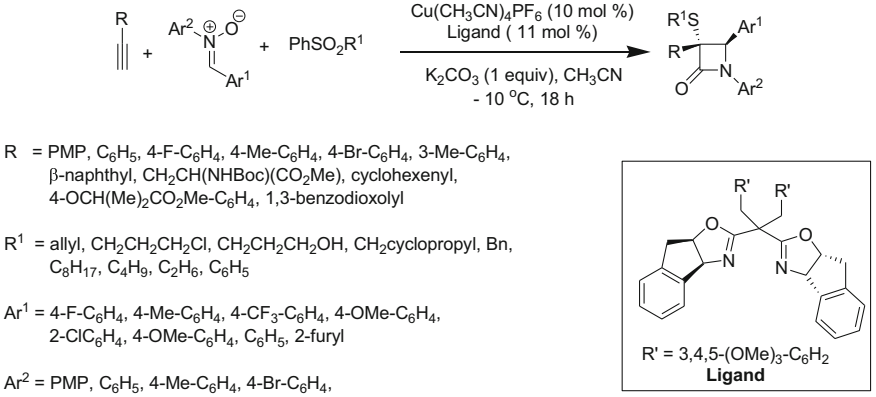


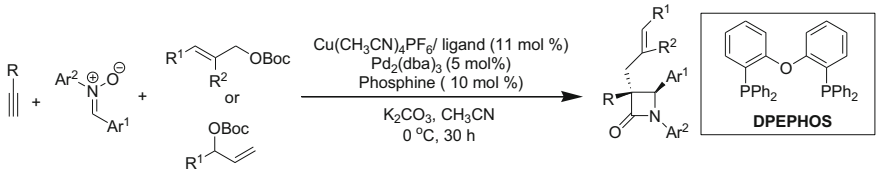
Fig. 7.12 Synthetic strategy towards thiol-functionalized β-lactams using Cu(I) catalyst

Among the plethora of such reactions, an intramolecular Kinugasa reaction to fabricate β- lactams was developed by Popik et al. (Popik et al. 2020). With the advantages of intramolecular reactions in mind, this group obtained excellent results in terms of diastereoselectivity using CuBr as the catalyst, and a carbohydrate-derived nitrone was chosen as the reactant for this purpose.

There are innumerable examples of bimetallic catalysis where the presence of a second metal enhances or controls the reactivity in the overall process (Lee et al. 2004; Park and Hong 2012) Sengupta et al. 2014). Pd/Cu has always been in the center of attraction due to their redox properties (Shih et al. 2013), and "Cu effect" in the Pd catalyzed cross-coupling reaction has received much attention (Espinet and Echavarren 2004; Peng and Li 2010). Muticomponent reactions (MCR) are very popular in the sphere of synthetic organic chemistry. In recent times, asymmetric MCRs have achieved much attention owing to the rising demand for optically pure compounds and one-step methodologies (Pellissier 2013; de Graaff et al. 2012; Touré and Hall 2009). Xu and coworkers developed a multicomponent strategy by utilizing the synergistic effect of the Pd/Cu system in the production of chiral β-lactams (Fig. 7.13) (Qi et al. 2021). Not only an interrupted Kinugasa allylic alkylation (IKAA) strategy was successfully developed with step- economy, but also a large number of substrates were tested for each component.

7.2.3 C–H Activation Reaction

C–H activation is a class of reaction that is so diversified in terms of substrates, catalysts, and functionalized compound synthesis that it has drawn the attention of chemists for ages (Yang et al. 2017; Newton et al. 2017; Wei et al. 2017; Yi et al. 2017). In the history of coupling reactions, Pd is an absolute dominant metal that is also popular in the field of metal-catalyzed C(sp^3/sp^2)–H activation (He et al. 2017;



R = C_6H_5, 4-F-C_6H_4, 4-Me-C_6H_4, 4-Cl-C_6H_4, 4-Ph-C_6H_4, β-naphthyl, $CH_2CH(NHBoc)(CO_2Me)$, cyclohexenyl, 4-$OCH(Me)_2CO_2Me$-C_6H_4, 1,3-benzodioxolyl

R^1 = H, 2-furyl, 2-thiophenyl, C_6H_5, 4-Y-C_6H_4 (Y = F, Cl, Br, CF_3, NO_2, OMe, Ph), 2-OMe-C_6H_3N, 2-OMe-C_6H_4, 3-CF_3-C_6H_4, 3-(1-Ts)indole

R^2 = H, C_6H_5

Ar^1 = 4-F-C_6H_4, 4-Me-C_6H_4, 4-Cl-C_6H_4, C_6H_5, β-naphthyl

Ar^2 = C_6H_5, 4-Me-C_6H_4,

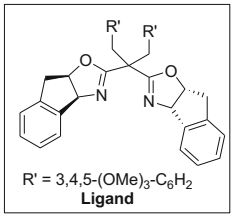
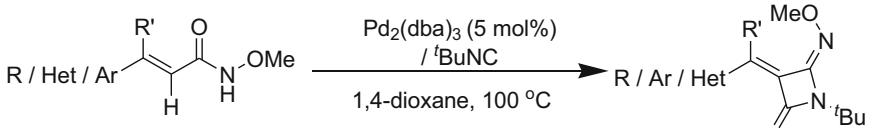


Fig. 7.13 An interrupted Kinugasa allylic alkylation (IKAA) reaction strategy using a combination of copper and palladium catalytic system



R' = H, Me, Ph
R = Me
Ar = C_6H_5, 2-Me-C_6H_4, 3-Me-C_6H_4, 4-Y-C_6H_4 (Y = Me, OMe, Br, Cl, CF_3), 2-naphthyl, -$(CH_2)_5$ -
Het = 3-pyridyl, 4-pyridyl, 2-furyl, 3-furyl, 2-thiophenyl, 1-Boc-3-indolyl

36 - 86 %

Fig. 7.14 $Pd_2(dba)_3$ promoted (sp^2)−H activation of α, β-unsaturated olefins: synthesis of 4-imino-β-lactam

Crabtree and Lei 2017). C–H functionalization was once considered a bizarre exception to the conventional rule, but today the role of such an influential strategy cannot be overlooked. In the development of synthetic strategies, it was evident that such C–H activation and functionalization have become popular in the rising demand for β-lactam antibiotics. Recent publications regarding such appealing works are covered under this section.

Yu and coworkers developed a protocol for the C(sp^2)−H activation of α, β-unsaturated olefins to fabricate 4-imino-β-lactam derivatives with excellent regioselectivity and *cis*-stereoselectivity (Fig. 7.14) (Kong et al. 2016). This Pd-catalyzed reaction offers good compatibility for diverse heterocyclic molecules as substrates.

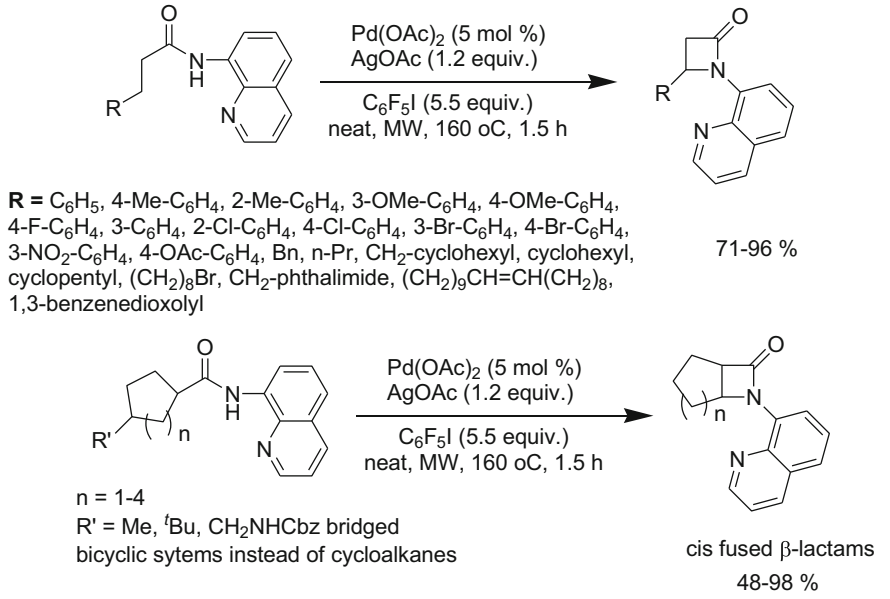
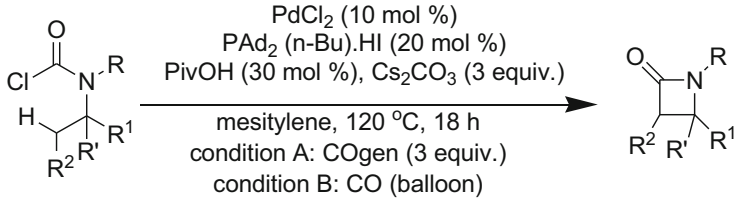


Fig. 7.15 Intramolecular amination by Pd-based catalyst-unactivated C(sp^3)−H activation

Apart from C(sp^2)−H activation, there are examples of C(sp^3)−H functionalization during the process of β-lactam synthesis (Zhang et al. 2016). One such illustration can be found in the work of Wu where C(sp^3)−H bond activation and intramolecular amination reaction had taken place in presence of a Pd-catalyst and Ag-salt at the β-position of carboxamides (Fig. 7.15). Scrutiny of the substrate scope allowed them to conclude that methylene group activation was favored over that of the tertiary CH and a methyl group at the β-carbon of carboxamides. This method is very useful for making β-lactams with 5/4, 6/4, 7/4, or 8/4 *cis*-fused ring systems, which would otherwise require lengthy synthetic sequences. Further, this protocol was extended, where reactions were performed to achieve various diazabicyclic β-lactams.

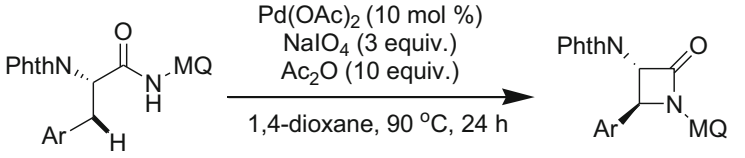
Another C(sp^3)−H functionalization was reported by Dailler et al. (2017) in the presence of a Pd(0) catalyst and utilizing carbamoylation with stoichiometric carbon monoxide (Fig. 7.16) (Dailler et al. 2017). They took precautionary measures to avoid the much-expected side reaction, i.e., decarbonylation, prior to the C–H activation step resulting in secondary amine. Primary, secondary, as well as tertiary C–H bonds responded well under the reaction conditions.

Amidst a vast array of these C–H activation reactions, another strategy of 'sequential C–H monoarylation and amidation was reported by Shi with the catalytic assistance of Pd-based catalyst (Fig. 7.17). Here, 5-methoxyquinolin-8-amine (MQ) auxiliary was used as the directing group, and it can be removed under mild reaction conditions. Moreover, the synthesis of orthogonally protected anti-α,-β-diamino acids is also feasible with this approach (Ling et al. 2017).



R = TMB, CH_2cylopentyl, CH_2-mesityl, Ph, CH_2Ph, CH_2CH_2Ph, $(CH_2)_3$Ph, $CH_2CH_2CO_2$Et, ^{i}Pr, cy $CH_2CH_2CH_2$NPhth and cyclic/ brigded bicyclic substituents
R' = H, Me, Et, Ph
R^1 = Me, ^{t}Bu, n-Hex, cy, CH_2OPiv, Ph, cyclopropyl
R^2 = Me and fused rings

Fig. 7.16 C(sp^3)–H carbamoylation by Pd(0) catalytic system



Ar = 2-thiophenyl, 1-Ac-3-indolyl, R-C_6H_4 [R = 4-Et, 3-Me, 3,4-diMe, 4-OMe, 3-OMe, 4-F, 3-F, (3-Cl, 4-F), 4-NHAc, (3-Cl, 4-NHAc), (4-NHAc, 3-NO_2), (3-OH-4-OMe-5-Me)]

Fig. 7.17 Stereoselective synthesis of α-amino-β-lactams mediated by $Pd(OAc)_2$: A sequential monoarylation-amidation

With so many examples of Pd-catalyzed C–H activation in hand, we can add another such successful attempt of Tong et al. for the asymmetric synthesis of β-lactams (Fig. 7.18). Starting with propanoic acid and aryl iodides through Pd-mediated intramolecular C(sp^3)–H amidation, this approach leads towards 94% ee (Tong et al. 2020). The utility of a 2-methoxy-5-chlorophenyl iodide oxidant was significant in controlling the competing pathways of reductive elimination for Pd (IV). Steric as well as electronic effects of the oxidant were liable for controlling the competing C–N vs C–C pathways.

A diastereoselective formation of β-lactams bearing 4-cyanoalkyl β-substituents was reported by He and coworkers with a copper-based catalytic system (Fig. 7.19). This C(sp^2)–H aminoalkylation comprises of aminoalkylation of unactivated alkenes with 5-iodo-8-aminoquinoline as the directing auxiliary and cyclobutanone oxime ester as alkyl radical donor (Zhang et al. 2021). A successful installation of both primary and secondary alkyl groups was achieved with selectivity for the C4 position of *cis*-internal or terminal 3-alkenamides.

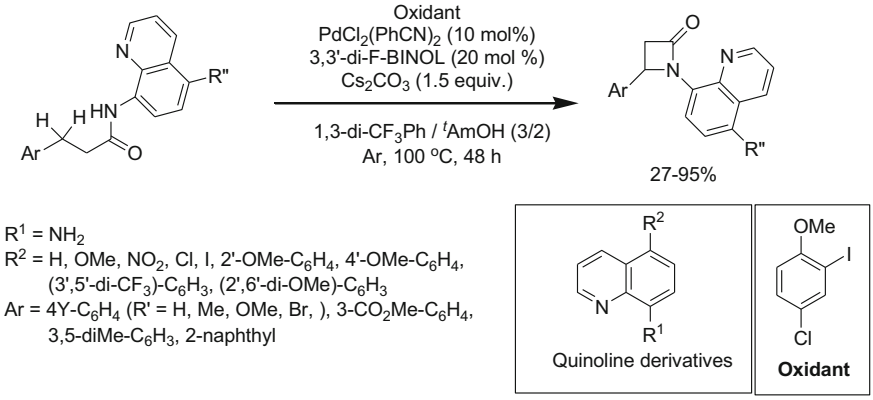


Fig. 7.18 Pd-mediated intramolecular C(sp^3)−H amidation towards the synthesis of β-lactams

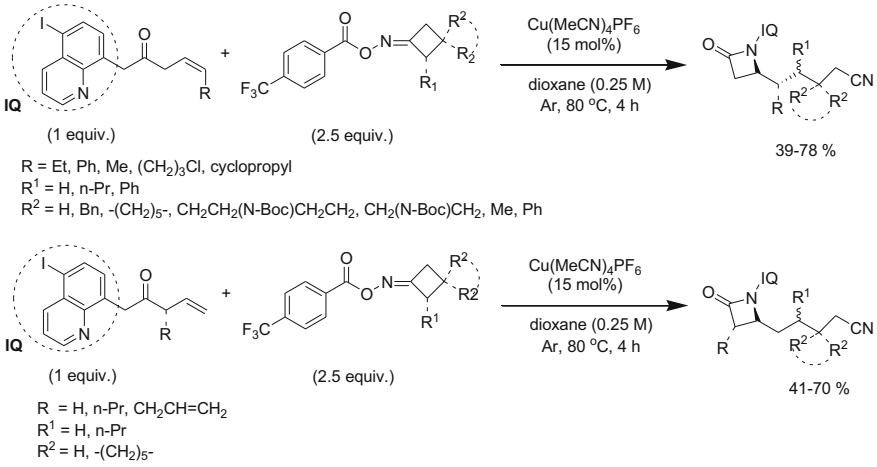


Fig. 7.19 Cu-catalyzed direct aminoalkylation of unactivated alkene to synthesize β-lactams scaffolds

A unique approach towards the synthesis of β-lactams by virtue of a Cu(I)-catalyzed cascade methodology involving radical carboamination of alkenyl carbonyl derivatives was delineated by Shi et al. (Fig. 7.20). With diverse substitution, alkenes were designed from vinylacetic acid and resulted in the corresponding β-lactams. The mechanistic study indicated a free radical mechanism with a Cu (I)/Cu (II)/Cu (III) catalytic cycle. A bidentate directing group assisted the catalytic process (Shi et al. 2019).

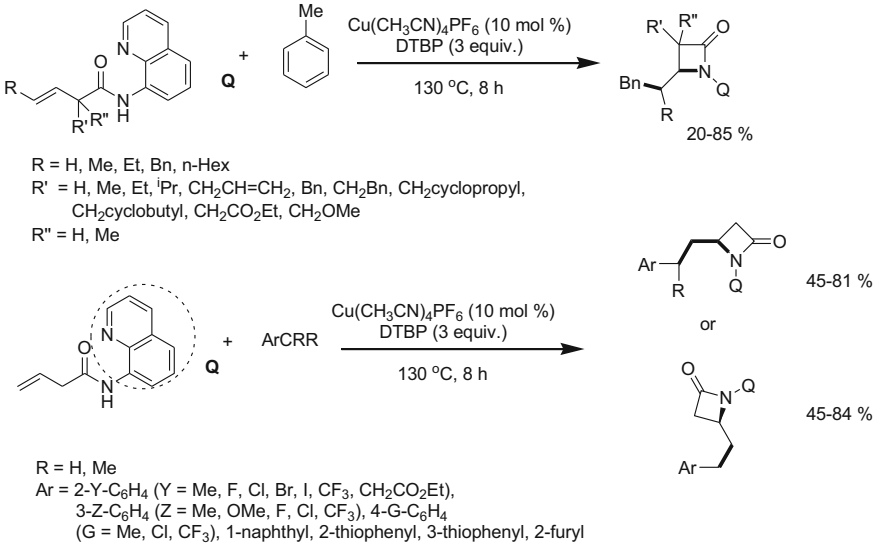


Fig. 7.20 Radical carboamination of alkenyl carbonyl compounds with a Cu catalyst

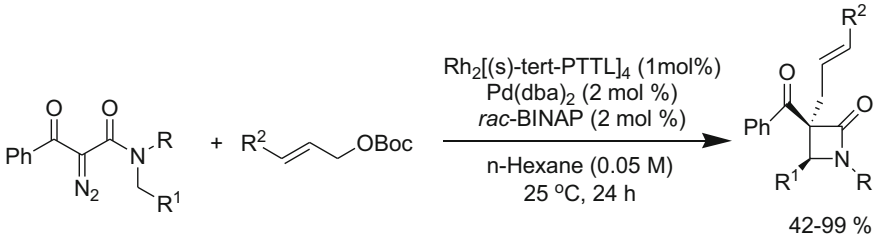


Fig. 7.21 Rh(II)/Pd(0) relay catalysis in α-quaternary chiral β-lactam synthesis

7.2.4 C–H Insertion

In the middle of plentiful approaches, an interesting one deserves to be mentioned, which is an example of a C–H insertion reaction (Dong et al. 2016). We have already focused on relay catalysis of the Pd/Rh system, and here we found another relay catalysis again with the Rh(II)/Pd(0) system reported by Huang et al. (Fig. 7.21). The limiting scope of β-lactam ring construction, despite a considerable advancement in this field, encouraged them to expand their previous work, and consequently, an asymmetric reaction was proposed to furnish α-quaternary chiral β-lactam scaffolds with a sequential enantioselective intramolecular C–H insertion reaction for the Rh (II) carbenoids derived from α-diazo acetamides, followed by Pd-mediated allyl alkylation. A wide variety of substrates (containing Me, OMe, Br, Cl, F, CF_3, NO_2,

CN groups at various positions) reacted under this reaction condition to provide high yield and enantioselectivity.

7.2.5 *Aza-Reformatsky Reaction*

Among diverse reaction strategies for the development of β-lactam antibiotics, Munck et al. showed an Aza-Reformatsky reaction starting with cyclic aldimines and ketimines for the synthesis of chiral β-amino esters (De Munck et al. 2016). Here $ZnMe_2$ was utilized as the zinc source and diaryl prolinol as chiral ligands. Now, these chiral β-amino esters can be further converted into the corresponding acids which are precursors or intermediates in the β-lactam synthesis. β-lactam derivatives through indirect metal-catalyzed stepwise synthesis were achieved in this work.

7.2.6 *Other Approaches*

Although the last 5 years have witnessed a plethora of cutting-edge approaches towards the synthetic procedures of β-lactam antibiotics (Isoda et al. 2015), this chapter mainly revolves around the metal-catalyzed synthetic strategies. Some other approaches regarding this include Ugi intramolecular cyclization (Ugi 1982; Cheibas et al. 2019), Gilman-Speeter reaction (Troisi et al. 2010; Sierra et al. 2001; Gilman and Speeter 1943), Alper reaction (Troisi et al. 2010), Torii Reaction (Troisi et al. 2010), photo irradiation reaction (Vaske et al. 2010), carbene insertion, etc. (Cainelli et al. 1996). Figure 7.1 showed the numbering in the β-lactam scaffold and recent literature reveal the synthetic strategies involving the formation of C3–C4 bond, N1–C2 and C3–C4 bonds, N1–C2 and C2–C3 bonds, C3–C4 and C4–N1 bonds, N1–C4 bond, and C2–C3 bond.

7.3 Conclusion

Over the course of time, conventional organic reactions have been preferentially substituted by newly invented modern approaches in the synthesis of β-lactam. Staudinger reactions and cyclization of β-amino acids were the basic synthetic methods for β-lactam synthesis for a long period. Years thereafter, the ligand-controlled transition metal catalysis became popular, like the Kinugasa reaction and Rh-assisted diazo activation/C–H bond insertion to name a few. To add to this list, NHC-catalyzed reactions, photocatalysis, and C–H activation/amidation entered the battlefield. The increasing demand for this scaffold in the pharmaceutical industry triggered the discovery, development, and prospects of β-lactam, investigating the unturned stones in this field.

An emerging problem in the field of drug design at the present time is the development of antimicrobial resistance (AMR) of pathogens towards the existing therapeutic medicines. This is primarily caused by the unwarranted overuse of antibiotics in modern lifestyle. Multifaceted interventions are required to counter this challenge, which may include the evolving modifications in the synthetic strategies of β-lactam, keeping pace with the evolving pathogens. Exploring the innovative vistas of metal-catalyzed synthesis of β-lactam antibiotics may be the key to opening the mighty Pandora's box of the pharmaceutical industry in this twenty-first century.

Acknowledgement None to declare.

Conflict of Interest None to declare.

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Chapter 8
Upgrading the Antibiotic Arsenal Against Gram-Positive Bacteria: Chemical Modifications of Vancomycin

Yash Acharya and Jayanta Haldar

Abstract Glycopeptides are one of the most prevalent classes of antibiotics against Gram-positive bacterial infections. A prominent member of this class, vancomycin, has been used as a last-resort drug for countering infections by drug-resistant Gram-positive superbugs. However, nearly thirty years after its discovery, resistance to vancomycin was reported initially in the clinic, and consequently, pathogenic vancomycin-intermediate resistant *Staphylococcus aureus* (VISA), vancomycin-resistant *S. aureus* (VRSA), and vancomycin-resistant *Enterococcus faecium* (VRE) were isolated. The obsolescence of vancomycin against Gram-positive superbugs posed a huge therapeutic challenge. This prompted the search for strategies to counter vancomycin resistance. Among these, the development of semi-synthetic derivatives, which can tackle vancomycin resistance in bacteria, has emerged as a powerful strategy. This chapter will focus on the mechanism of action of vancomycin, emergence of vancomycin resistance, and effective chemical strategies for the development of semi-synthetic and synthetic analogues of vancomycin with restored activity against vancomycin-resistant Gram-positive bacteria.

Keywords Antibiotic resistance · Antibiotic-sugar conjugates · Biofilms · Cell-wall biosynthesis inhibitors · Dormant cells · Glycopeptide antibiotics · Gram-positive bacteria · Gram-positive infections · Lipophilic-cationic · Membrane-active compounds · Multivalency · Peptide-vancomycin conjugates · Persisters · Semi-synthetic derivatives · Stationary-phase bacteria · Vancomycin · Vancomycin derivatives · Vancomycin-resistant · VRE · VRSA

Abbreviations

CBP	Chlorobiphenyl
D-Ala-D-Ala	D-Alanine-D-alanine
D-Ala-D-Lac	D-Alanine-D-lactate

Y. Acharya · J. Haldar (✉)
Antimicrobial Research Laboratory, New Chemistry Unit and School of Advanced Materials, Jawaharlal Nehru Centre for Advanced Scientific Research, Bengaluru, Karnataka, India
e-mail: jayanta@jncasr.ac.in

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D-Ala-D-Ser	D-Alanine-D-serine
FDA	US Food and Drug Administration
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-susceptible *Staphylococcus aureus*
NAG	*N*-Acetyl glucosamine
NAM	*N*-Acetyl muramic acid
UDP-NAG-pp	Uridine diphosphate-*N*-acetyl glucosamine-pentapeptide
VISA	Vancomycin intermediate-resistant *Staphylococcus aureus*
VRE	Vancomycin-resistance *Enterococcus faecium*
VRSA	Vancomycin-resistant *Staphylococcus aureus*
WHO	World Health Organization

8.1 Introduction

Vancomycin is one of the most well-known antibiotics discovered in the golden era of antibiotic discovery. The first of the glycopeptide class of antibiotics, vancomycin, was discovered by the chemist Edmund Kornfeld in 1952, while working for the company Eli Lily Inc (Butler et al. 2014). It was isolated from the Actinomycetes, *Streptomyces orientalis* (now renamed as *Amycolatopsis orientalis*), and demonstrated activity against *S. aureus* bacteria, including penicillin-resistant strains (Levine 2006). The activity against penicillin-resistant strains provided hope to researchers, and a vigorous process of isolation, characterization, and purification was adopted. Contrary to penicillin, this new drug displayed a negligible propensity for resistance. Consequently, the US Food and Drug Administration (FDA) approved the use of vancomycin for infections of penicillin-resistant bacteria in 1958 (Butler et al. 2014). However, initial difficulties with purification and the presence of impurities, leading to side effects such as nephrotoxicity and ototoxicity, coupled with the approval of methicillin in the same year, were responsible for the relegation of vancomycin to use only in rare conditions of β-lactam-resistant bacterial infections (Levine 2006). In the subsequent decades, the sudden emergence of methicillin-resistant *S. aureus* (MRSA) in the United States, the effectiveness of vancomycin in tackling multiple drug-resistant bacterial infections, and the slow propensity of resistance development restored the importance of vancomycin as a one-of-its-kind drug against Gram-positive bacterial infections.

Vancomycin is a Class I tricyclic glycopeptide and possesses five amino acids with an aromatic side chain and two amino acids with an aliphatic side chain (Fig. 8.1). This antibiotic targets a metabolite, as opposed to other antibiotics which target enzymes (Reynolds 1989). This leads to slower resistance development. It was three decades after its discovery that the first reports of vancomycin resistance were observed in *E. faecium* in 1988 (Bager et al. 1997). Vancomycin resistance in *S. aureus* was also observed a few years later. This was a red alert for

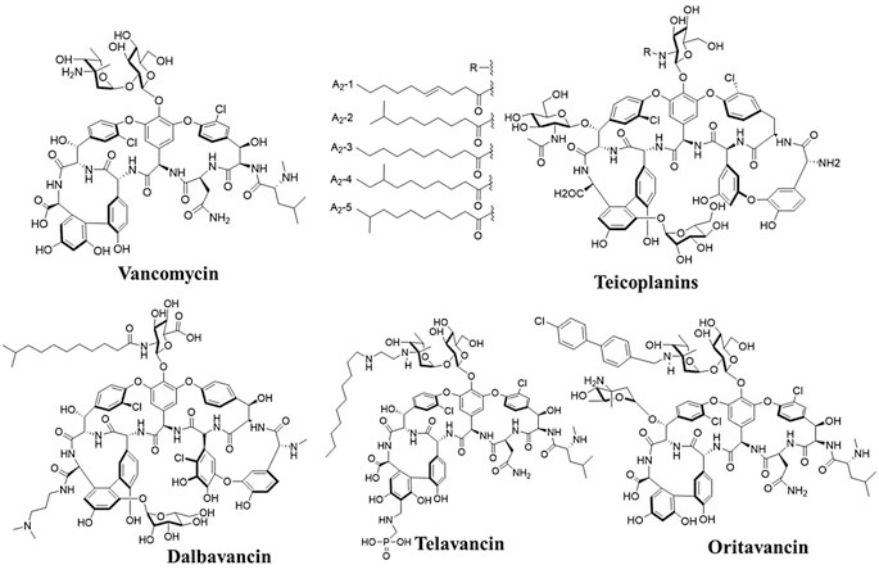

Fig. 8.1 Structures of approved glycopeptide antibiotics

researchers. In fact, concurrent with the increased use of vancomycin, we entered the era of emerging and intensifying vancomycin resistance. In the present times, vancomycin-resistant pathogens are a severe cause of concern for clinics all over the world. Since the available treatment options for vancomycin-resistant bacteria are limited, vancomycin-resistant *E. faecium* (VRE) and vancomycin-intermediate and resistant *S. aureus* (VISA and VRSA) were classified as pathogens of high priority by the World Health Organization (WHO) in its 2017 report on antimicrobial resistance (Dhanda et al. 2019). This stark and disturbing reality has jolted medicinal chemists to search for treatments and strategies for countering vancomycin resistance. This search has adopted various directions, many of which have shown promising results.

After vancomycin, another naturally occurring glycopeptide, teicoplanin, was isolated from the actinobacterium species *Actinoplanes teichomyceticus* (Fig. 8.1). Teicoplanin is a Group IV glycopeptide antibiotic, which possesses a hydrophobic side chain (Butler et al. 2014). Naturally occurring teicoplanin is a mixture of different components, with varying side chains. Approved for treatment of bone infections, pneumonia, joint infections, urinary tract infections, endocarditis, and bacteraemia caused by MRSA and enterococci in Europe in 1988, commercially available Teicoplanin (marketed as TARGOCID©) comprises of five major components (teicoplanin A_2-1, A_2-2, A_2-3, A_2-4, and A_2-5) and four minor components (teicoplanin R_S-1, R_S-2, R_S-3, and R_S-4).

Currently, three semi-synthetic derivatives of glycopeptides, telavancin, dalbavancin, and oritavancin, possessing activity against both vancomycin-sensitive

as well as vancomycin- resistant bacteria, have been developed. All of them have been approved for clinical use by drug-control agencies. They are lipoglycopeptides, possessing a core of glycopeptide, with long lipophilic moieties linked to it (Fig. 8.1). These moieties allow for better anchoring to the membrane and binding to the substrate, or dimerization in the case of oritavancin, thereby making the derivatives more potent (Higgins et al. 2005; Malabarba et al. 1995). Further, the enhanced lipophilicity provides better pharmacokinetic/pharmacodynamic stability (Higgins et al. 2005). Briefly, telavancin is a vancomycin derivative approved for complicated skin and skin structure infections (cSSSi) treatment in 2009 and hospital-associated pneumonia caused by *S. aureus*, in 2013, by the US FDA (Sarkar and Haldar 2019). Dalbavancin, a derivative belonging to the teicoplanin family, is active against a range of vancomycin-sensitive and vancomycin-resistant Gram-positive pathogens (Candiani et al. 1999). It was approved by the FDA in 2014 for use against MSSA, MRSA, and vancomycin-sensitive *E. faecuim*, involved in skin and skin structure infections. It has a long half-life of 149 to 250 hours (Zhanel et al. 2010). The third semi-synthetic glycopeptide, oritavancin, is a chloroeremomycin derivative. While it possesses activity against MRSA, VRSA, and VRE, it is approved by the US FDA along with dalbavancin, against skin and tissue infections caused by MSSA, MRSA, and vancomycin-sensitive *E. faecium* (Arhin et al. 2009). This drug, owing to its hydrophobicity, possesses an unusually long half-life of 393 hours (Saravolatz and Stein 2015).

However, the ever-increasing resistance, coupled with the shrinking list of active antibiotics, has led researchers to look for other strategies to counter vancomycin resistance. In this chapter, we attempt to provide a comprehensive overview of one such successful strategy, semi-synthetic modifications of vancomycin, and synthetic glycopeptides. Semi-synthesis, for multiple reasons, is an attractive tool for developing new antibiotics. Given the complexity of total synthesis, the arduous and time-consuming nature of the search for novel antibiotics, and the ease of working with known and approved drugs, semi-synthetic modification has evolved as one of the best strategies to upgrade and expand our antibiotic arsenal. The chapter briefly discusses the mechanism of action of vancomycin, the origin and chemistry of vancomycin resistance, and a multitude of chemical strategies adopted to semi-synthetically modify vancomycin with the aim of restoring its activity in vancomycin-resistant bacteria. The reader will be introduced to the rationale of different approaches, successes in the field, and future prospects and challenges in developing semi-synthetic and synthetic analogues of vancomycin.

8.2 Mechanism of Action and Resistance

Vancomycin is a glycopeptide antibiotic, which acts on the process of cell-wall synthesis in bacteria. This antibiotic is slightly different from the other classes of cell-wall biosynthesis antibiotics, as it binds to a precursor of a cell wall, and not to an enzyme (Reynolds 1989). Bacterial cell walls are composed of peptidoglycan.

This peptidoglycan is a sugar-based polymer, with peptide cross-links. The monomers of peptidoglycan possess alternating sugars, *N*-acetyl glucosamine (NAG) and *N*-acetyl muramic acid (NAM), linked through the β-(1,4) glycosidic linkage (Liu and Breukink 2016). To the NAM sugar, a species-specific short peptide sequence is attached through the carboxyl group. Gram-positive bacteria possess an L-alanine-D-glutamine-L-lysine-D-alanine-D-alanine peptide sequence (L-lysine is replaced by m-diaminopimelate in Gram-negative bacteria). In the *S. aureus* bacterium, an additional pentaglycyl chain is attached to the L-lysine (Vollmer et al. 2008). In the process of cell-wall synthesis, peptidoglycan monomers are added to a growing chain to form peptidoglycan (Liu and Breukink 2016). This action is performed by the bacterial enzyme transglycosylase. This enzyme adds a monomer unit (NAG-NAM-peptide) to the free -OH terminal of the NAG unit of a growing peptidoglycan chain. However, the polymer is strengthened by the process of cross-linking individual chains through a peptide linkage. This is achieved by the bacterial enzyme, transpeptidase, which facilitates the reaction involving the formation of a peptide bond between the penultimate D-alanine and a peptide on another chain (Liu and Breukink 2016).

Vancomycin binds to the nascent peptide, specifically to the D-Ala-D-Ala terminal (Reynolds 1989). Through its binding, it encapsulates the dipeptide, preventing the approach of the transpeptidase enzyme and hence the process of transpeptidation (Fig. 8.2). This binding happens through hydrogen bonding interactions (Williams and Bardsley 1999). Primarily, five hydrogen bonds contribute to the stability of the vancomycin-D-Ala-D-Ala complex (Fig. 8.2).

Thus, vancomycin binding inhibits the process of cell wall synthesis. Hence, bacteria cannot divide effectively, leading to their eventual death. Most of the glycopeptides act in a similar manner, by inhibiting cell-wall biosynthesis through binding to the peptidoglycan precursor peptide (Sarkar et al. 2017). However, the approved semi-synthetic derivatives also possess additional mechanisms of action.

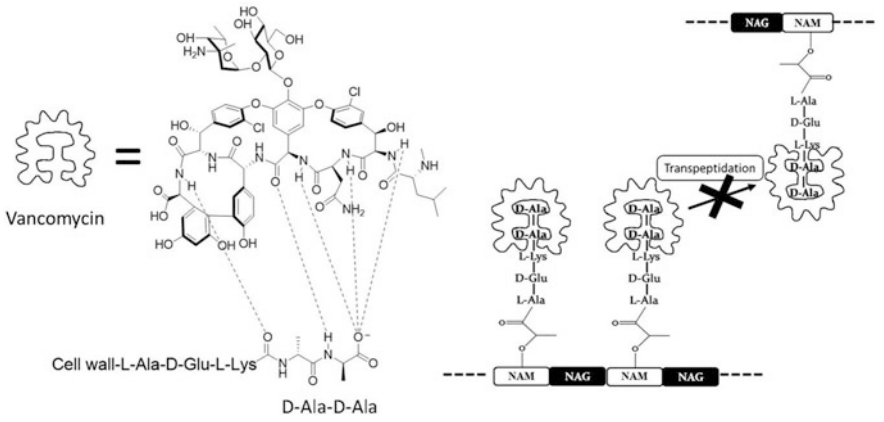

Fig. 8.2 Binding of vancomycin to cell-wall biosynthesis precursor peptide and mechanism of action of vancomycin

Telavancin and dalbavancin display membrane anchoring properties (Higgins et al. 2005). Dalbavancin forms dimers, which leads to enhanced binding to target peptides. Oritavancin also forms dimers due to the biphenyl moiety, and along with inhibiting transpeptidation, it also prevents transglycosylation (Malabarba et al. 1995).

Since the emergence of vancomycin-resistant bacteria like VRE, VISA, and VRSA, the genetic factors responsible for vancomycin resistance have been studied in detail. It was found that intermediate resistance to vancomycin arises in VISA due to the thickening of the cell wall (Hiramatsu 2001). Similar thickening is also observed in the cell walls of VRSA. Relatively recent studies have also shown that the VISA cell wall possesses less cross-linking, thereby possessing free D-Ala-D-Ala terminals, which further reduces vancomycin activity. The primary cause of resistance in VRE and VRSA, however, is the modification of the target peptide (Hiramatsu 2001; Evers et al. 1996; Arthur and Courvalin 1993). In both of these resistant strains, the terminal dipeptide for the peptidoglycan precursor is modified, from D-Ala-D-Ala to D-Ala-D-Lac (or D-Ala-D-Ser) leads to an ~1000-fold (or ~sevenfold decrease) in the binding constant (McComas et al. 2003; McKessar et al. 2000). A large operon, comprising of seven to eight genes, is responsible for these different precursors being used. To date, 11 gene clusters have been identified, which are responsible for vancomycin resistance (Dhanda et al. 2019). These gene clusters can be divided into two groups, based on the precursor sequence that they generate. The *vanA*, *vanB*, *vanD*, *vanF*, *vanI*, and *vanM* clusters generate the D-Ala-D-Lac precursors, while *vanC*, *vanE*, *vanG*, *vanL*, and *vanN* produce the D-Ala-D-Ser precursors. The D-Ala-D-Lac variant is the most common, arising due to the *vanA* and *vanB* clusters. It is generated from the D-Ala-D-Ala dipeptide, which is cleaved by a D-D-dipeptidase enzyme, *vanX* into the constituent amino acids. For this D-Ala, the amino acid D-Lac (synthesized from pyruvate by a D-lactate dehydrogenase *vanH*) is ligated by the *vanA* ligase (Arthur et al. 1996). Other genes contributing to activation of the vancomycin resistance pathway in response to the presence of vancomycin or other glycopeptides in the system have been identified. This happens through the VanS kinase, which autophosphorylates in response to the perturbation of cell-wall precursors by vancomycin.

This prompts phosphorylation of VanR (a transcriptional activator and downstream response regulator), which increases expression of *vanS* and *vanR*, and also binds to the *vanHAX* regulatory core. This modulates the expression of the *vanA* and *vanB* operons (Arthur et al. 1996, 1992, 1998; Evers and Courvalin 1996). Apart from the *vanA*, *vanB*, *vanH*, and *vanX* genes described above, the operons also contain the *vanY* gene, which codes for a D,D-carboxypeptidase which cleaves the C-terminal D-Ala present on late membrane-bound precursors (Arthur et al. 1996). Another gene, *vanZ*, responsible for teicoplanin resistance, is also a part of the *vanA* and *vanB* operons (Arthur et al. 1995). Recently, strains producing a dual-functional VanXY enzyme, with properties of both D,D-dipeptidase and D,D-pentapeptidase, have been identified (Podmore and Reynolds 2002; Reynolds et al. 1999).

8.3 Strategies to Overcome Vancomycin Resistance

Vancomycin offers multiple functional groups, which can be modified chemically or enzymatically. They include the acid and the amine terminals, along with the hydroxyl groups of the vancosamine sugars and the phenolic hydroxyl groups of the peptide backbone. The following section describes the detailed chemical strategies and modifications which have been adopted for designing semi-synthetic and synthetic vancomycin derivatives to tackle increasing vancomycin resistance in drug-resistant Gram-positive pathogens.

8.3.1 Modifications that Increase the Binding Efficacy to Target Peptide

Vancomycin modifications, leading to enhanced binding to target peptide, can be grouped into three chemical strategies, namely, synthetic analogues with modifications to the peptide backbone, modifications that enhance H-bonding, and modifications that confer multivalency.

8.3.1.1 Synthetic Analogues Involving Modifications to the Core Peptide Backbone

The structural reasons responsible for the reduced binding affinity of vancomycin to the target have been studied by the Boger group (James et al. 2012). Their study disclosed that the reduced number of hydrogen bonds in resistant target peptide and vancomycin was less significant as compared to the introduction of lone pair-lone pair repulsion between the carbonyl oxygen and ester oxygen atoms of vancomycin and the resistant target peptides, respectively. Since this repulsion was ten times more significant than the loss of H-bonding, it would be logical that a strategy which eliminates this repulsion is bound to deliver higher activity against the vancomycin-resistant bacteria. Following this, vancomycin aglycon analogues with core-peptide modifications were developed by the Boger group through total synthesis (Xie et al. 2011, 2012; Crowley and Boger 2006). The best compound of these synthetic vancomycin analogues had the 4-position carbonyl replaced by an amidine group (Fig. 8.3). The modified groups' ability to act as a hydrogen-bond donor as well as acceptor allowed the analogue [Ψ[C(=NH)NH]Tpg^4] to bind to both unaltered and resistant peptides (binding constants of 7.3×10^4 M^{-1} and 6.9×10^4 M^{-1}, respectively). The MIC of this analogue was >5 μg/mL against both vancomycin-resistant and sensitive strains (Xie et al. 2012). The Boger group also incorporated peripheral hydrophobicity into the amidine and thioamide analogues, by modifying the *N*-terminal of the sugar and introducing a (4-chlorobipenyl) methyl group (CBP) at that position (Okano et al. 2014). The resulting derivatives displayed higher activity and

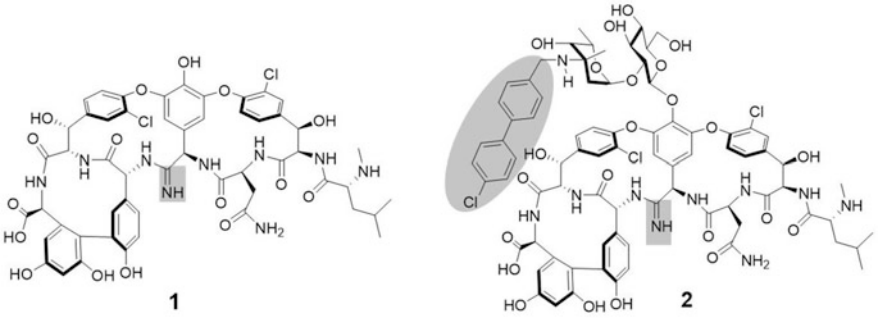

Fig. 8.3 Structures of synthetic vancomycin analogues with core peptide backbone modifications

binding affinity than the analogues without hydrophobicity. The best of the series, an amidine derivative from the CBP group (Fig. 8.3), displayed an MIC of 0.005 μg/mL against the VRE VanA strain. The high activity and binding constant suggested synergistic action of the core and peripheral modifications. Further investigations revealed that the CBP group inhibits cell wall synthesis, by inhibiting transglycosylation (Okano et al. 2017). Many such core modifications were performed, where the first, third, and fourth residues of the vancomycin core peptide were modified. However, few were successful in conquering vancomycin resistance. Further, as this process relied on total synthesis of the vancomycin aglycon, it was marred by complex synthetic challenges and the limitations of time.

8.3.1.2 Modifications to Impart H-Bonding Affinity

The study of the crystal structure of complexed vancomycin and target peptide by Nitanai et al. (2009) found that a water molecule is present as a bridge between the carboxylate groups of vancomycin and the target peptide. This indicated that incorporation of moieties which will introduce additional hydrogen bonding can lead to higher binding affinity to resistant target peptides. Thus, a strategy was developed in our group, where different cyclic and acyclic sugars such as maltose, cellobiose, gluconic acid, lactobionic acid, etc. were appended to the C-terminal of vancomycin (Yarlagadda et al. 2015a). While most of the derivatives displayed similar results as vancomycin, the lactobionic acid and gluconic acid derivatives were found to be highly active against the VRE VanA phenotype (MIC = 36 μM for the lactobionic acid derivative) (Fig. 8.4). This was further corroborated by the ~150-fold increase in binding affinity of the derivative to the resistant D-Ala-D-Lac target peptide (binding constant = 8.8×10^4 M^{-1}). The binding constant for the unmodified target peptide was in a range similar to vancomycin (2.1×10^5 M^{-1}).

To increase the antibacterial activity of this lactobionic acid-vancomycin conjugate, different alkyl chains (from C_8 to C_{12}) were attached to the amino terminal of the lactobionic acid-vancomycin conjugate (Yarlagadda et al. 2015a). This modification, resulting in alkyl-lactobionic acid-vancomycin conjugates, imparted the

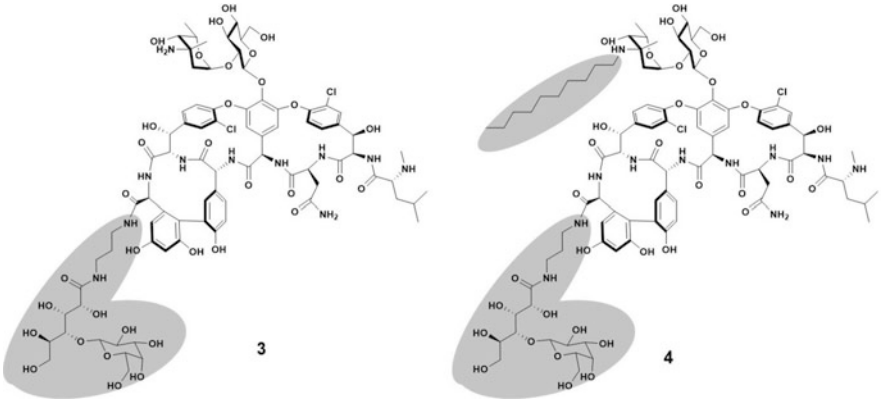

Fig. 8.4 Structures of vancomycin derivatives with modifications that enhance the hydrogen-bonding capacity of the derivative

additional property of membrane anchoring. The decyl analogue was identified as the most selective one from the series, with low toxicity and high antibacterial activity (Fig. 8.4). This analogue displayed a 1000-fold and 250-fold increase when compared with vancomycin, against VanA and VanB strains of VRE. The activity was found to be very high, with MIC ~ 0.7 μM against VRE VanA and ~1 μM against VRE VanB phenotype. This increase in activity was attributed to enhanced interactions with the membrane, owing to the presence of the alkyl chain. The in vivo potential of this analogue was tested in a neutropenic murine thigh infection model, where it led to ~6 log reduction in the VISA count. Further, the pharmacokinetic/pharmacodynamic properties of the analogue were found to be improved, as compared to vancomycin (Yarlagadda et al. 2015b).

8.3.1.3 Modifications Involving Multivalency

Vancomycin possesses various functional groups, which allow for the formation of hydrogen bonds, as well as hydrophobic interactions. These interactions lead to spontaneous dimerization of the glycopeptide in an aqueous medium (Nicolaou et al. 1999). It has been observed that these non-covalent aggregated dimers of vancomycin display a cooperative effect in the binding to the peptide (Mackay et al. 1994). This result has prompted researchers to design covalently bound dimers and trimers of vancomycin, connected by a variety of linkers, such as alkyl moiety, triamines, disulphide alkyl moieties, platinates, etc., which are appended to different positions on the vancomycin aglycon. The first dimeric bis(vancomycin)carboxamides were synthesized by the Sundram et al. utilising three different linkers (Sundram et al. 1996). Promising increase in activity was seen for these derivatives against VRE, as compared to vancomycin. The best derivative, possessing the disulphide ethyl linker, is capable of binding to the modified D-Ala-D-Lac peptide with a dissociation

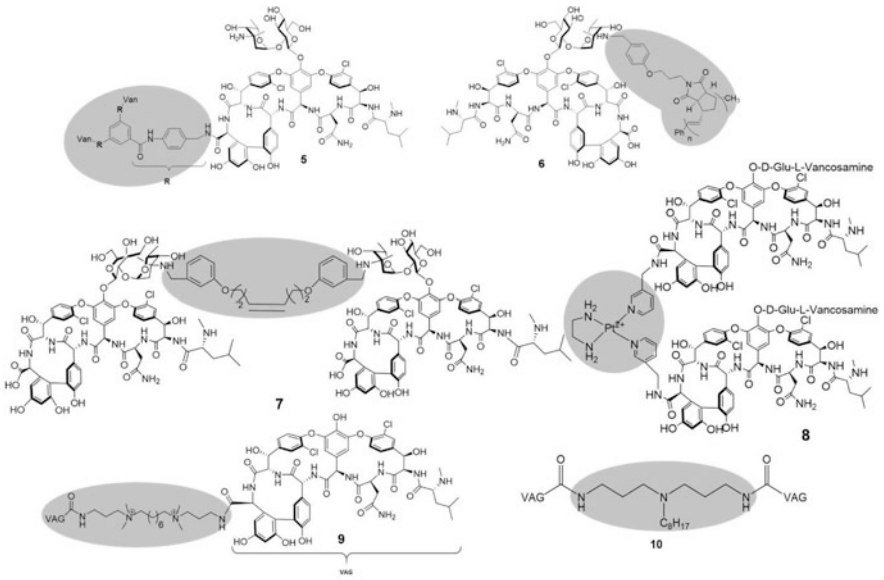

Fig. 8.5 Structures of multimeric vancomycin analogues

constant of 440×10^{-6} M. This good affinity translates to a ~60-fold enhancement in the MIC against VRE (MIC = 11 μM) as compared to vancomycin. At about the same time, in 1998, trivalent vancomycin derivatives, which possessed enhanced binding affinity, were developed by the Whitesides group (Rao et al. 1998). In these derivatives, a benzene-1,3,5-tricarboxamide linker connects three vancomycin aglycones, through amidation at the C-terminus of vancomycin (Fig. 8.5). This idea was further expanded by Arimoto and co-workers, who extended multivalency to polyvalency (Arimoto et al. 1999). This group conjugated vancomycin to a norbornene dicarboximide-based polymer backbone through ring-opening metathesis polymerization (Fig. 8.5). This polyvalent vancomycin derivative displayed an MIC of 31 μg/ml against the VRE VanA strain (60-fold increase) and a potent activity with MIC = 2 μg/ml against the VRE VanB strain.

In a later report by the Nicolaou group in 2000, a combinatorial synthesis strategy was used to select the best dimeric analogues (Nicolaou et al. 2000). It was observed that olefinic linker conjugated dimers, with an *N*-methyl leucine substitution (Fig. 8.5), were the most active against VRE, with an MIC = 1–2 μg/mL. It was noted that having a flexible organic linker compromises the advantage of multivalency and results in a loss of conformational entropy. To overcome this shortcomings, Bing Xu and co-workers came up with the idea of linking two vancomycin molecules through a metal complex, which will ensure rigid geometry (Xing et al. 2003). The resultant design possessed a platinate complex [$Pt(en)_2(H_2O)_2$], where two vancomycins were coordinated to the metal centre through a pyridine moiety, at a position cis to each other (Fig. 8.5). This rigid dimer

displayed a very low MIC = 0.8 μg/mL against the VanA phenotype of VRE, with a dramatic 720-fold rise in activity as compared to vancomycin.

Haldar and co-workers employed a simple chemical strategy of dimerising vancomycin aglycon, with varying hydrophobicity and charge between the two glycopeptide moieties (Fig. 8.5) (Yarlagadda et al. 2015c). The octylene-linker derivative with two positive charges was found to be highly active against VISA with a 130-fold increase (MIC = 0.1 μM) and active against VRE with a 15-fold increase in activity (MIC = 48 μM). Our group also worked on developing dimeric analogues of vancomycin aglycon with membrane anchoring properties (Fig. 8.5) (Yarlagadda et al. 2015c). To impart this membrane anchoring property, a triamine linker with a dangling octyl chain on the central amine was used to connect the vancomycin aglycons. This derivative displayed potent activity against VRE with MIC = 2.5 μM (300-fold increase when compared to vancomycin).

In recent years, Sharpless group developed a click chemistry-based method to bridge two vancomycin scaffolds bearing different C-terminal functionalized alkynes and azides, through a triazole linker (Silverman et al. 2017). These derivatives displayed good activity against the VRE VanB strain (0.8 μg/mL). The same study also reported heterodimers of vancomycin and alkylated vancosamine-vancomycin derivatives. These derivatives displayed reduced efficacy against MRSA but improved efficacy against VRE, as compared to vancomycin.

8.3.2 Modifications that Confer Membrane-Interacting Properties

Vancomycin primarily targets the process of peptidoglycan biosynthesis in the cell wall of an actively dividing bacterium. Near the bacterial cell wall, another important bacterial structure is present, the plasma membrane. Modifications which can allow vancomycin to interact with this cell membrane have a good chance of overcoming vancomycin resistance. Different semi-synthetic strategies have been adopted by leading researchers to confer these interactions on vancomycin. The membrane-interacting and disrupting vancomycin derivatives can be grouped into the following classes, on the basis of the nature of modification.

8.3.2.1 Alkylated Vancomycin Derivatives

The idea of appending alkyl groups to vancomycin owes its origins to the structure and activity of teicoplanin. A second-generation glycopeptide, teicoplanin, displays higher activity compared to vancomycin. This is generally attributed to the presence of a lipophilic chain appended to the primary amino terminus of the vancosamine sugar through an amide bond. Inspired by this, Thorson and co-workers first attached a lipophilic 6-azido glucose through chemoselective enzymatic synthesis (Fig. 8.6)

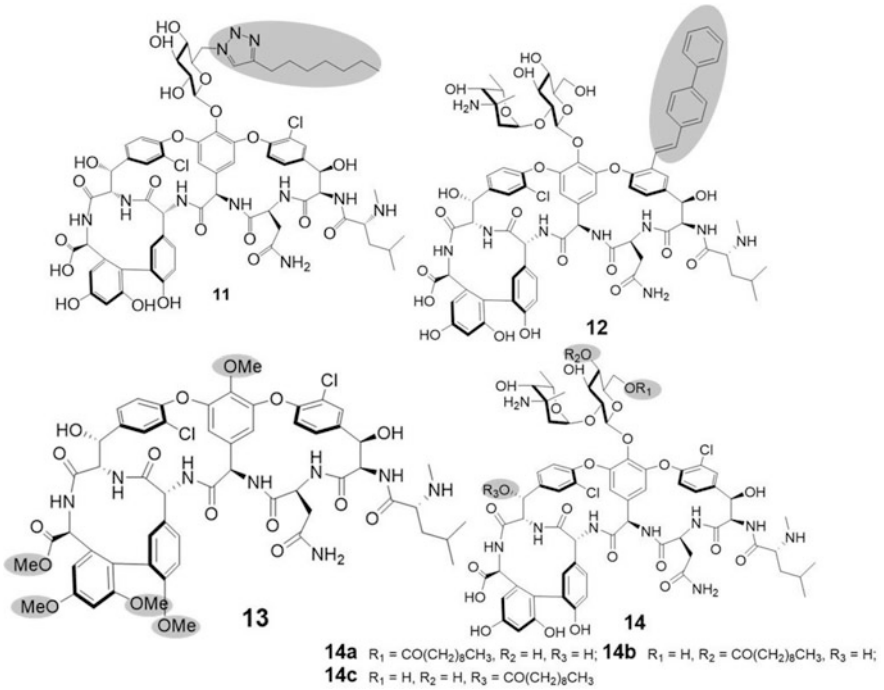

Fig. 8.6 Structures of alkylated vancomycin derivatives

(Fu et al. 2005). This derivative displayed high activity (MIC = 1 μg/mL) against the VanB strain of VRE. This betterment in activity, as compared to vancomycin, was attributed to the heptyl chain displaying better membrane interaction and disruption. In a slightly different strategy, the Arimoto group developed a vancomycin derivative where the 2-amino acid chloro group was selectively replaced by a biphenyl-containing lipophilic moiety (Fig. 8.6) (Nakama et al. 2010). Suzuki-Miyayura coupling was used to perform the reaction of replacing the chloro group with hydrocarbon substituents. This moiety aided in enhancing the activity of the derivative against the VRE VanB phenotype (MIC = 0.5 μg/mL), while no enhancement was observed against the VanA phenotype. When this approach was expanded and a similar lipophilic moiety was introduced at the sixth amino acid residue of the glycopeptide, activity against vancomycin susceptible strains was lost too.

The Boger group introduced alkyl groups at different positions within the glycopeptide and generated methyl ether derivatives of vancomycin aglycon, with modification at different aryl hydroxy positions (Crane et al. 2010). One of the tetramethyl ether derivatives, which was also esterified at the C-terminus, displayed excellent activity against the VRE strain with the VanB phenotype, with an MIC in the range of 1.6 μg/mL (Fig. 8.6). Recently, the Miller group adopted a regioselective catalyst-based synthetic strategy which selectively substituted one of the three aliphatic hydroxyl groups on vancomycin with a decanoyl lipid chain

(Fig. 8.6) (Yoganathan and Miller 2015). The subsequently generated three lipidated vancomycin analogues were highly active against VRE VanB and VanS phenotypes (MIC ~ 0.25 μg/mL).

8.3.2.2 Cationic-Lipophilic Vancomycin Derivatives

The idea of incorporating membrane disruptive property through the inclusion of a lipophilic moiety in vancomycin, as seen in the previous section, yielded positive results. This concept was taken further by our group, and the novel strategy of appending cationic lipophilicity to vancomycin was developed to enhance the interaction of these derivatives with negatively charged bacterial cell membranes. Our group developed cationic-lipophilic vancomycin derivatives with different alkyl chains, by conjugation at the carboxylic acid group of vancomycin (Yarlagadda et al. 2015d, 2014). With this series of molecules, activity against vancomycin-resistant strains was found to increase with increase in the length of the hydrocarbon chain. One of the first compounds of this series, the octyl chain-bearing derivative, displayed a ~320-fold jump in activity against VRSA (MIC = 3 μM), as compared to vancomycin (Fig. 8.7). It also displayed activity against VISA and VRE. This derivative was potent in the in vivo setting too, leading to a ~3 log reduction in MRSA titre upon treatment of infected mice. The derivative displayed little intravenous toxicity, with an LD50 ~78 mg/kg. The tetradecyl derivative displayed the highest activity, with an MIC = 0.7 μM against VRE VanA phenotype (1000-fold enhancement in activity as compared to vancomycin) (Fig. 8.7). Preliminary investigations towards the mechanism of action illustrated the role of bacterial membrane depolarization and permeabilization in the enhanced activity of this derivative, even against vancomycin-resistant strains. Activity of this derivative was observed against intracellular MRSA also (Yarlagadda et al. 2016b). This molecule was also active against sturdy biofilms of MRSA, displaying >5 log reduction in biofilm cell count. In a recently published work from our group, the synthesis of cationic lipophile-vancomycin conjugates which possess an amide linker between charge and hydrophobicity was reported (Fig. 8.7) (Sarkar et al. 2020). The molecule displayed good activity against VISA (MIC = 0.1 μM), VRSA (MIC = 0.2 μM), as well as VRE VanA and VanB phenotypes (MIC = 2.2 μM and 4 μM, respectively). Intracellular activity was also observed for this molecule against MRSA.

With a view of achieving lower MIC values, due to higher binding to target peptide, two different approaches were coupled, where the lactobionic acid-vancomycin vancosamine analogues, discussed in the earlier section, were appended with a cationic lipid moiety (Fig. 8.7) (Yarlagadda et al. 2015d). This resulting cationic lipophile-vancomycin-lactobionic acid conjugate, possessing dual properties, displayed an 8000-fold increase in the activity against VRE (MIC = 0.09 μM), as compared to vancomycin. This derivative also displayed *in vivo* activity against VRE in a murine renal infection model. It displayed ~6 log reduction in the bacterial count upon treatment with 12 mg/kg of the derivative. Further, negligible toxicity was displayed as the LD_{50} was found to be more than 100 mg/kg in mice. It also

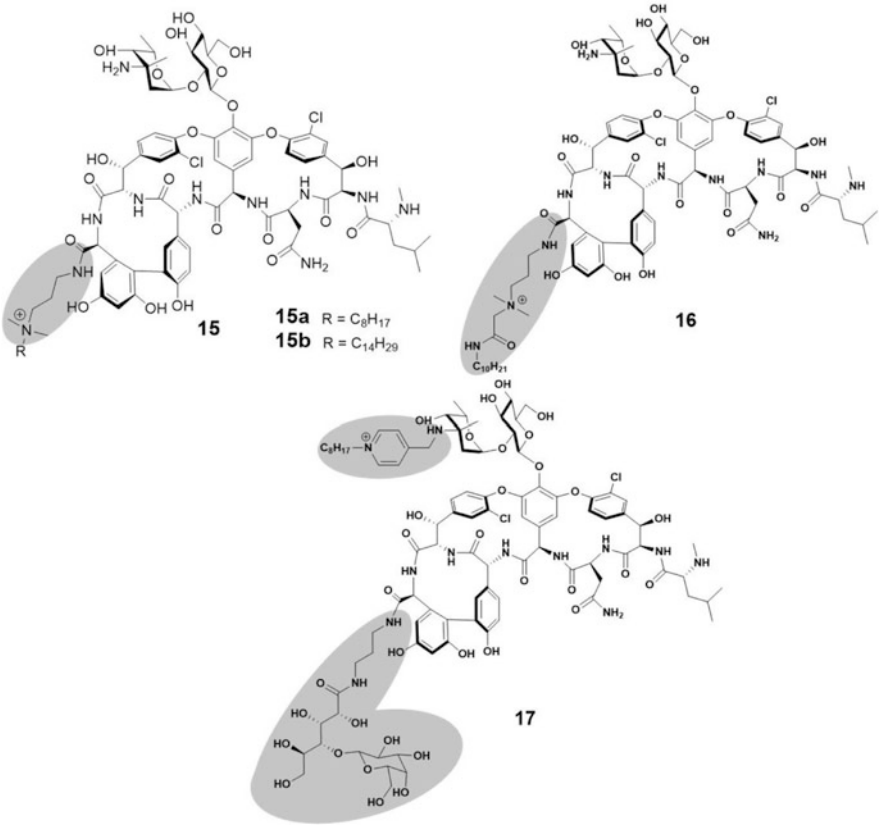

Fig. 8.7 Structures of cationic-lipophilic vancomycin derivatives

possessed anti-biofilm properties. All the derivatives have demonstrated higher accumulation of cell-wall precursor, indicating the inhibition of cell-wall synthesis. Similarly, all of these membrane active derivatives of vancomycin displayed higher activity against vancomycin-resistant bacterial strains, even in an intracellular setting. The propensity of resistance development against these derivatives was found to be negligible too, as no substantial increase in MIC values was observed after multiple serial passages. A recent work by Boger group involved the attachment of the CBP group to the previously discussed amidated core-modified synthetic vancomycin analogue, [Ψ[CH_2NH]Tpg4], to the disaccharide, along with a membrane-interacting component at the C-terminal (Okano et al. 2017). This double modified analogue, capable of inhibiting transglycosylation (through CBP) and displaying membrane-disruption (through cationic lipophilic moiety), displayed a whopping >10,000-fold increase in activity against the VRE VanA phenotype, as compared to vancomycin.

8.3.2.3 Peptide-Vancomycin Conjugates

Vancomycin resistance can be overcome through conjugation of short peptides to the glycopeptide, since these peptides often contain cationic side chains (lysine, arginine, etc.), which offer enhanced interaction with the negatively charged cell membrane of bacteria. Further, attaching a hydrophobic membrane-disrupting moiety also leads to better activity. This strategy has been used by various groups. One of the earliest reports was from the Cegelski group, where cell-associating molecular transporter peptides (octa-^{D}Arginine moiety) were attached to the carboxylic acid terminal of vancomycin (Fig. 8.8) (Antonoplis et al. 2018). This peptide-vancomycin conjugate displayed dual action and, due to the presence of the peptide, facilitated enhanced access to and association with bacterial cell envelope constituents. This conjugate displayed good efficacy against MRSA, in both planktonic and biofilm forms. Most importantly, it was found to possess activity against VRE, with a >100-fold improvement in activity, as compared to vancomycin. In a study by Jelinkova et al., vancomycin was conjugated to a natural antibacterial/cell-penetrating peptide, Hecate, through the carboxylic acid terminal (Jelinkova et al. 2018). This is a 23-amino acid long peptide, with lysine as a major component. The conjugation of vancomycin and peptide aided in tackling the drawbacks of either components. The presence of Hecate helped with better penetration of vancomycin, while attachment to vancomycin led to enhanced selectivity of the peptide for bacteria, thereby reducing toxicity. The conjugate displayed an MIC of 2.5 μM against MRSA and 5 μM against VRSA. From the individual components, both vancomycin and the peptide had an MIC >80 μM against VRSA. Similarly, no haemolysis was observed for the conjugate at higher concentrations, while only the peptide displayed ~15% haemolysis at sub-MIC concentration. These results indicate the synergy of this vancomycin-peptide conjugate. Recently, Blaskovich et al. have developed a vancomycin-peptide-lipophile conjugate, vancapticin, which possesses membrane disruptive properties (Blaskovich et al. 2018).

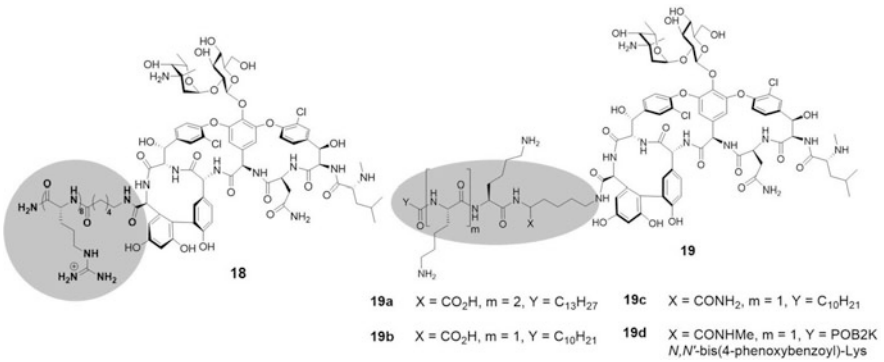

Fig. 8.8 Structures of peptide-vancomycin conjugates

In vancapticins, vancomycin is linked to a membrane-interactive moiety, composed of an electrostatic effector peptide sequence (EEPS) and a lipophilic membrane-insertive element (MIE) (Fig. 8.8). The EEPS is composed of an ε-lysine linker, followed by an oli+go-lysine unit. It terminates in the MIE, which possessed long alkyl chains, or bis-phenoxybenzyl lysine (POBK2). These vancapticins resulted in membrane depolarization and displayed good activity against vancomycin-resistant bacteria. The MIC for the POBK2-derivative was 0.125 μg/mL, 0.08 μg/mL and 0.05 μg/mL against VISA, VRSA, and VRE VanA strain, respectively. One of the alkyl derivatives was tested for its in vivo potential against MRSA. Treatment with the conjugate lead to ~6 log reduction in MRSA count in a murine thigh infection model.

8.3.3 *Alternative Strategies of Modification of Vancomycin to Overcome Resistance*

Peptidoglycan synthesis
involves the transport of the precursor disaccharide, *N*- acetylglucosamine-*N*-acetylmuramic acid pentapeptide (NAG-NAM-pp), which is synthesized in the cytosol, to the outer side of the bacterial membrane. This transport is undertaken by a membrane lipid, bactoprenol (C_{55}-lipid) (peptidoglycan precursor transporter). This membrane spanning lipid also facilitates the synthesis of the cell-wall precursor and its translocation from the inner side of membrane to the outer side. An interesting strategy to overcome vancomycin resistance, thus, would be to inhibit this precursor recruitment, by incorporating a moiety which can bind to this component. Our group has worked in this direction, and we have developed a novel vancomycin analogue, dipicolyl-1,6-hexadiamine conjugated vancomycin derivative (Dipi-van). This analogue, upon binding with a Zn2+ ion, targets the phosphate group of the bactoprenol (C_{55}-lipid), which is responsible for ferrying the peptidoglycan precursor to the site of peptidoglycan synthesis, from the interior of the cell (Fig. 8.9) (Yarlagadda et al. 2016c). Here, the zinc (II) cation-binding dipicolyl moiety chelates to the abundant Zn^{2+} ions present in the vicinity of infected tissue.

This Dipi-van-Zn^{2+} then further complexes with the pyrophosphate groups of the peptidoglycan precursor transporter. This will also lead to inhibition of cell wall biosynthesis, in addition to some effect of vancomycin. This inhibition has been confirmed through *in vitro* studies of intracellular accumulation of UDP-*N*-acetylmuramyl-pentadepsipeptide (UDP-NAG-pp). This new inhibition of precursor transport, along with the conventional vancomycin binding to the target peptide, has led to a high activity against vancomycin-resistant *E. faecium* (VanA type), with a 275-fold increase as compared to vancomycin. Further, no propensity for resistance development in MRSA has been observed, as the MIC remained constant after multiple serial passages. The Dipi-van analogue works against VRE in the in vivo setting too, where bacterial burden has been reduced by ~5 log upon treatment with

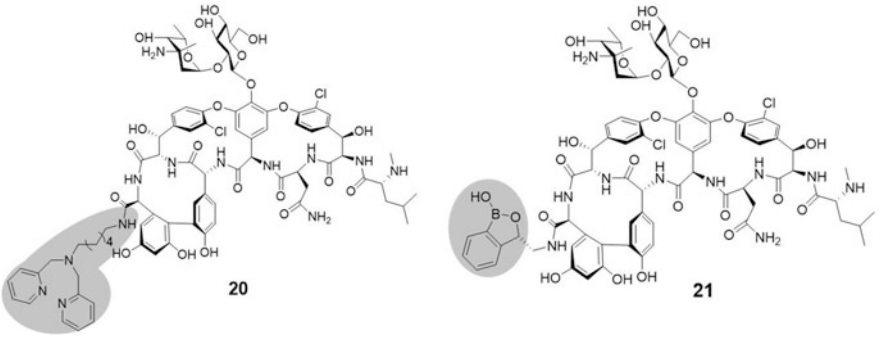

Fig. 8.9 Structures of vancomycin analogues with alternative strategies to overcome resistance

12 mg/kg of analogue, in a murine model of VRE kidney infection. The analogue did not show any toxicity at the active concentration, as the LD_{50} value is more than 100 mg/kg. Another unique strategy was used by the Zhang group, where they introduced moieties which show interaction with saccharides present on the bacterial envelope. As the target site of vancomycin is present near the cell membrane, moieties which can adhere to other components of the cell envelope would be able to stabilise the drug, aiding in the restoration of its activity against vancomycin-resistant pathogens. In this strategy, the benzoxaborole group was appended to the *C*- or *N*-terminal of vancomycin and various other glycopeptides (Fig. 8.9) (Printsevskaya et al. 2013). It is proposed that this group interacts with the 1,2- and 1,3-diols of the cell-surface saccharides. This additional interaction can lead to enhancement in the activity of the drug, which is reflected in the activity of these molecules against VISA, where they have an MIC = 1–2 μM, which is considerably less than that of vancomycin.

8.3.4 Modifications Involving Conjugation of Vancomycin of Other Antibiotics

Antibiotic conjugates, involving different antibiotics which are covalently linked to each other, offer an alternative approach to conquering antibiotic resistance. These conjugates also aid in delaying resistance to individual antibiotics, owing to the multimodal mechanism of action. This strategy has been tested with vancomycin and two other classes of antibiotics. In one study, the antimicrobial peptide nisin was conjugated to the *N*- or *C*-terminus of vancomycin through its C-terminus (Arnusch et al. 2008). Nisin binds to pyrophosphate of lipid II, thereby inhibiting the translocation of peptidoglycan precursors to the site of cell wall synthesis. It was hypothesized that simultaneous binding of both the antibiotics to lipid II can lead to enhanced binding of vancomycin through the chelate effect. Positively, such an effect was observed, where one of the synthesized conjugates displayed enhanced

activity against VRE, with an MIC = 2.3 μM (40-fold increase as compared to vancomycin).

Similar to this example, it was conjectured that a conjugate of vancomycin and a β-lactam antibiotic will be extremely potent, given the vicinity of the target and site of action of both the classes. Accordingly, a vancomycin-cephalosporin conjugate, called Cefilavancin was developed. It has shown successful results in the treatment of complicated skin and skin-structure infections (Stryjewski et al. 2012). This candidate was recruiting for phase III trials as of 2016; however, no updates are available. Along with cefilavancin, TD-1607, another such conjugate developed by Theravance Biopharma Inc., is also undergoing clinical trials (Long et al. 2008). While both of these molecules display enhanced activity against vancomycin-sensitive bacteria, unfortunately, they are completely inactive against vancomycin-resistant strains. However, this strategy is still being tested, and there is scope for the development of conjugates of vancomycin with various other classes of antibiotics.

8.4 Future Prospects

Vancomycin has been one of the most successful antibiotics for the treatment of Gram-positive bacterial infections, right from its discovery. Further, due to its different mechanism of action, whereby it targets a metabolite, resistance development has been relatively slow. However, the ever sturdy and resilient bacteria have caught up and evolved to possess various operons which confer vancomycin-resistance. Due to extensive use of vancomycin, post its emergence, vancomycin resistance too has spread rapidly through the clinic, and to multiple bacterial species. We are, therefore, to this date, plagued by dangerous Gram-positive pathogens such as VRSA and VRE. This has prompted scientists from different fields of medicinal chemistry to look for solutions and alternatives to overcome vancomycin resistance. One of the major directions of this research has been towards the development of highly active semi-synthetic derivatives of vancomycin. We have discussed here the multidirectional strategies employed by researchers to develop novel synthetic and semi-synthetic glycopeptide analogues to overcome vancomycin resistance. Vancomycin and many glycopeptides, owing to their peptidic nature, display high binding to proteins present in the blood plasma (Chen et al. 1992). The upcoming derivatives also display adverse effects due to the modifications. While promising in vitro activities are reported, very little or no data has been created with respect to their in vivo efficacy and toxicity. Finally, a recurring problem with vancomycin derivatives is the issues faced in their synthesis and purification. Synthetic analogues of vancomycin are extremely challenging to obtain, given the large number of synthetic steps involved. Similarly, purification of the analogues of choice also is a complicated affair. More importantly, while the target analogues can be obtained on a small scale in the laboratory, large scale synthesis often faces multiple issues. Because of these issues, these derivatives face multiple challenges, irrespective of their activity profile. While significant attention has been accorded to overcoming vancomycin

resistance, to truly update our arsenal would mean that we need to start acknowledging other contemporary and pressing issues related to complex infections and look for solutions to them in the domain of vancomycin and vancomycin analogues too. Future designs of vancomycin derivatives need to factor in various issues, apart from synthesis and purification issues. Today, infections are increasingly becoming difficult to treat due to their association with bacterial biofilms and dormant bacterial populations such as stationary phase and persister cells. Vancomycin is ineffective against both. Similarly, vancomycin cannot be used to treat intracellular infections, due to its poor permeability. Thus, future designs of vancomycin analogues should aim to address a few, if not all, of these problems. Already, we have derivatives which are displaying antibiofilm and anti-stationary phase cell properties. Similarly, the development of more potent vancomycin analogues with activity against Gram-negative bacteria would truly result in expanding the horizons for vancomycin.

Hitherto, vancomycin was and still continues to be used for treating Gram-positive bacterial infections. With the emergence of vancomycin resistance and approval of other glycopeptides such as dalbavancin, oritavancin, and telavancin, there is speculation that vancomycin will slowly be phased out of the clinic. However, recent reports hint towards the broad-spectrum applicability of vancomycin analogues, apart from its conventional use for treatment of bacterial infections (Yarlagadda et al. 2016a). The ongoing COVID-19 pandemic has reiterated the significance of repurposing, and vancomycin and its derivatives may well be one of the lucky candidates which can make their mark as antimicrobial agents for other pathogenic microorganisms and indications. Hence, we believe that this blockbuster antibiotic, vancomycin, possesses a lot of potential is yet to be explored.

8.5 Conclusions

Vancomycin, the blockbuster antibiotic of the twentieth century, has emerged and evolved as a formidable antibiotic for the treatment of infections caused by the dangerous MRSA, among other Gram-positive superbugs. However, increasing use, misuse, and overuse of glycopeptide antibiotics, particularly in the bovine setting, has led to the development of resistant bacteria, against which no effect of vancomycin is observed. These lethal pathogens, such as VRE, VISA, and VRSA, have started appearing in the last couple of decades in clinics and hospitals all over the world. This sparked a riot in the medicine community, as very few options are available for treatment of infections caused by vancomycin-resistant bacteria. To address this challenge, medicinal chemists have explored various strategies in the chemical sphere. One of the prime successful strategies involves undertaking semi-synthetic modifications of vancomycin, to develop new derivatives, as well as synthetic analogues which display enhanced or restored activity against vancomycin-resistant bacteria. Through this chapter, we have tried to summarize the multitude of chemical approaches which have been reported over the past two decades, leading to the development of vancomycin analogues with potent activity

against different species and strains of vancomycin-resistant bacteria. Different research groups from around the world, including our group, have been consistently contributing to this field of semi-synthesis and development of vancomycin derivatives. Today, we have a large number of highly active vancomycin derivatives, which have been synthesized through a multitude of chemical modifications. However, compared to the number of preclinical candidates, a miniscule number of semi-synthetic vancomycin derivatives, and synthetic analogues in particular, are present currently in the clinical pipeline. This slow progress can be attributed to a few major issues that are encountered in the development of effective drugs, such as challenging synthetic chemistry, extensive purification, complexity in large-scale synthesis, unwanted side effects, such as sepsis, etc. Upcoming research needs to address most of these challenges to effectively contribute to the antibacterial pipeline. In this direction, the field of semi-synthesis has a lot to offer. This strategy, which weds the knowledge of organic synthesis, with the understanding of microbiology, physiology, and pharmacokinetics/ pharmacodynamics, is a promising one, and will successfully contribute to the antibiotic pipeline in the coming days!

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Conflict of Interest The authors declare no competing conflict of interest.

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Chapter 9
Heterocyclic Scaffolds in Novel Synthetic Antibacterial Agents

Bipransh Kumar Tiwary and Kiran Pradhan

Abstract The emergence of antibiotic resistant bacteria is becoming a serious global problem. The situation will look very grim in the near future if proper strategies for tackling this menace are not put in place. The use of synthetic drugs for treating microbial diseases decreased after the discovery and development of antibiotics. A paradigm shift in therapeutics for treating bacterial diseases took place with the discovery of penicillin and subsequent advances in the development of other antibiotics. Of late, the emergence of antibiotic resistant variants of the earlier sensitive bacteria and the emergence vis-a-vis development of new infectious diseases have led to a reduction in the effectiveness of antibiotics against bacterial infection. Various strategies have been and are currently investigated and developed to reduce and/or delay bacterial resistance. Nevertheless, there is a tremendous amount of pressure on the scientists and researchers working in the area of medicinal chemistry to deliver novel antimicrobials. At this juncture, it would be helpful if we revisit the classical approach to drug design. Since heterocyclic scaffolds are present abundantly in natural products, they are good targets for the design and synthesis of novel antimicrobial compounds due to their broad range of biological activities and plentiful applications in the extensive fields of pharmacy. Statistically, 75% of the top 200 branded drugs in the world contain heterocyclic moieties in their structures. They have already provided a platform for the rapid growth of research in the areas of organic, pharmaceutical, analytical, and medicinal chemistry. In this chapter, some elementary information about heterocyclic structures in accordance with their ring sizes and their biological and clinical applications has been discussed with the firm belief that the strategic application of modern chemical synthesis to antibacterial drug discovery might play a critical role in the search for novel alternatives to antibiotics.

B. K. Tiwary
Department of Microbiology, North Bengal St. Xavier's College, Rajganj, Jalpaiguri, West Bengal, India

K. Pradhan (✉)
Department of Chemistry, University of North Bengal, Darjeeling, Rajganj, West Bengal, India
e-mail: kpradhan@nbu.ac.in

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Abbreviations

EDG	Electron-donating group
EWG	Electron-withdrawing group
MRSA	Methicillin-resistant *Staphylococcus aureus*
SARS	Structure-activity relationship study

9.1 Introduction

Antibacterial therapy, as of today, has become a challenging field in the world of medicine because of the emergence of multidrug-resistant bacteria (Yoneyama and Katsumata 2006; Molton et al. 2013). The emergence of such pathogens, many of which are Gram-negative bacteria, is largely due to overconsumption and misuse of antibiotics and exposure to infections in hospitals (Breijyeh et al. 2020). These pathogenic microorganisms have over the years developed mechanisms to resist the actions of most of the first- and second-generation antibiotics. Since the search and development of antibiotics for the new drug resistant bacteria is time-consuming, mankind's arsenal against these microbial pathogens needs to be revamped now and then. If mankind is to sustain and win this war against these microbes, newer strategies for antibacterial therapy need to be adopted. Earlier approaches to antibacterial therapy required the discovery of a compound that killed the organism. The exact molecular target of the drug compound was not investigated. Limitations of this approach have become apparent with the emergence of multidrug-resistant strains that are immune to the best antibiotics. The most recent World Economic Forum Global Risks reports have listed antibiotic resistance as one of the greatest threats to human health. To overcome it, a thorough understanding of antibiotic resistance as well as new antibacterial targets is required. This is true for many of today's drugs which effectively combat the symptoms of disease, rather than rectify the causes, leading to the development of the condition. The world will undoubtedly see an alarming rise in the rate of infections in the years to come unless the scientific community steadfastly continues with some significant research to design and synthesize novel compounds possessing a broad spectrum of antibacterial activities.

Over the years, various strategies have been developed to counter these microbial infections. While ancient literature reveals the use of some metals and metal oxides to combat infections, compounds like azo dyes were put to use before the advent of chemotherapeutic treatment. The discovery of the potency of organic compounds as antimicrobial agents paved the way for chemotherapy. This strategy was subsequently replaced by the advent of antibiotics, which had very effective antibacterial activities. Alternative strategies also included the use of natural products which had phytochemicals present in them that exhibited potent activities. These agents are nonantibiotic drugs and can act alone or in combination with antibiotics to enhance the antibacterial activity against a wide range of bacteria (Khameneh et al. 2019). Another effective strategy involves the use of a combination therapy, where two or more drugs are used to increase the efficacy of both drugs against bacterial pathogens that are resistant to ordinary antibiotics. Yet another new and emerging strategy in the field of antimicrobial chemotherapy involves the use of nanoparticle-based materials which has emerged as an alternative to control multidrug-resistant bacterial infections (Beyth et al. 2015). The growing interest in nanomaterials is largely due to their promising antibacterial activity in combating multidrug-resistant mutants and biofilm. Apart from multidrug-resistant bacteria, biofilms produced by bacteria have become equally threatening. A study carried out in 2001 (Donlan 2001) put the estimates due to biofilm formation at 65% of all human bacterial infections. Hence these newer strategies like the use of nanoparticle-based materials might be able to fill the gaps where antibiotics frequently fail (Pelgrift and Friedman 2013; Zhang et al. 2010).

9.2 Natural to Synthetic

The greatest challenge for the present generation of researchers is to prevail over the impediment due to the emergence of microbial resistance to conventional antimicrobial agents. Chemistry research catering to the drug and pharmaceutical industry has in the past derived ideas from nature and tried to mimic nature's mechanisms. It has modulated a vast majority of chemical compounds occurring naturally that have been found to be effective in the treatment of many kinds of diseases (e.g., the Ayurvedic system of treatment). There are various drugs which are in use today to fight many infectious diseases whose predecessors have been ones that were derived directly from plants. Synthetically made drugs are more convenient to use, and their side effects and contraindications are known. Though, some structural modifications are usually seen in the synthesized drug, but that does not lead to a reduction of its biological activity. A very familiar example would be that of the natural drug for malaria, quinine, and its synthetic counterpart, chloroquine. The scientific community has also derived drugs that have been used by other organisms as a defense mechanism against pathogens (the discovery of antibiotics). Since then, microbial natural products have been the most important source of antibiotic lead compounds. And over the last four decades, natural product-derived antibacterials have been a

major part of all new chemical entities. But of late, the antibiotic resistance of pathogens whose infections are difficult to treat with conventional antibiotics has driven the scientific community involved in medicinal chemistry research to move from natural to synthetic.

9.3 Heterocyclic Scaffolds as Antimicrobial Agents

The antimicrobial activity shown by various drugs is usually due to the presence of a structural feature in the molecular structure of the particular compound which acts as a drug. These structural features called "pharmacophores" are usually organic moieties. The organic moieties may be a part of a carbocyclic system or a heterocyclic system.

The first antimicrobial agent in the history of mankind was a synthetic compound, an organoarsenic compound, Salvarsan (synthesized by Ehrlich in 1910), which was an antibacterial agent used for the treatment of syphilis. Two decades later, it was replaced by another synthetic organic compound, sulfanilamide (previously used as Prontosil, an azo dye), as an antibacterial agent with lesser side effects. Sulfonamides are bacteriostatic (they do not kill bacteria but can stop them from reproducing). These discoveries were significant milestones in chemotherapy. Sulfa drugs were widely used as antibacterial agents, till the development of penicillins as antibiotic agents. Both, Salvarsan and sulfanilamides are non-heterocyclic systems. The significance of a heterocyclic moiety in the drug candidates of antimicrobial agents is evident from the structure of the first antibiotic, penicillin, which contains a β-lactam ring and a thiazolidine heterocyclic moiety. The large-scale fermentative production of penicillin and the synthetic modification of the molecule led to the emergence of semi-synthetic penicillins as antibiotic agents. It also opened up an a priori unexplored area of research: the extraction of substances from microorganisms which were able to inhibit the growth of another microbe. The extraction of streptomycin and chlortetracycline from soil bacteria was thus subsequently achieved and effectively put into use.

Nevertheless, various antimicrobial compounds containing non-heterocyclic moieties like chloramphenicol, tigecycline, chlorhexidine, and tetracycline, to name a few, have been in prolonged use over the years as very effective antimicrobial agents. However, over the last few decades, heterocycles have been found to play a much bigger role in the modern repertoire of medicinal chemists. According to statistics, more than 85% of all biologically active chemical entities contain heterocycles (Jampilek 2019). Very recently, a database of all U.S. FDA-approved pharmaceuticals was compiled, and the analysis of that has revealed that 59% of unique small-molecule drugs contain nitrogen heterocycles (Vitaku et al. 2014). Because heterocycles are the core elements of a wide range of natural products such as nucleic acids, amino acids, carbohydrates, vitamins, and alkaloids, medicinal chemistry efforts often evolve around simulating such structural motifs. Moreover, drug properties like potency, selectivity, lipophilicity, polarity, hydrogen-bonding

capacity, and solubility can be easily modulated by modifying the heterocyclic moiety in the molecule, resulting in the optimization of the ADME/Tox properties of drug candidates.

There have been remarkable advances in research by medicinal chemists in the search for synthetic analogues, and a plethora of organic moieties have been constructed and tested for antimicrobial activities to increase the arsenal of medicinal chemists in the fight against microbial infections. This is a short review of a few synthetic compounds with heterocyclic scaffolds which have been found to show potent antibacterial activities.

9.3.1 The Azirine and Aziridine Scaffolds

These three-membered nitrogen-containing heterocycles have been found to possess many biological activities especially antitumor and antibacterial ones, due to the presence of the aziridine ring. The natural azirine-2-carboxylic acids, containing the heterocyclic azirine scaffold, isolated from bacteria and marine sponges were the first azirine compounds known to display broad-spectrum antibacterial activity against Gram-positive and Gram-negative bacteria. Aziridines are powerful alkylating agents, and their in vivo potency is based primarily on toxicity rather than specific activity. Several important natural products, such as mitomycin C, are well known in the literature as biologically active agents (Wakaki et al. 1958; Zang and Gates 2000; He et al. 2018). Over 130 biologically active aziridine-containing compounds demonstrate confirmed pharmacological activity including antitumor, antimicrobial, and antibacterial effects (Ismail et al. 2009). Mitomycin C and its derivatives (Fig. 9.1) are the best-known drugs of all the aziridinyl quinines. From the structure-activity relationship studies of about seventy mitomycin derivatives, it was concluded that the aziridine, quinone, and methylurethan groups apparently were required for full mitomycin antibacterial activity (Miyamura et al. 1967).

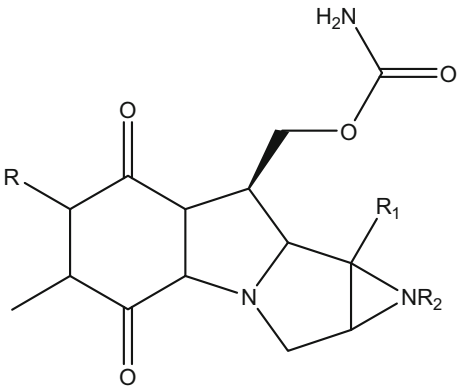


Fig. 9.1 Structure of mitomycin derivatives. (Miyamura et al. 1967)

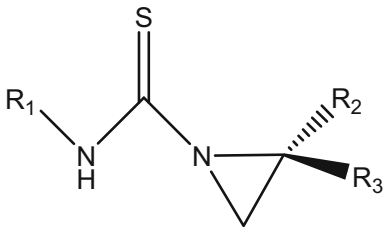


Fig. 9.2 Aziridine-thiourea derivatives. (Kowalczyk et al. 2018)

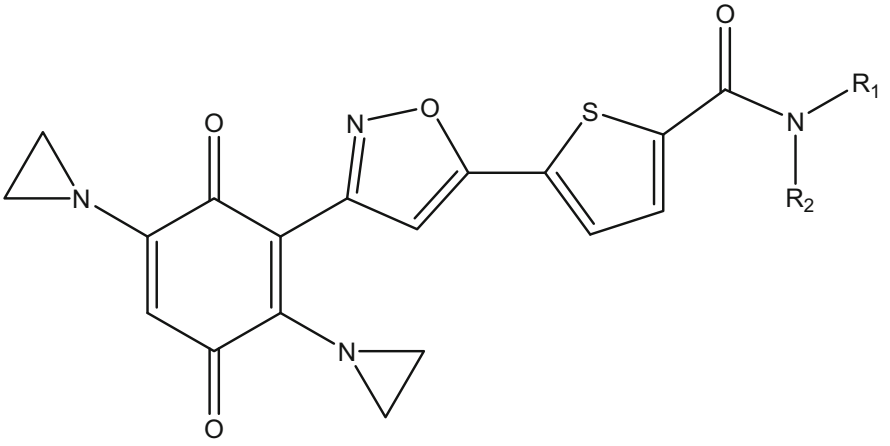


Fig. 9.3 Diaziridinyl benzoquinone isoxazole derivatives. (Swapnaja et al. 2016)

The effectiveness of synthesized aziridine derivatives has also been verified on a broad spectrum of bacteria. A recent publication by Kowalczyk disclosed promising antibacterial activity by a series of novel synthetic aziridine-thiourea derivatives (Fig. 9.2) against a representative panel of bacteria, i.e., *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *S. epidermidis*, *Enterococcus faecalis*, *Proteus vulgaris*, and *Proteus mirabilis*, using nitrofurantoin, ampicillin, and streptomycin as reference drugs (Kowalczyk et al. 2018).

Previously, Swapnaja et al. had also reported a series of novel diaziridinyl quinone isoxazole hybrids (Fig. 9.3) to be promising agents against reference strains of *E. coli*, *S. aureus*, and *S. epidermidis*. The enhanced antibacterial activity of molecular hybrids and conjugates arises from dual targeting, since antimicrobial hybrids are molecules containing two discrete functional elements, both having antibacterial activity, linked through a spacer (Rossi and Ciofalo 2020). Six of the tested compounds appeared to show good antibacterial activity against different Gram-positive and Gram-negative bacterial strains (Swapnaja et al. 2016). The synthesized compounds were also found to exhibit promising anti-biofilm activity and antifungal activity.

9.3.2 *The Azetidin-2-One (β-Lactam) Scaffold*

The azetidine-2-one ring system is the central core of many clinically relevant antibiotics, such as penicillins, cephalosporins, cephamycin, carbapenems, etc., the historical and biological significance of which have already been highlighted (Fig. 9.4). 2-Azetidinone skeleton is well established as the pharmacophore of β-lactam antibiotics, the most widely employed class of antibiotics.

Synthesis and investigation of antimicrobial activities of numerous compounds containing the azetidine-2-one moiety have since been a widely researched area. Sharma et al. reported moderate to good antibacterial, antifungal, and antitubercular activity of a series of N-[2-(10*H*-phenothiazinyl)ethyl]-4-(phenyl)-3-chloro-2-oxo-1-iminoazetidine compounds (Fig. 9.5). It was also revealed that the activity of the synthesized compounds depended on the electron withdrawing nature of the substituent, R on the phenyl ring. The sequence of the activity was as follows: $NO_2 > Cl > Br > OH > OCH_3 > CH_3$ (Sharma et al. 2011).

Recently, a series of novel phenyl substituted azetidine-2-one sulphonyl derivatives (Fig. 9.6) were found to show significant antibacterial activity against the entire set of tested bacterial strains as compared to ampicillin (Mandal et al. 2020). The structure-activity relationship study (SARS) suggested that the inhibitory activity was greatly influenced by the presence of electron-withdrawing groups (EWGs) on the phenyl group, while the presence of their donating counterparts (EDGs) showed

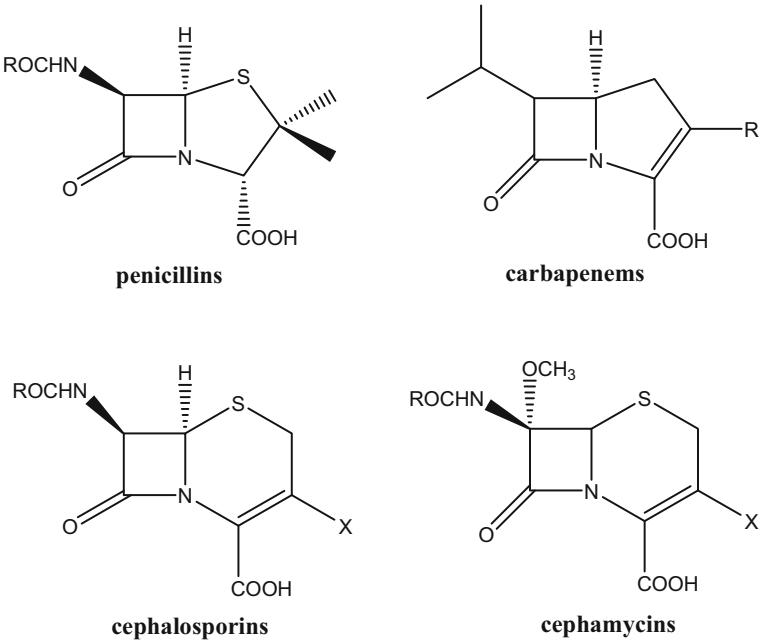


Fig. 9.4 Some clinically relevant antibiotics containing the azetidine-2-one scaffold

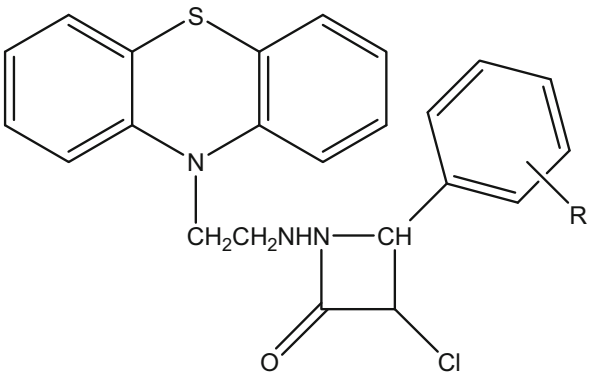


Fig. 9.5 2-oxo-azetidine derivatives of phenothiazine. (Sharma et al. 2011)

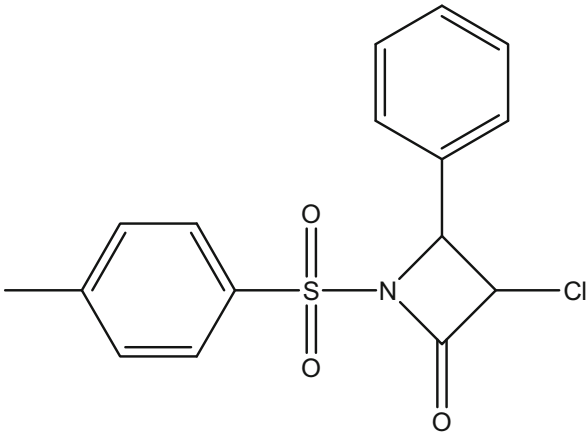


Fig. 9.6 4-(R-phenyl)-3-chloro-1-[(4-methylphenyl) sulfonyl] azetidin-2-one derivatives. (Mandal et al. 2020)

mild to moderate activity. Moreover, the absence of phenyl substitution on azetidine-2-one arm was detrimental to its antibacterial activity.

Very recently, a series of β-lactam-anthraquinone hybrids were synthesized and tested as potential antibacterial and antifungal agents. Among them, the thio-methyl substituent at C-3 position and the 3,4,5-trimethoxy phenyl group at the C-4 position of the β -lactam ring scaffold were found to exhibit the most potent antibacterial activity (MIC = 0.25 g/mL) against the *S. aureus* bacterial strain, compared with the standard ciprofloxacin (MIC = 0.5 g/mL) (Fig. 9.7). The same compound displayed equal antifungal activity (MIC = 4 g/mL) as the reference ciclopiroxolamine (MIC = 4 g/mL) against the *Candida albicans* strain (Mohamadzadeh et al. 2020).

Similarly, another series of β-Lactams and their derivatives had been synthesized (Kuskovsky et al. 2019) and evaluated for antimicrobial activity against *Mycobacterium tuberculosis*, M.tb, and *Moraxella catarrhalis*, M.cat. Among them, the derivative with meta-CF_3 of the phenylthiol ring and the achiral carbamyl group at the lactam nitrogen (Fig. 9.8) showed potent activity against M.tb (MIC = 25 g/mL) and M.cat (MIC = 1.5 g/mL).

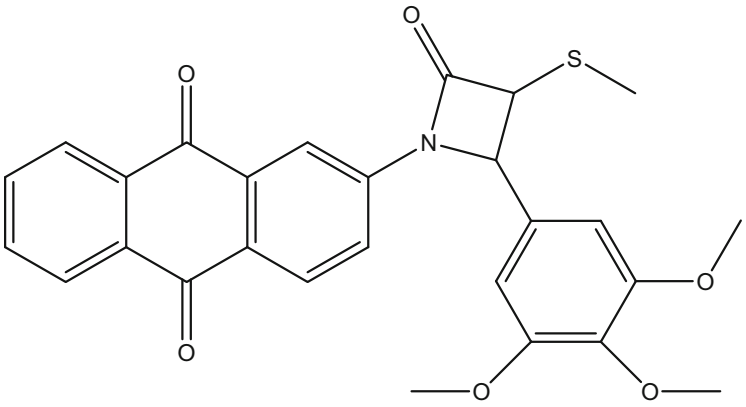
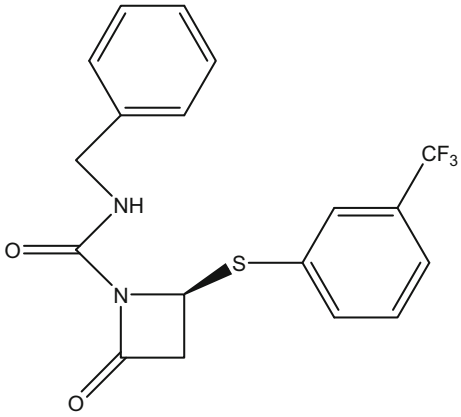


Fig. 9.7 2-(3-(Methylthio)-2-oxo-4-(3,4,5-trimethoxyphenyl)azetidin-1-yl)anthracene-9,10-dione. (Mohamadzadeh et al. 2020)

Fig. 9.8 The most potent C4-phenylthiolactam. (Kuskovsky et al. 2019)



9.3.3 The Furan/Pyrrole/Thiophene Scaffolds

Despite being found to exhibit a wide range of biological properties that could provide an increasing number of therapeutic applications, the three heterocyclic scaffolds still do not feature in the top five most commonly used five-membered heterocycles in FDA-approved drugs. The top five five-membered aromatic heterocycles appear in a total of 101 drugs, which is 9% of the total number of unique U.S. FDA-approved small molecules. All the five-membered heterocycles have additional nitrogen (imidazole, tetrazole, and benzimidazole) or sulfur (thiazole) atoms except indole, which contains a single heteroatom (Vitaku et al. 2014).

Nevertheless, recently, a very potent DNA gyrase B inhibitor bearing the furan heterocyclic moiety (Fig. 9.9) was reported. The compound is a furan-2-carboxamide derivative. DNA gyrase B is one of the most important enzymes

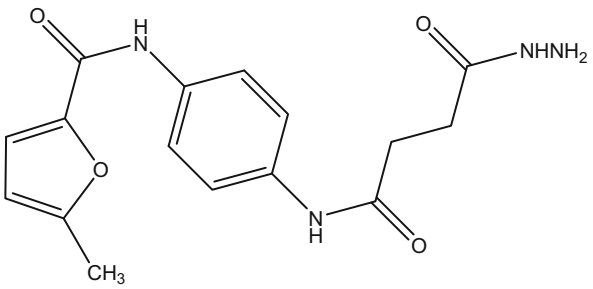


Fig. 9.9 The most potent furan-2-carboxamide derivative. (Janupally et al. 2015)

among the DNA topoisomerases which are well-validated targets in microorganisms (Janupally et al. 2015).

9.3.4 The Imidazole and Benzimidazole Scaffold

The unique structural features of the five-membered imidazole and benzimidazole scaffold distinguish them as important heterocycles, and these structures are part of many natural products and synthetic compounds. The electron-rich nature of the imidazole-based derivatives is useful to freely bind with various receptors and enzymes in the biological profile, thus showing broad biological activities. The presence of the imidazole scaffold lends unique bioactivity patterns to the drug molecules. Imidazole-based compounds have been found to show varied biological activities ranging from antioxidant, anti-inflammatory, analgesic, and neuroprotective to chemotherapeutic and anti-HIV. Imidazoles and in particular their salts are found to possess favorable properties such as excellent bioavailability, good tissue penetrability, and permeability, on the one hand, and a relatively low incidence of adverse and toxic effects, on the other. By far, the most important imidazole drug, metronidazole, an antimicrobial agent, had been initially used for the treatment of parasitic infections. But of late, the compound has also played an important role in the treatment of infections caused by a variety of anaerobic and microaerophilic bacteria.

Heerding et al. have revealed that appropriately functionalized 1,4-disubstituted imidazoles (Fig. 9.10) are effective inhibitors of FabI. The results showed that representative compounds had antibacterial activity against both Gram-positive and Gram-negative bacteria and provided further validation of FabI as an antibacterial target. The results of their study suggested that the antibacterial activity was primarily due to inhibition of FabI, and it further revealed that the presence of the 1,4-disubstituted imidazole ring was critical for FabI activity (Heerding et al. 2001).

Some pyrazole, isoxazole, pyrrolotriazine, imidazolothiadiazole, and imidazolopyrazole derivatives were synthesized and the compounds screened for activity against bacterial and fungal strains. Among them, the imidazolopyrazole and

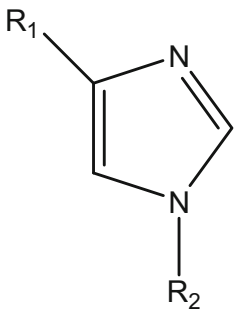
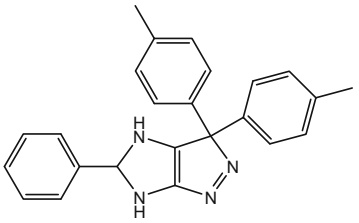
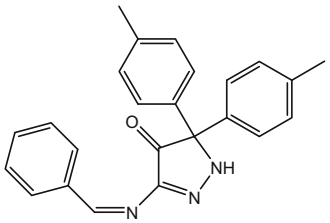


Fig. 9.10 1,4-Disubstituted imidazoles. (Heerding et al. 2001)



3,3-Di(4-methylphenyl)-5-phenyl-4,5-dihydroimidazo[4,5-c]pyrazole



3-(Benzylideneamino)-5,5-di(4-methylphenyl)-1H-pyrazol-4(5H)-one

Fig. 9.11 The most potent imidazolopyrazole and pyrazolone derivatives. (Azab et al. 2015)

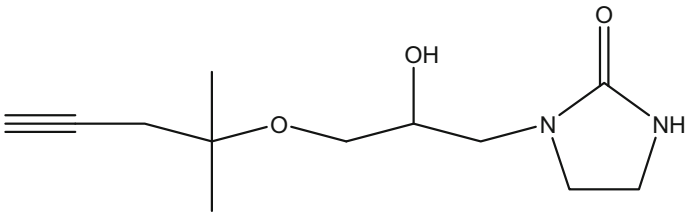


Fig. 9.12 1-[3-(1,1-Dimethylbut-3-ynyloxy)-2-hydroxypropyl]imidazolidine-2-one. (Movsumzade et al. 2018)

pyrazolone derivatives (Fig. 9.11) were found to have antimicrobial activity higher or almost equal to the reference compounds, viz., ampicillin, streptomycin, and nystatin (Azab et al. 2015).

Recent advances in the synthesis of heterocyclic antimicrobial agents include the synthesis of a 1,3-imidazolidin-2-one heterocycle (Fig. 9.12), which was found to exhibit very remarkable antimicrobial activity against *S. aureus*, *E. coli*, *P. aeruginosa*, and *Candida* fungi (Movsumzade et al. 2018).

Skocibusi et al. synthesized and evaluated five novel N-substituted imidazole 2-aldoximes and their six quaternary salts in the search for a new class of potential antimicrobial agents (Fig. 9.13). The antimicrobial activity was assessed against a panel of representative Gram-positive and Gram-negative bacteria, including multidrug-resistant bacteria. All compounds demonstrated potent in vitro activity

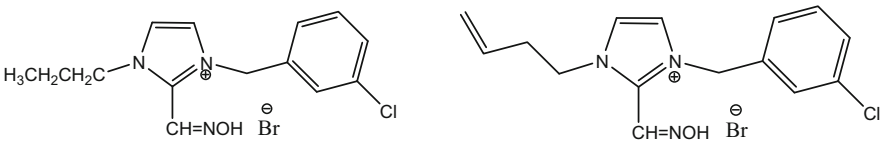


Fig. 9.13 The most potent imidazole-2-aldoximes. (Skocibusic et al. 2018)

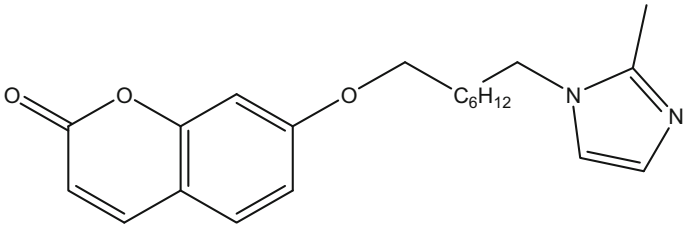


Fig. 9.14 The most potent coumarin-imidazole derivative. (Hu et al. 2018)

against the tested microorganisms, with MIC values ranging from 6.25 to 50.0 μg/mL. Among the tested compounds, two quaternary compounds (N-but-3-enyl- and meta- or para-N-chlorobenzyl imidazolium 2-aldoximes) displayed the most potent and broad-spectrum activity against both Gram-positive and Gram-negative bacterial strains (Skocibusic et al. 2018).

Coumarin derivatives containing the imidazole skeleton have also been found to be very promising antibacterial scaffolds acting through enoyl-acyl carrier protein reductases inhibition. The most potent compound (Fig. 9.14) showed broad range activity in all in vitro experiments for the inhibitory activity against FabI and FabK (Hu et al. 2018).

In the first example of its kind, Valls et al. (2020) have reported the synthesis of imidazole and imidazolium compounds derived from L-valine and L-phenylalanine containing different hydrophobic groups. According to the research group, the antibacterial activity of imidazole and imidazolium salts is highly dependent upon their lipophilicity, which can be tuned through the introduction of different hydrophobic substituents on the nitrogen atoms of the imidazole or imidazolium ring of the molecule. The compounds were tested for their antibacterial activity against two model bacterial strains, Gram-negative *E. coli* and Gram-positive *B. subtilis*. Overall, the results indicated that they could serve as potent antibacterial agents with low cytotoxicity to human cell lines.

9.3.5 The Triazole Scaffold

The 1,2,3-triazole scaffold is the main pharmacophore system among the nitrogen-based molecules and is a privileged building block in the discovery of various new

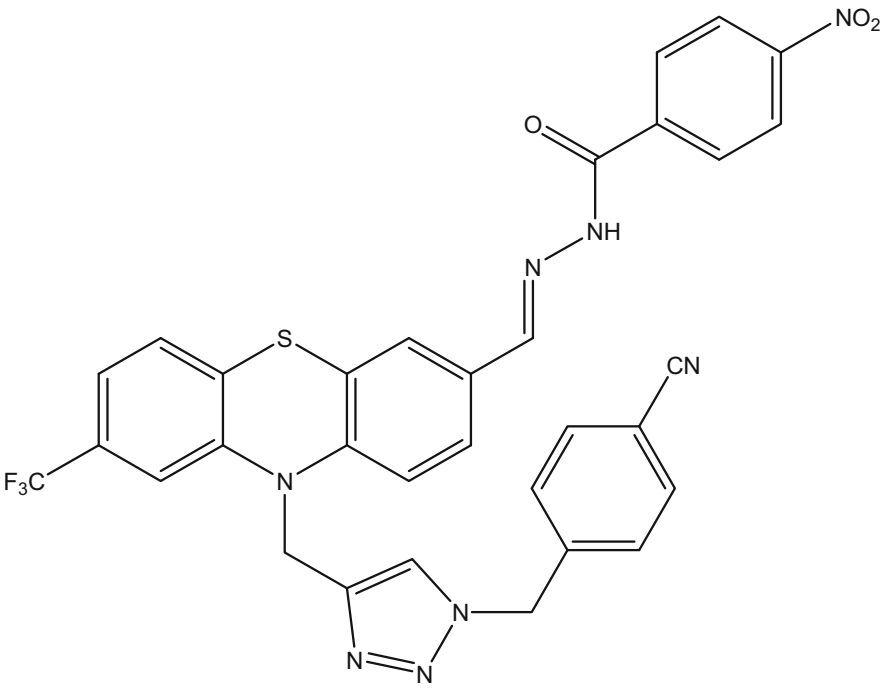


Fig. 9.15 The most potent phenothiazine-1,2,3-triazole conjugate. (Reddyrajula et al. 2019)

biological targets. These compounds can interact with different biological targets through hydrogen bonding, non-covalent and van der Waals interactions, as well as dipole-dipole bonding interactions. Furthermore, the strong dipole properties of the triazole unit make it a very important moiety in the field of medicinal chemistry, as it binds to the biological target with high affinity.

A phenothiazine conjugate with a 1,2,3-triazole linker and a few of its analogues were developed (Fig. 9.15) and the conjugates screened for antitubercular activity against *M. tuberculosis* H37Rv strain. All the compounds exhibited relatively good in vitro antitubercular activity as demonstrated by the compound bearing a 4-nitro group that showed significant antitubercular activity. The potent compound signified a novel hybrid for the development of potential antitubercular agents (Reddyrajula et al. 2019).

9.3.6 The Pyrazole Scaffold

Pyrazole is a well-known five-membered nitrogen-based heterocycle and exhibits a broad spectrum of synthetic and biological applications. A large number of heterocycles containing the pyrazole scaffold have been synthesized and found to exhibit

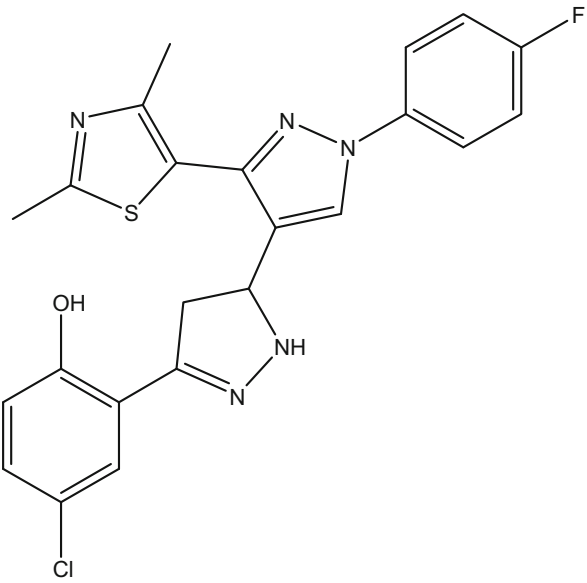


Fig. 9.16 Most potent hybrid of 2-(3′-(2,4-dimethylthiazol-5-yl)-1′-(4-fluorophenyl)-3,4-dihydro-1'H,2H-[3,4′-bipyrazol]-5-yl) phenols. (Takate et al. 2019)

antimicrobial activity. A pyrazole-linked thiazole hybrid substituted with a meta-chloro group on the phenyl ring was found to exhibit significant M. tuberculosis activity (Fig. 9.16). These hybrids are potential candidates for the progress of antitubercular agents (Takate et al. 2019).

9.3.7 The Quinoline Scaffold

Quinoline derivatives are among important compounds previously reported to have a wide array of biological activities (Navneetha et al. 2017). Therefore, the introduction of different functional groups onto the quinoline scaffold is a very good idea for the development of new drugs. The discovery of nalidixic acid in 1962, along with its introduction for clinical use in 1967, marks the beginning of decades of quinolone development and use. Since then, various fluoroquinolones have been synthesized and used to treat various bacterial infections for decades (Emmerson and Jones 2003). Oxolinic acid, pipemidic acid, and nalidixic acid belong to the first generation and were used for the treatment of urinary tract infection caused by the majority of Gram-negative bacteria; however, all of them have a short lifetime (Sharma et al. 2009a, b). Enoxacin, ofloxacin, lomefloxacin, norfloxacin, and ciprofloxacin belong to the second generation, have longer half-life, and improved activity against Gram-negative bacteria compared to the first generation (Sarkozy 2001). Sparfloxacin, grepafloxacin, and temafloxacin belong to the third generation (Riahifard et al. 2017) and are used as oral broad-spectrum antibacterial agents in the treatment of acute bacterial exacerbation of chronic bronchitis and mild-to-moderate pneumonia (Hong

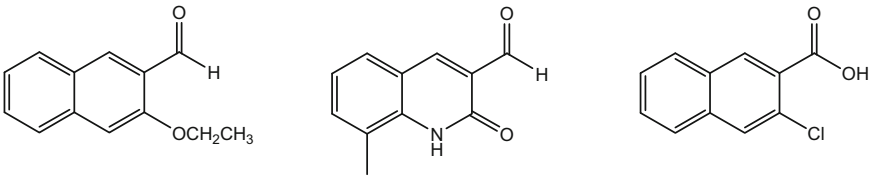


Fig. 9.17 Quinoline-3-carbaldehyde derivatives. (Zeleke et al. 2020)

2001). Gatifloxacin, clinafloxacin, trovafloxacin, and moxifloxacin belong to the fourth generation of fluoroquinolone drugs, which display extended activity against both Gram-negative and Gram -positive bacteria strains; additionally, they are active against anaerobes and atypical bacteria (Farghaly et al. 1990). Besides fluoroquinolone-based drugs, quinoline-3-carbaldehyde derivatives have also been synthesized and tested for their antibacterial and radical scavenging activities. Of all the synthesized compounds, 2-ethoxyquinoline-3-carbaldehyde and 8-methyl-2-oxoquinoline-3-carbaldehyde were found to show maximum activity against *P. aeruginosa,* while 2-chloroquinoline-3-carboxylic acid (Fig. 9.17) exhibited maximum activity against *E. coli* (Zeleke et al. 2020).

9.3.8 The Quinazoline Scaffold and the Quinazoline-4-(3H)-Ones

Quinazolines and quinazolinones are heterocyclic compounds that are of considerable interest because of the diverse range of their biological activities (Asif 2014). The quinazoline-4(3H)-one and its derivatives constitute an important class of fused heterocycles that are found in more than 200 naturally occurring alkaloids. This fused bicyclic compound was earlier known as benzo-1,3-diazine.

The stability of the quinazolinone nucleus has inspired medicinal chemists to introduce many bioactive moieties to this nucleus to synthesize new potential medicinal agents.

Most importantly, we find in the literature that 4(3H)-quinazolinones with 3-substitution has been associated with antimicrobial properties (Nagarajan and Kavimani 2010).

Various substituted phenyl ring moieties, bridged phenyl rings, heterocyclic rings, and aliphatic systems have been incorporated as substituent in the 3-substituted quinazolinones. It is also reported that hydrazine-derived Schiff's bases have potential antibacterial activity. In another study, 3-(arylideneamino)-2-phenylquinazoline-4(3H)-ones were synthesized by placing two potential bioactive sites, a quinazolone moiety as well as a Schiff's base in the system to increase biological activity. The compounds were found to inhibit the growth of both Gram-positive and Gram-negative bacteria. The authors have proposed the promising

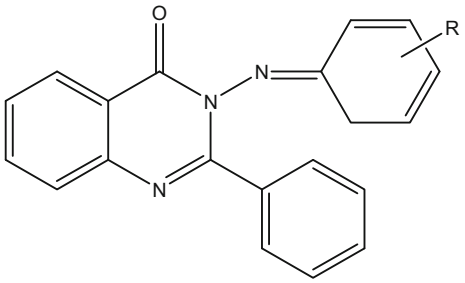


Fig. 9.18 3-(arylideneamino)-2-phenylquinazolin-4(3H)-ones. (Nanda et al. 2007)

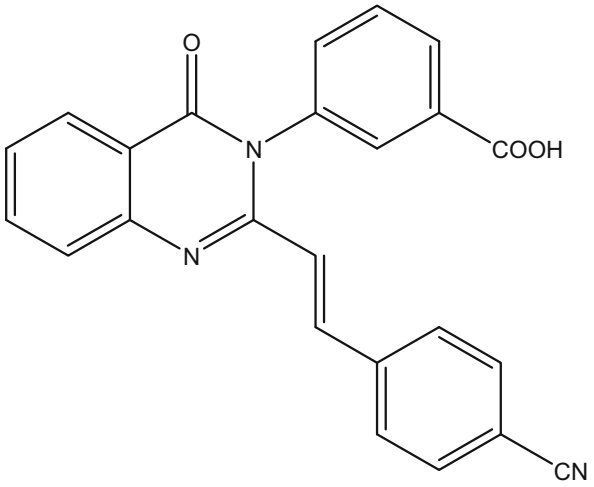


Fig. 9.19 (E)-3-(3-carboxyphenyl)-2-(4-cyanostyryl)quinazolin-4(3H)-one. (Bouley et al. 2015)

effect of such compounds against multiple-antibiotic-resistant Gram-negative enteric bacteria which could lead to the development of new drugs. In this study, authors have reported that 3-amino-2-aryl-4(3H)-quinazolinone (Fig. 9.18) were highly potent against the multiple-antibiotic-resistant bacteria (Nanda et al. 2007).

Recently, Bouley et al. (2015) have discovered E)-3- (3-carboxyphenyl)-2-(4-cyanostyryl)quinazolin-4(3*H*)-one (Fig. 9.19) as an antibiotic, effective in vivo against methicillin-resistant *S. aureus* (MRSA). They also found that this antibiotic damages cell-wall biosynthesis by binding penicillin-binding protein (PBP) 2a. They proposed this as a promising antibiotic in fighting MRSA infections (Bouley et al. 2015).

A new series of novel 2-methyl-3-(1,3,4-thiadiazol-2-yl)-4-(3*H*) quinazoline (Fig. 9.20) have been synthesized by reacting 2-amino-5-aryl/alkyl- 1,3,4-thiadiazoyl with 2-substituted benzoxazin-2-one. These compounds have been found to possess antibacterial activity against *S. aureus*, *Bacillus subtilis*, and *E. coli*. Antifungal activity was screened against *C. albicans*, *Aspergillus niger*, and *Curvularia lunata*. Interestingly, the synthesized compounds have shown both antibacterial and antifungal activity (Jatav et al. 2008).

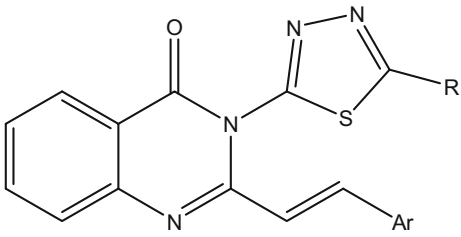


Fig. 9.20 3-[5-Substituted 1,3,4-thiadiazole-2-yl]-2-styryl quinazoline-4(3H)-ones. (Jatav et al. 2008)

Recently, Gatadi et al. reported the antibacterial activity of a series of new 3-phenylquinazolin-4(3H)-one derivatives against ESKAP (*E. coli*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeroginosa*) (Gatadi et al. 2018). Chang et al. designed and synthesized 3-(2-(4-ethynylstyryl)-4-oxoquinazolin-3(4H)-yl) benzoic acid and were found highly effective against gram positive bacteria *S. aureus* (MRSA) (Chang et al. 2019). Gatadi et al. summarized in a review that phenyl, 3-arylideneamino, 3-((2-oxoindolin-3-ylidene)amino, 3-(2-(benzylideneamino) ethyl groups at position N-3 are essential for antibacterial activity, whereas methyl, substituted phenyl, substituted benzyl, and 2-methylene hydrazinecarbothioamide groups at position C-2 are essential for antibacterial activity. Further, substitutions at position 6/8 showed promising antibacterial activity, while substitutions at position 5/7 did not exhibit antibacterial activity (Gatadi et al. 2019).

9.4 Future Prospects

In order to counter infections caused by drug-resistant bacteria, there are few drugs that have been approved by the FDA recently, and some of the drug candidates are undergoing clinical trials. Heterocyclic scaffolds are considered very valuable in the current crisis of novel alternatives to antibiotics. Literature suggests that more than 85% of all biologically active compounds are heterocycles or consist of heterocycles and, commonly, nitrogen heterocycles as a backbone in their complex structures. These facts explicate the vital role of heterocycles in medicinal chemistry. Common heterocycle scaffolds are present in most natural products and marketable medicines which are currently used for the treatment of bacterial infections. Interestingly, approximately 60% of FDA drugs are nitrogen-based heterocycles, thus revealing its importance in drug design and drug discovery. Due to their essential resourcefulness and diversity, the synthetic innovation in heterocyclic synthesis lends greater opportunity to discovering new lead compounds.

9.5 Conclusion

With current concerns related to the emergence of MDR bacteria, the scientific community is required to provide more diverse and effective alternatives to antibiotics. Within this context, synthetic organic compounds may once again become the cornerstone of a sustainable antibacterial therapy. Moreover, synthetic analogues of a variety of biologically active natural products, antibiotics, and newer heterocyclic compounds may be added to the existing arsenal of biologically potent molecules to ameliorate the present condition. This chapter presents the impact of heterocycles in medicinal chemistry and interesting common structural patterns within each heterocyclic subcategory. The studies concerning the synthesis, characterization, and evaluation of the antibacterial activities of azirine and aziridine scaffolds, azetidin-2-one (β-lactam), furan/pyrrole/thiophene, pyrazole, imidazole and benzimidazole, triazole, quinoline, and quinazoline are summarized. This book chapter is prepared to attract the attention of organic synthetic chemists, biologists, as well as pharmacists and general practitioners and specialists.

Acknowledgments None to declare.

Conflict of Interest The authors declare that there is no conflict of interest.

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Chapter 10
Antibacterial Metal-Organic Frameworks

Sandeep Kumar Dey and Arghya Basu

Abstract The continuous evolution of complex resistance mechanisms in pathogens is intimidating the efficacy of current antibiotic therapy in medical and health care systems. Thus, the continuous emergence of drug-resistant bacteria has turned out to be a global concern. For the most effective measure against drug-resistant bacteria, the development of bioactive agents having fewer side effects is of utmost necessity. In this connection, porous coordination polymers, popularly known as metal-organic frameworks (MOFs), are well thought-out to be the next-generation antibacterial bioactive agents and drug delivery materials. Recently, the possibilities of MOF nanoparticles (NPs) and MOF nanocomposites (NCs) as alternative treatment methods to combat drug-resistant microbes have gained momentum. Compared to the commonly used antibacterial materials (metal salts, metal oxides, metal nanoparticles, and organic pharmaceutical compounds), MOFs are blessed with exceptional properties such as high specific surface area, structural flexibility, tunable pore size, and high drug loading capacity with sustained release capabilities of bioactive substances. Hence, MOFs can directly be employed as antibacterial agents or they can possibly be used as potential carriers of antibacterial agents such as metal/metal oxide NPs, antibiotics, therapeutic agents, and phytochemicals. The existence of easily hydrolysable multiple coordination bonds in MOFs established them as suitable materials for the sustained release of metal cations, bioactive ligands, and encapsulated antibacterial compounds at the site of action, which is a prerequisite for the treatment of many intercellular bacterial infections. Moreover, post-synthetic modification and surface modification techniques can also be employed to make MOFs and their nanocomposites more bioactive and less toxic to human cells.

S. K. Dey (✉)
Materials Science and Technology Division, CSIR-North East Institute of Science and Technology, Jorhat, Assam, India
e-mail: sandeep@neist.res.in

A. Basu
Amity School of Applied Sciences, Amity University Mumbai, Mumbai-Pune Expressway, Somathne, Navi Mumbai, Maharashtra, India

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This book chapter provides a comprehensive description and significant developments in the bactericidal applications of different bioactive MOFs and their nanocomposites. Herein, we mainly focus on the antibacterial efficacy of silver- (Ag^{+}), zinc- (Zn^{2+}), and copper (Cu^{2+})-based MOFs. The effective drug encapsulation and delivery and surface modifications of MOFs to attain better bactericidal and therapeutic action have been discussed with suitable examples. Towards the end of this chapter, a section is included to comprehend the mode of antibacterial action of MOFs and their nanocomposites. We believe that this chapter not only will fill the gap in current knowledge on MOFs but also will help the readers to understand how the MOFs could possibly be applied as potential next-generation antibacterial materials.

Keywords Antibacterial · Antibiotic · Bactericidal · Bioactive · Biocompatible · Biomaterials · Cytotoxic · Carboxylic acid · Coordination · Composite · Drug · Frameworks · Ligand · Microbes · Mechanism · Metal oxide · Nitric oxide · Nanoscale · Nanoparticle · Porous · Polymers · Pathogens · Pore volume · Surface area · Therapy

Abbreviations

BDC	Benzene dicarboxylate
BTC	1,3,5-Benzene tricarboxylic acid
CUSs	Coordinatively unsaturated metal sites
DCBP	4,4′-Dicarboxy-2,2′-bipyridine
DHFRs	Dihydrofolate reductase inhibitors
DHTP	Dihydroxy terephthalic acid
DMF	Dimethyl formamide
FA	Folic acid
HA	Hyaluronic acid
HIP	5-Hydroxyisophthalic acid
HRTEM	High-resolution transmission electron microscopy
HzBA	4-Hydrazine benzoic acid
MAA	Mercaptoacetic acid
MBCs	Minimal bactericidal concentrations
MDR	Multidrug-resistant
MH	Mueller–Hinton
MIm	2-Methylimidazole
MOFs	Metal-organic frameworks
MRSA	Methicillin-resistant *Staphylococcus aureus*
NCs	Nanocomposites
NPs	Nanoparticles
ONBA	*Ortho*-nitro benzaldehyde
PBA	3-Phosphonobenzoic acid

PDC	Pyridine-3,5-dicarboxylic acid
PSM	Post-synthetic modification
PTA	1,3,5-Triaza-7-phosphaadamantane
ROS	Reactive oxygen species
TEA	Triethylamine
TiO_2	Titanium dioxide
ZIFs	Zeolitic imidazolate frameworks
ZnO	Zinc oxide

10.1 Introduction

The continuous emergence of drug-resistant microbes has become a major public health concern worldwide (Prestinaci et al. 2015). This has encouraged chemists and biologists to synthesize and evaluate the antibacterial efficacy of new organic and metal-organic chemical compounds having specific intracellular action. Most bacteria are capable of developing compensatory mechanisms to resist the antibacterial drugs in common use and eventually form multidrug-resistant (MDR) strains. The emergence of MDR bacteria has outpaced the antibacterial drugs in the earlier decades, justifying the discovery of more potent and safer, biocompatible organic and metal-organic based antibacterial drugs. Thus, the most important challenge towards the development of new strategies for controlling healthcare-associated microbial infections is the synthesis and evaluation of highly effective antimicrobial drugs and materials for the treatment of infectious diseases (Gandra et al. 2014).

Intracellular bacterial infections can exist in the host for extensive periods of time and can even reduce the antibiotic efficacy during the treatment period. The major concerns of antibacterial therapy are antibiotic resistance and adverse side effects, but noncytotoxic materials with controlled release of bioactive agents can considerably reduce the side effects and achieve targeted use of drugs at the precise time and location. The need for effective and safer antibacterial drugs against MDR bacteria is a real challenge, not only for the systemic treatment of infections but also for topical treatments (burns and wounds) and for prophylactic purposes (e.g., surface treatment of medical devices). In order to successfully counter the MDR bacteria, the synthesis of new antibacterial drugs and drug delivery systems with fewer side effects are being pursued by many researchers in the world.

Several transition metal oxides and metal nanoparticles (NPs) of silver (Ag), copper (Cu), and zinc (Zn) have gained significant applicability in recent times as substitutes for traditional antibacterial drugs. Recently, there is an emerging interest in the synthesis of new bioactive nanomaterials to meet pharmacological and biological requirements for antibacterial applications. This also comprises the design of suitable framework materials through the search for new types of bioactive organic linkers capable of coordination (binding) to bioactive metal cations such as zinc, copper, and silver. The design and synthesis of bioactive metal-organic

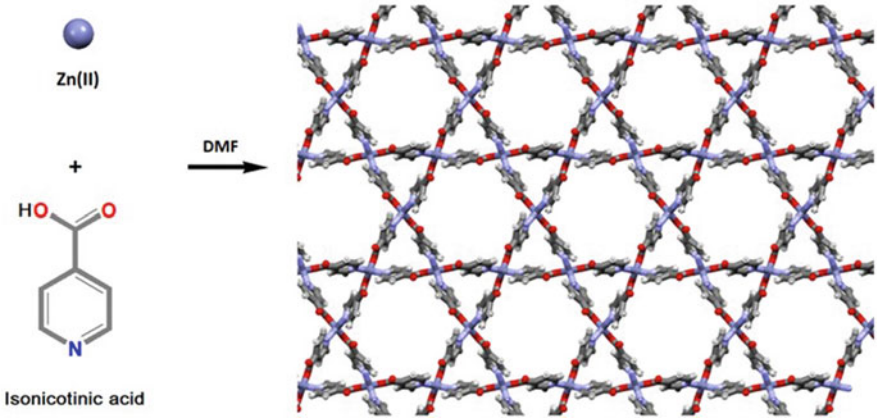

Fig. 10.1 A representative example of a nanoporous metal-organic framework (MOF) obtained from the self-assembly of isonicotinic acid and zinc (II) salt in dimethyl formamide (DMF)

frameworks (MOFs) is a significant step towards the finding of newer materials for the control of healthcare associated microbial infections (Horcajada et al. 2012). Due to the difficulty of efficiently crossing the bacterial cell membranes for most antibiotics (due to drug resistance), porous nanomaterials having lower cytotoxicity and functionalized surfaces have attracted great attention as nanocarriers of active pharmaceutical compounds for bacterial therapy.

The development of new functional materials has been a subject of considerable attention during the last two decades. Coupling inorganic clusters and organic molecules to form MOFs opens up the numerous possibilities of creating new functional materials with fascinating structures and interesting properties to explore (Fig. 10.1). The preparation of MOFs is fundamentally governed by coordination-driven molecular self-assembly. MOFs are typically obtained as microcrystalline powders through hydrothermal and solvothermal synthesis. In MOFs, the organic ligand (also called linker or spacer) and metal cations can be selected considering a specific application in need. The prospect of using a range of chemical building blocks and metal clusters/ions in MOFs enables the construction of porous networks with desired properties. Research in the field of functional porous materials including MOFs has now moved beyond the evaluation and tuning of their physical and chemical properties in the context of bulk applications like gas storage/separation and heterogeneous catalysis (He et al. 2014). Researchers are now continuously engaged in exploring the electronic and magnetic properties of porous frameworks for solid-state devices (Stassen et al. 2017) and also in biomedical applications (Rojas et al. 2019).

In particular, a pronounced interest has emerged in the development of new antimicrobial MOFs, popularly known as bio-MOFs, as possible alternatives to conventional antimicrobial drugs that are becoming more microbe resistant (Wyszogrodzka et al. 2016). In addition to the highly desirable antibacterial properties, well-defined pores in MOFs are capable of storing bioactive molecules and of

releasing them at the appropriate rate for bactericidal action. The biochemical properties, size, and shape of MOF particles, together with zeta potential, are the most important factors which determine the bactericidal activity of porous materials. Structural diversity, post-synthetic surface modification, biodegradability, and capability to accommodate bioactive compounds within the pores enable nanoscale MOFs with greater advantages as compared to common antibiotics in use. MOFs can work as drug release carrier materials by encapsulating bioactive substances such as active pharmaceutical compounds or metal/metal oxide nanoparticles within their porous structures or by incorporating the bioactive molecule as a constitutive entity of the framework. This latter strategy permits the utilization of therapeutically active molecules bearing multiple coordinating groups and bioactive metal cations to build the bio-MOFs and then deliver the active compounds via framework hydrolysis. To fulfil the ever-increasing demand for bacterial infection therapy and other microbial infections, advancement in research has been directed towards the search for bioactive and biocompatible MOFs and other nanocarriers to precisely control the release of active pharmaceutical substances for effective drug delivery.

The chapter discusses the important developments on the antibacterial applications of MOFs, mainly focusing on the inherent antibacterial efficacy of silver- (Ag^+), zinc- (Zn^{2+}), and copper (Cu^{2+})-based MOFs, and MOFs as promising nanomaterials for drug delivery applications. Surface modification of MOF nanocomposites by biocompatible molecules to facilitate their permeability through the microbial cell membranes to counter intracellular infections has also been discussed. Further, a brief note on the post-synthetic modification (PSM) of MOFs for the controlled release of bioactive nitric oxide gas for antimicrobial therapeutic action has been added. Finally, based on the examples of antibacterial MOFs discussed in this chapter, some important factors that are accountable for the bactericidal properties of different MOFs have been summarized, and also the strategies adopted to greatly enhance their bactericidal properties are discussed in brief.

10.2 Synthesis of Metal-Organic Frameworks (MOFs)

The different syntheses and applications of MOFs have attracted a lot of attention in the past three decades. The possibility to obtain a wide range of microporous and mesoporous structures by the judicious choice of molecular building blocks (ligands or linkers), metal salts, and solvents established MOFs as new-generation materials for various applications in chemical and material sciences (Stock and Biswas 2012). The conventional way of synthesizing crystalline and porous MOFs is by heating a mixture of metal salt and an organic linker in a suitable solvent under mild to moderate conditions, preferably below 200 °C in a closed reaction vessel. The most common organic solvents used in the solvothermal synthesis of MOFs are dimethylformamide, diethylformamide, ethanol, and acetonitrile, sometimes in the presence of water to facilitate crystallization. In the hydrothermal synthesis of

MOFs, water is used as the only solvent. In their synthesis, the kinetics of crystallization has to be favourable to allow nucleation and facilitate growth of the highly desirable crystalline phase. The most common hurdle in the synthesis of MOFs is to establish and then to reproduce the synthesis conditions by using the right amount of an appropriate solvent (or solvent mixture), reaction temperature, duration of the reaction, and cooling rate of the reaction. The nature of the solvent, reaction time and temperature, subsequent cooling rate, and the presence or absence of a mineralizer (additive) can significantly influence the particle size and morphology, crystallinity, and porosity of the resulting MOFs. In solvothermal and hydrothermal synthesis, the reaction time can range from a few hours to several days to obtain MOFs of high crystallinity and purity. However, there are examples of imidazole-based MOFs called zeolitic imidazolate frameworks (ZIFs) which can be synthesized at room temperature under ambient conditions to obtain the desired porous crystalline materials within 24 h.

The concepts of solvothermal and hydrothermal reaction conditions and microwave-assisted synthesis have originated from zeolite chemistry. It has also been observed that different synthesis methodologies may lead to the formation of different MOFs from the same reaction mixture. Also, different synthesis methodology may strongly influence the yields, crystallinity and porosity, particle size and morphology, reaction time, and temperature of the same reaction mixture. Activation of the synthesized MOFs is considered a crucial step in exploring the full potential of MOFs and is also important for post-synthetic modification reactions to significantly reduce the possibility of any side reactions. In addition to room temperature and solvothermal synthesis, several other synthesis methods have been developed and successfully applied to synthesize different types of MOFs. Syntheses of MOFs by microwave heating, electrochemistry, mechanochemistry, and ultrasonic methods have also been employed. These newly developed synthesis methods helped in controlling the desirable properties and chemical functionalization of MOFs, which is very important to achieve targeted performances of the synthesized MOFs. Thin films of MOFs are generally prepared by the layer-by-layer technique in which a functionalized organic ($-$COOH, $-$OH, etc.) surface is immersed in the solutions of metal ions and organic linkers one after another in a sequential manner.

In mechanochemical synthesis of MOFs, a solvent free mixture of a metal salt (or a metal oxide) and an organic linker is grinded in a mortar pestle or preferably in a ball mill under ambient conditions to obtain the desired MOF. The mixture is then collected and gently heated to remove the volatile by products such as water and acetic acid. The applied mechanical force breaks the intramolecular bonds in the reactants and allows the chemical transformations by metal-ligand coordination bond formation. This method is environment friendly and moderate to high yields of MOFs can be obtained within a short reaction time (10–60 min). When a small amount of solvent is added during the grinding process, the process is then called liquid-assisted grinding and is known to accelerate the mechanochemical reactions by increasing the mobility of the reactants and providing structure-directing properties.

In microwave-assisted synthesis, the required activation energy for the reaction comes from microwave radiations, and this method has wide applications in the fast synthesis of MOFs. Microwave synthesis has several advantages such as phase selectivity and purity, higher nucleation rate, and narrow particle size distribution in addition to crystallinity and porosity. In sonochemical synthesis, ultrasonic radiation (20 kHz–10 MHz) is used for the rapid synthesis of MOFs under solvent-free conditions at room temperature and low pressure. The MOFs synthesized by this energy-efficient method show significantly smaller particle sizes than those by the conventional solvothermal synthesis.

In electrochemical synthesis, a potential or current is applied to the metal electrode (anode) which is immersed in a solution containing an organic linker along with a supporting electrolyte. When a potential is applied, the metal electrode gets oxidized, and metal ions are continuously released into the reaction medium, which then reacts with the organic linker near the electrode surface to form MOFs. This method was developed with the objective of excluding the anions (nitrate, sulfate, etc.) from the synthesis and running a continuous process of preparing MOFs.

10.3 Bioactive Silver-Organic Frameworks

It is widely known that silver and its simple salts are effective antimicrobial agents which are known to display a broad-spectrum bactericidal property. Among the bioactive metals, Ag/Ag^+ exhibits the highest toxicity to most bacterial strains (and also to some protozoa, viruses, and fungi), while simultaneously showing a relatively low toxicity to eukaryotic and mammalian cells. Over the years, several bioactive Ag^+ coordination compounds have been tried and tested as bactericidal agents (Sim et al. 2018). However, in spite of their significant bactericidal activity, the frequent use of these Ag^+ coordination compounds as potential drugs, creams, and wound dressings is restricted by their light sensitivity and insolubility in aqueous media. It is observed that Ag^+ forms weak coordination bonds with O− and N − donor ligands and shows lesser solubility in aqueous media than coordination compounds possessing rather strong $Ag^+ - S$ or $Ag^+ - P$ bonds. Further, recent research has shown that Ag^+ N-heterocyclic carbene complex [Ag-1] having an $Ag^+ - C$ bond exhibits efficient bactericidal properties because of the gradual release of Ag^+ in solution (Berchel et al. 2011). Along this line, the synthesis of Ag^+ MOFs by the judicious choice of ligands containing either nitrogen, oxygen, sulfur, and phosphorus donor atoms or mixed donor atoms is worth evaluating for their potential bactericidal properties.

The antibacterial efficacy of 3-phosphonobenzoic acid (PBA)-based Ag^+ MOF $[Ag_3{\cdot}(PBA)]$ has been evaluated using three different strains, namely, *Staphylococcus aureus* (the laboratory strain RN4220, the clinical strains Newman, and MRSA N315), two strains of *Pseudomonas aeruginosa* (clinical strains PA240709 and PA130709), and an *Escherichia coli* strain (MG1655) (Berchel et al. 2011). Both carboxylic acid and phosphonic acid groups are classified as hard bases (based on

Table 10.1 MBCs (μg/mL) of [Ag-1], [Ag_3·(PBA)], and antibiotics kanamycin and ampicillin

Strains	[Ag-1]	[Ag_3·(PBA)]	Kanamycin	Ampicillin
S. aureus-RN4220	200	50	25	1
S. aureus-Newman	20	75	10	1
S. aureus-N315	200	50	>500	100
E. coli-MG1655	100	50	5	25
P. aeruginosa-130,709	50	20	200	>500
P. aeruginosa-240,709	75	30	100	>500

HSAB principle) and form weak $Ag^+ - O$ coordination bonds providing moderate stability to the Ag^+ MOF, which would favour the release of Ag^+ at a slower rate by framework hydrolysis under physiological conditions. [Ag_3·(PBA)] displays broad-spectrum bactericidal properties against both Gram-negative and Gram-positive strains. Minimal bactericidal concentrations (MBCs) were determined using liquid broth micro-dilution assays, and MBCs of two commonly used antibiotics, namely, kanamycin and ampicillin, were also determined following the same experimental conditions. It is evident from Table 10.1 that [Ag_3·(PBA)] showed superior bactericidal effects at lower concentrations against *S. aureus* N315 and against both the strains of *P. aeruginosa*, when compared to kanamycin, ampicillin, and N-heterocyclic carbene complex [Ag-1]. The bacterial membranes were severely ruptured upon contact with Ag^+, resulting in the loss of cellular cohesion followed by leakage of bacterial cytoplasm. It's important to mention here that vancomycin, a last-generation glycopeptide antibiotic, is active against *S. aureus* strains between 1 and 10 μg/mL. Haemolysis assays performed using human red blood cells showed that both [Ag-1] and [Ag_3·(PBA)] did not exhibit any significant cytotoxicity up to 500 μM concentrations.

Two dicarboxylic acid-based Ag^+ MOFs, [Ag_2(HIP)(H_2O)(H_3O)] and [Ag_5(PDC)$_2$(OH)], were synthesized under the hydrothermal conditions using 5-hydroxyisophthalic acid (HIP) and pyridine-3,5-dicarboxylic acid (PDC), respectively. Both of these MOFs showed superior antibacterial efficacy against *E. coli* and *S. aureus* when compared to [Ag_3(PBA)] (Lu et al. 2014). Both the MOFs showed the release of bioactive Ag^+ gradually by framework hydrolysis, resulting in the long-lasting antibacterial activities against the tested strains. High-resolution transmission electron microscopy (HRTEM) images revealed that the dicarboxylate based Ag^+ MOFs can effectively damage the bacterial membranes, ensuing in their death after 24 h. The minimum inhibitory concentrations (MICs) of [Ag_2(HIP)(H_2O)(H_3O)] (5–15 μg/mL) and [Ag_5(PDC)$_2$(OH)] (10–20 μg/mL) are also lower and, hence, better than the silver sulfadiazine salt, but a little higher than silver tris(pyrazolyl)borates.

1,3,5-Triaza-7-phosphaadamantane (PTA) and PTA derivatives (PTA = O and PTA = S) are versatile building blocks for the synthesis of MOFs (and nonporous CPs) attributable to the presence of four coordinating sites and the diamond-like geometry. Although numerous organic ligands (typically N–donor polypyridine, or O–donor polycarboxylate derivatives) are used as linkers for the synthesis of MOFs,

Table 10.2 MICs (μg/mL) of PTA = X (X = O/S) derived silver-organic frameworks

Strains	[Ag(PTA = O)] NO_3	[Ag_2(PTA = O)] SO_4	[Ag(PTA = S)] NO_3	[Ag_4(PTA = S)$_2$] $(SO_4)_2$
E. coli	6	6	4	20
P. aeruginosa	7	6	5	20
S. aureus	20	20	20	40
C. albicans	30	20	30	60

the search for new water-soluble MOFs for antibacterial applications is highly desirable. Complexation of PTA = O (Kirillov et al. 2011) and PTA = S (Jaros et al. 2013) with $AgNO_3$ and Ag_2SO_4 yielded four distinct 3D MOFs, which exhibit moderate to good antimicrobial efficacy against *S. aureus*, *P. aeruginosa*, *E. coli*, and *Candida albicans*. These studies were performed by the serial dilution method using the Antibiotic Broth, and the antimicrobial efficacy is expressed as MICs (μg/mL) of compounds that fully inhibit the microbial growth. The observed antimicrobial efficacy of PTA = X (X = O/S)-derived Ag^+ MOFs is associated with the bioactive silver nodes, since the antimicrobial efficacy of PTA = O and PTA = S ligands is very low in comparison to the Ag^+ MOFs (Table 10.2). Three of these Ag^+ MOFs showed very promising antibacterial properties against *P. aeruginosa* and *E. coli*, and the MICs are found to be lesser than $AgNO_3$ or $Ag(CH_3CO_2)$ salts (Tables 10.2 and 10.3).

An interesting approach to improving the antimicrobial efficacy of Ag^+ MOFs consists of applying a mixed-ligand strategy involving both carboxylic acid and amino-phosphine building blocks. Coordination polymers of 1,3,5-triaza-7-phosphaadamantane (PTA) and PTA derivatives are water-soluble and air-stable, which makes them good candidates as antimicrobial agents. The antimicrobial efficacy of PTA and carboxylic acid-based Ag^+ MOFs was evaluated against *S. aureus*, *P. aeruginosa*, *E. coli*, and *C. albicans* (Jaros et al. 2014; Jaros et al. 2016). Mixed-ligand Ag^+ MOFs which were synthesized using silver oxide (Ag_2O), PTA, and a dicarboxylate linker such as succinic acid (2), malonic acid (3), phenylmalonic acid (4), and dimethylglutaric acid (5) are water-soluble materials and show excellent antimicrobial properties against the probed pathogens. The MICs of Ag^+ MOFs and Ag salts were evaluated in vitro using the Antibiotic Broth serial dilution method in each case (Table 10.3). The MICs of [Ag_2·(PTA)·3] and [Ag_2·$(PTA)_2$·$(4)_2$] against *P. aeruginosa* and *C. albicans* (yeast) were determined to be significantly lower than silver acetate ($AgCH_3CO_2$) and silver nitrate ($AgNO_3$).

Silver-organoboron frameworks were also evaluated as bactericidal materials against the growth of *E. coli* and *S. aureus*. The MICs of Ag^+–organoboron frameworks (with different counter anions) against the tested pathogens are found to be in the range of 293–308 μg/mL, and the frameworks can give a steady and prolonged release of bioactive Ag^+ in biocidal concentrations for several months showing long-lasting effectiveness (Liu et al. 2010). 1,2,4-Triazole-based Ag^+ MOF [Ag·(TAZ)] was tested against two Gram-negative strains, *E. coli* and *P. putida*, and comparative studies have been conducted with the antibacterial efficacy of two

Table 10.3 MICs (μg/mL) of PTA and carboxylic acid-based (mixed ligand) silver-organic frameworks

Strains	$[Ag_2(PTA)_2 \cdot 2]$	$[Ag_2(PTA) \cdot 3]$	$[Ag_2(PTA)_2(4)_2]$	$[Ag(PTA) \cdot 5]$	$Ag(CH_3CO_2)$	$AgNO_3$
E. coli	6	7	5	9	4	9
P. aeruginosa	20	6	5	10	9	9
S. aureus	6	8	10	10	4	20
C. albicans	40	30	30	50	90	40

cobalt imidazolate MOFs, ZIF-67, and Co-SIM-1 (Aguado et al. 2014). The antibacterial efficacy of Co-SIM-1 was evaluated to be slightly better than [Ag·(TAZ)], followed by ZIF-67.

10.4 Bioactive Copper-Organic Frameworks

The antimicrobial properties of copper and its compounds are well recognized, and several studies on Cu-based coordination compounds showed remarkable antibacterial efficacy against various strains (Wang et al. 2021). While the mechanism of antibacterial action of metallic copper and its alloy materials is complex and diverse, it is known that Cu^{+}/Cu^{2+} ions on metallic surfaces can seriously damage the cellular membranes of bacteria. The antibacterial efficacy of Cu compounds is due to the electrostatic attraction between the positively charged Cu^{+}/Cu^{2+} ions and the negatively charged cellular membranes of several strains, which results in their death by damaging the bacterial membranes. However, excessive release of Cu ions from Cu compounds can be harmful to tissues, which often hinders their use for medical applications. To resolve this critical issue, Cu^{2+} MOFs are receiving significant attention owing to their tunable and robust structures, lower cytotoxicity, and most importantly gradual and sustained release of Cu^{2+} ions, similar to the bactericidal Ag^{+} MOFs.

A mixture of 4,4′-dicarboxy-2,2′-bipyridine (DCBP) and $Cu(CH_3COO)_2$ in the presence of an organic additive (triethylamine or acetic acid) in ethanol-water (1:1) solvent system yielded Cu^{2+} MOFs of identical structure $[Cu{\cdot}(DCBP)(H_2O)_4]$. However, morphologies of the $[Cu{\cdot}(DCBP)(H_2O)_4]$ MOFs were examined to be different when synthesized in the presence of triethylamine (TEA) and acetic acid (CH_3COOH). The antibacterial efficacy of $[Cu{\cdot}(DCBP)(H_2O)_4]$ MOFs of different morphologies was studied against *Bacillus subtilis, S. aureus, Salmonella enteriditis, E. coli, P. aeruginosa*, and *P. vulgaris* by determining the MICs, (μg/mL) by a colorimetric method (Wang et al. 2011a). The MOF particles obtained as rhombus-lump morphology in the presence of acetic acid showed better bactericidal properties against Gram-negative *E. coli*, *P. aeruginosa*, and *P. vulgaris* in comparison to those obtained in the presence of triethylamine as rhombus-layer morphology (Table 10.4), possibly because of their smaller size. In contrast, the MOF particles obtained in the presence of TEA and SDS mixture showed bread-like morphology and have negligible antibacterial effects to the tested bacterial strains. Although the exact mechanism of the antibacterial action of these Cu^{2+} MOFs could not be concluded, but it is obvious from the results that the particle morphology and particle size of $[Cu{\cdot}(DCBP)(H_2O)_4]$ can significantly influence the bactericidal properties. Accordingly, it is very much likely that antibacterial MOFs with higher surface area and smaller particle size will possibly show superior bactericidal properties. Decrease in particle size results in significant increment of the external surface area and enhanced density of edges (coordinated metal centres) on the nanoscale MOFs. However, the antibacterial efficacy of $[Cu{\cdot}(DCBP)(H_2O)_4]$ is much inferior

Table 10.4 MICs (μg/mL) of $[Cu{\cdot}(DCBP)(H_2O)_4]$ with identical structure but different morphology

Bacterial strains	$[Cu{\cdot}(DCBP)(H_2O)_4]$ (rhombus-layer like NPs)	$[Cu{\cdot}(DCBP)(H_2O)_4]$ (rhombus-lump like NPs)	$[Cu{\cdot}(DCBP)(H_2O)_4]$ (bread-like NPs)
B. subtilis	12.5	12.5	>50
S. aureus	12.5	12.5	>50
S. enteritidis	25	25	>50
E. coli	12.5	6.25	>50
P. vulgaris	25	12.5	>50
P. aeruginosa	25	6.25	>50

in comparison to the Ag^+ MOFs of 1,3,5-triaza-7-phosphaadamantane (PTA), and its derivatives PTA = O and PTA = S (Fig. 10.2).

A mixed-ligand strategy was adopted to synthesize four robust Cu^{2+} MOFs by hydrothermal synthesis. Mixed-ligand Cu^{2+} MOFs having the general formula $[Cu_2(Glu)_2(L)]{\cdot}(H_2O)_x]$ (L = bipyridine ligands) have been synthesized using cupric nitrate trihydrate, glutaric acid (Glu), and bipyridine ligands such as 4,4′-bipyridyl, 1,2-bis(4-pyridyl)ethane, 1,2-bis(4-pyridyl)ethylene, and 1,2-bis(4-pyridyl)propane in water. The antibacterial efficacy of the four Cu^{2+} MOFs was studied by the determination of their minimal bactericidal concentrations (MBCs) against five bacterial strains, *E. coli, Klebsiella pneumonia*, *P. aeruginosa, S. aureus,* and *methicillin-resistant S. aureus (MRSA)*, which showed more than 99% of bactericidal effect during an incubation time of 24 h at 37 °C (Jo et al. 2019). The antibacterial efficacy of the bridging ligands was observed to increase in the order of 1,2-bis(4-pyridyl)ethylene<1,2-bis(4-pyridyl)propane < glutaric acid <4,4-′-bipyridyl <1,2-bis(4-pyridyl)ethane. MOF degradation tests in deionized water showed that the $[Cu_2(Glu)_2(L)]{\cdot}(H_2O)_x]$ MOFs are largely hydrophobic because of the bipyridine organic linkers, and Cu^{2+} ions are slowly released from the framework by hydrolysis of the robust structures. Thus, the coordinated Cu^{2+} centres in the MOFs can be considered as the active sites for their exceptional bactericidal properties. All four $[Cu_2(Glu)_2(L)]{\cdot}(H_2O)_x]$ MOFs displayed similar bactericidal effects, and the MICs against the five tested strains were found to be 20 μg/mL.

HKUST-1 or MOF-199 having the chemical composition $[Cu_3(BTC)_2(H_2O)_3]$ is a well-known commercially available Cu^{2+} MOF of 1,3,5-benzenetricarboxylic acid (BTC), which showed notable antibacterial efficacy at the interface when attached to the solid surfaces. An antibacterial film of chitosan (CS), a natural polysaccharide and HKUST-1, has been demonstrated for local infection therapy (Rauf et al. 2019). HKUST-1/CS film showed lower cytotoxicity with a potential antibacterial efficacy against *S. aureus* and *E. coli* due to the slow and sustained release of Cu^{2+} ions in comparison to the Cu^{2+}/CS film. In vivo experimental results showed that the HKUST-1/CS film could kill the microbes and simultaneously promote vessel regeneration, which resulted in an improved wound healing rate in the local infection therapeutic treatment. The MICs of HKUST-1/CS film against *S. aureus* and *E. coli* were determined to be between 200–300 μg/mL depending upon the MOF particle

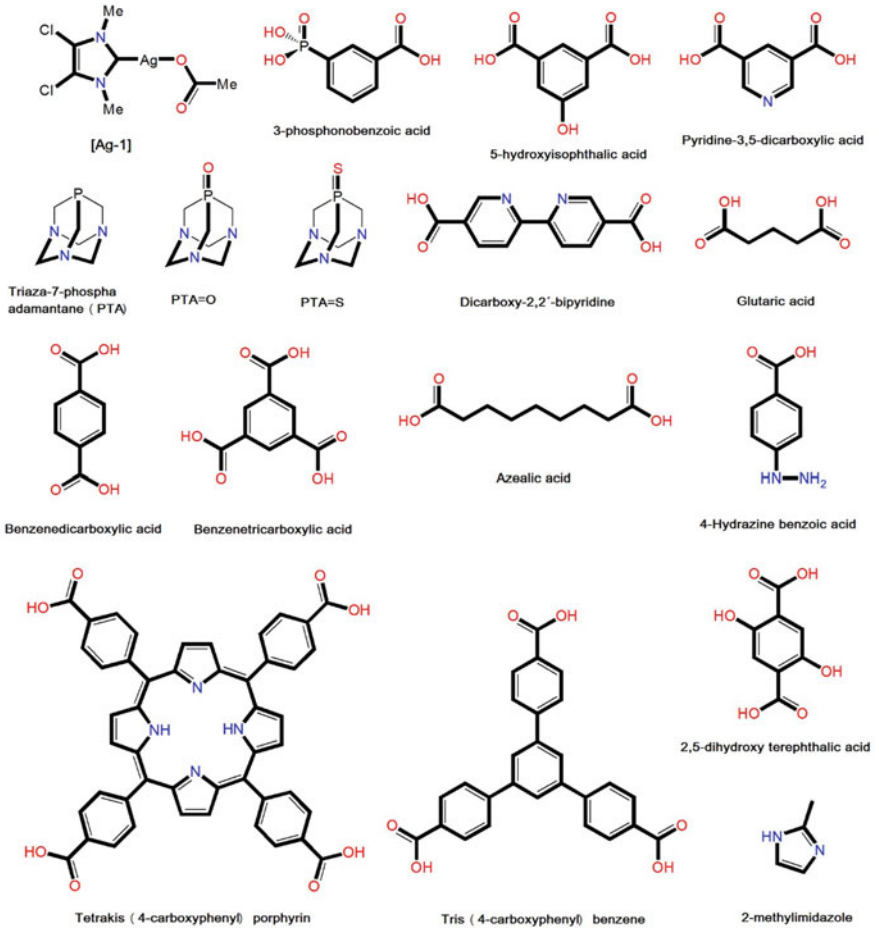

Fig. 10.2 Molecular structures of organic ligands (linkers) used for the synthesis of bioactive MOFs

morphology (nanofibers/macroparticles) and showed 96–99.9% inhibition of microbial growth.

HKUST-1 immobilized over cellulosic fibres showed a strong antibacterial activity against *E. coli* (Rodriguez et al. 2014). However, the antibacterial efficacy of HKUST-1 and the cellulosic substrate was found to be inferior. The specific method used in the synthesis of HKUST-1 crystalline NPs immobilized over the cellulosic substrates provides a strong bond between the cellulosic fibres and MOF particles, allowing the MOF-based textile to be reused after washing. This will provide a new avenue to fabricate fabrics with antibacterial properties.

10.5 Bioactive Zinc-Organic Frameworks

Zinc is an inexpensive nontoxic metal commonly used in pharmaceuticals and cosmetics. Simple zinc salts and zinc oxide are well recognized for their antidandruff, astringent, anti-inflammatory, and antimicrobial properties. Several Zn^{2+}-based bioactive MOFs have been prepared, and their antibacterial activities were studied against both Gram-negative and Gram-positive strains. The most potent antibacterial Zn^{2+} MOF is BioMIL-5 (Bioactive Materials from Institute Lavoisier), which has been synthesized by hydrothermal process using $Zn(NO_3)_2$ and azelaic acid (Tamames-Tabar et al. 2015). Azelaic acid and Zn^{2+} both have good antibacterial, dermatological, and anti-inflammatory properties, but without any controlled release in physiological conditions. The MICs of BioMIL-5 against *S. epidermidis* and *S. aureus* were evaluated to be 1.7 μg/mL for both the strains, which is similar to the MICs of azelaic acid (1.5 μg/mL). Unlike Zn^{2+} salts where the release of Zn^{2+} cannot be controlled because of their high solubilities in water, BioMIL-5 degrades slowly and progressively in both aqueous medium and MHCA bacteria broth to release its therapeutic constitutive molecules (azelaic acid and Zn^{2+}) for bactericidal action. BioMIL-5 degradation in water was observed to be around 8% and 55% after 7 and 70 days, respectively, whereas in MHCA bacteria broth (a complex medium composed of many inorganic and organic nutrients such as amino acids, sugars, inorganic salts, etc.), BioMIL-5 degradation was around 25% and 95% after 7 and 70 days of incubation, respectively. Such a slow delivery of the bioactive therapeutic constituents by hydrolysis of BioMIL-5 framework allows control over the rapid growth of a Gram-positive bacterial culture for more than 7 days. The exact mechanism of zinc toxicity to bacterial cells has not been fully explained, although the irreversible binding to some surface adhesins may explain the bacterial susceptibility to Zn^{2+} ions. It is to be noted that the MICs of Zn^{2+} for *S. aureus* are in the range of 65–130 μg/mL, which suggests that the slow release of azelaic acid is mainly attributable to the bactericidal effects of BioMIL-5.

The antibacterial efficacy of another dicarboxylate-based Zn^{2+} MOF, $[Zn{\cdot}(DCBP)(H_2O)_6]$, was studied against five bacterial strains. The MOF was prepared at room temperature by mixing 4,4′-dicarboxy-2,2′-bipyridine (DCBP) and Zn $(CH_3COO)_2$ in ethanol-water solvent system in the presence of an additive (Wang et al. 2011b). The size and shape of the resulting MOF particles were found to be dependent on the quantity of solvent and the molar ratio of the reactants used in the synthesis. MOF particles obtained as nanorods in the presence of acetic acid (using a 2:3 ligand-to-metal ratio) showed better antibacterial efficacy against Gram-negative strains *E. coli, P. aeruginosa*, and *P. vulgaris* in comparison to Gram-positive strains *S. aureus* and *B. subtilis*. The MICs (μg/mL) of $[Zn{\cdot}(DCBP)(H_2O)_6]$ against Gram-negative strains were evaluated to be 12.5 μg/mL, and the MICs were > 50 μg/mL for Gram-positive strains. Since the ligand DCBP has no antibacterial properties, the MICs of $[Zn{\cdot}(DCBP)(H_2O)_6]$ against Gram-positive strains are considerably higher in comparison to BioMIL-5. $[Zn{\cdot}(DCBP)(H_2O)_6]$, when synthesized in the absence of any additive or in the presence of TEA as an additive, showed much higher MICs,

and this weaker antibacterial efficacy is attributable to the distinct crystal morphologies of the MOFs. Hence, it is noteworthy that the crystal morphology and size of the MOF particles can significantly influence the antibacterial properties.

The antibacterial efficacy of 4-hydrazine benzoic acid (HzBA)-based Zn^{2+} MOF $[Zn_2(HzBA)_4(H_2O)_4]$ was tested against drug-resistant bacterium *S. aureus* (Restrepo et al. 2017). The MOF particles showed remarkable antibacterial effects by inhibiting the metabolic activity and growth of the Gram-positive strain, due to the sluggish release of the bioactive ligand (HzBA). Zn^{2+} ions released as a consequence of the framework breakdown do not influence the antibacterial efficacy of the MOF significantly. HzBA is a molecule of biological interest and is used for the synthesis of a chelating agent called Deferasirox (Desferral), which is commonly employed for the treatment of iron overloads in humans.

10.6 Bioactive MOF Nanocomposites

MOFs can efficiently be employed as the carriers of bioactive metal/metal oxide nanoparticles (NPs) because of their highly ordered porous crystalline structures and large internal surface areas which can accommodate guest species. It is observed that the surface functionalization of MOFs impregnated with metal/metal oxide NPs further enhances their bactericidal properties. NPs can be stabilized by non-covalent interactions to prevent aggregation, and MOFs have the ability to stabilize NPs by encapsulation within their porous framework. Along this line, a metalloporphyrin-based Cu^{2+} MOFs have been shown to encapsulate Ag NPs, and the antibacterial efficacy of the Ag NPs impregnated MOF [Ag@Cu·(TCPP)] was demonstrated by in vivo and in vitro experiments (Ximing et al. 2017). The [Ag@Cu·(TCPP)] nanocomposite was synthesized using 5,10,15,20-tetrakis (4-carboxyphenyl) porphyrin (TCPP) and $Cu(NO_3)_2$ trihydrate in the presence of Ag-NPs by the solvothermal process. The antibacterial efficacy of [Ag@Cu·(TCPP)] was tested against *S. aureus*, *E. coli*, and *B. subtilis* by using the standard serial dilution (twofold) method recommended by the Clinical and Laboratory Standards Institute. The MICs of [Ag@Cu·(TCPP)] revealed that the nanocomposite is a more potent antibacterial material against *S. aureus* and *B. subtilis* in comparison to *E. coli* and mixed bacterial strains (Table 10.5). The observed antibacterial activities may be attributable to the functional groups present on the bacterial cell surface and the

Table 10.5 MICs (μg/mL) of [Ag@Cu·(TCPP)] nanocomposite and its comparison with Ag NPs, Ag^+ salt, and penicillin

Strains	[Ag@Cu·(TCPP)]	Ag NPs	Ag^+	Penicillin
E. coli	12.50	3.12	3.12	3.12
B. subtilis	6.25	1.56	1.56	6.25
S. aureus	6.25	3.12	3.12	6.25
Mixed strains	12.50	3.12	3.12	6.25

membrane structure of *B. subtilis* and *S. aureus*. However, the MICs of [Ag@Cu·(TCPP)] against *S. aureus* and *B. subtilis* were determined to be higher than the controls including Ag^+ salts and Ag NPs and equivalent to that of penicillin antibiotic (Table 10.5). The antibacterial mechanism of [Ag@Cu·(TCPP)] is attributable to the oxidation of Ag NPs to Ag^+ by the reaction with dissolved O_2 and protons (H^+) present in the surrounding medium, accompanied by the gradual release of Ag^+ from the hydrolysis of MOF. However, the MICs of [Ag@Cu·(TCPP)] are higher than the Ag^+ and Ag NPs, but the cytotoxic effect of [Ag@Cu·(TCPP)] is considerably lower than the Ag^+ and Ag NPs. In vivo experiments revealed that [Ag@Cu·(TCPP)] not only showed excellent bactericidal properties and low cytotoxicity in infected mice but also encouraged the wound healing faster than the Ag^+ ions and Ag NPs, which was examined by using the HaCat cell scratch wound assays.

1,4-Benzene dicarboxylic acid (BDC)-based Ni^{2+} MOF has been loaded with Ag-NPs to obtain $[Ag@Ni_3(OH)_2(BDC)_2(H_2O)_4]$ nanocomposite, and this was tested as an antibacterial material against *E. coli*, *B. subtilis*, and *P. aeruginosa* (El Salam et al. 2018). The experimental results showed that the bactericidal effects of the MOF $[Ni_3(OH)_2(BDC)_2(H_2O)_4]$ can be greatly enhanced by immobilizing traces of Ag NPs on the surface of the MOF particles, which were obtained in the form of nanosheets. The growth of both Gram-positive and Gram-negative bacteria could be inhibited at the MICs of 27–136 μg/mL using the MOF $[Ni_3(OH)_2(BDC)_2(H_2O)_4]$. The MOF particles exhibited higher antibacterial efficacy against *E. coli* and *P. aeruginosa* than *B. subtilis*, possibly due to the thick wall of the Gram-positive *B. subtilis*. Each Gram-negative bacterial strain when treated with $[Ag@Ni_3(OH)_2(BDC)_2(H_2O)_4]$ at a concentration of 2.5 μg/mL showed the complete inhibition of bacterial growth after 120 h of incubation. The oxidation of well-dispersed Ag-NPs to Ag^+ on the MOF feasibly promote the contact of Ag^+ with the microbes and successively resulted in the superior bactericidal properties. Further, benzene dicarboxylate (BDC) released by the hydrolysis of $[Ni_3(OH)_2(BDC)_2(H_2O)_4]$ could bind with calcium and magnesium ions of the bacterial cells and may result in the fragmentation of the bacterial cell DNA via generation of reactive oxygen species (ROS).

1,3,5-Benzene tricarboxylic acid (BTC)-based Cu^{2+} MOF has been shown to encapsulate Fe_3O_4 NPs, and the antibacterial efficacy of Fe_3O_4-loaded MOF $[Fe_3O_4@Cu·(BTC)]$ was evaluated against *S. aureus* and *E. coli* (Zhang et al. 2020). The core-shell magnetic microspheres of $[Fe_3O_4@Cu·(BTC)]$ were synthesized via a layer-by-layer growth process by dispersing mercaptoacetic acid (MAA)-functionalized Fe_3O_4 NPs in $Cu(CH_3COO)_2$ solution (in ethanol) and then added into a BTC solution (in ethanol) under ambient conditions. Bacterial inhibition rate of greater than 90% has been achieved using 30 μg/mL of $[Fe_3O_4@Cu·(BTC)]$ against both the strains after 2 h of incubation, which is better than the pure Fe_3O_4 NPs and [Cu·(BTC)] MOF particles. The antibacterial efficacy of $[Fe_3O_4@Cu·(BTC)]$ is attributable to the controlled and sustained release of Cu^{2+} by framework hydrolysis and excellent photocatalytic performance by generation of the reactive oxygen species (ROS). Thus, considering the superparamagnetic properties of Fe_3O_4

NPs, the nanocomposite under the influence of an external magnetic field can be enriched at the infected site for antibacterial therapeutic applications.

The antibacterial efficacy of 2-methylimidazole-based Zn^{2+} MOF, ZIF-8 (Zeolitic imidazolate frameworks-8), in combination with zinc oxide (ZnO) NPs, was tested against four strains, *E. coli*, *K. pneumoniae*, *P. mirabilis*, and *S. aureus* (Redfern et al. 2018). ZnO nanorods were immobilized on the surface of ZIF-8 particles to obtain a [ZnO@ZIF-8] nanocomposite. A minimum bactericidal concentration (MBC) of 250 μg/mL was determined for each bacterium using [ZnO@ZIF-8], while ZnO nanorods showed an MBC of 800 μg/mL against the four strains. [ZnO@ZIF-8] nanocomposites embedded in silicones (2–4 wt.% ZnO@ZIF-8-loading) demonstrated excellent antibacterial properties by eliminating the microbes from the surfaces within 24 h and are considered suitable in the pursuit to reduce catheter-associated urinary tract infections. Further, the ability of the [ZnO@ZIF-8] nanocomposite (2 mg/mL concentration) to inhibit the well-established biofilms of bacteria (containing 10^7–10^9 CFU/mL) below the detection limit in 24 h broadens the applicability of the nanocomposite for antimicrobial catheters.

Recently, titanium dioxide (TiO_2) and zinc oxide (ZnO) NPs have attracted a lot of attention because of their ability to produce ROS which exert bactericidal effects. Zeolite-A loaded with ZnO and TiO_2 NPs showed bactericidal effects against *S. aureus, E. coli*, *Listeria monocytogenes,* and *P. fluorescens,* (MICs 1–2 mg/mL). Zeolite-A has no antibacterial activity, but the release of ZnO and TiO_2 NPs could produce bioactive metal ions and ROS simultaneously, and a synergistic bactericidal activity will result in the inhibition of bacterial growth (Azizi-Lalabadi et al. 2019). In another study, zeolite-A loaded with Cu^{2+} and cuprous oxide (Cu_2O) NPs demonstrated an antibacterial efficacy of greater than 96% against *E. coli* (Du et al. 2017). It is noteworthy that CuO, ZnO, NiO, and Sb_2O_3 NPs are known to exhibit toxicity to bacteria (*S. aureus, E. coli*, and *B. subtilis*), with CuO being the most toxic of all the antibacterial metal oxides (Baek and An 2011).

Well-defined core-shell [Ag@ZIF-8] nanowires were prepared with a ZIF-8 shell thickness in the range of 30–100 nm by varying the amount of Ag nanowires. The [Ag@ZIF-8] nanowires showed superior antibacterial activities than the Ag nanowires and ZIF-8 nanocrystals when assessed against *B. subtilis* and *E. coli* (Guo et al. 2018). Complete inhibition of *B. subtilis* was observed after 8 h by using 200 μg/mL concentration of [Ag@ZIF-8], while complete inhibition of *E. coli* was noted after 12 h by using 300 μg/mL concentration of [Ag@ZIF-8]. In comparison, ZIF-8 nanocrystals and Ag nanowires showed weaker antibacterial efficacy with 49% and 85% inhibitions, respectively, at a concentration of 200 μg/mL. The antibacterial efficacy of [Ag@ZIF-8] nanocomposite can possibly be explained by the gradual release of Ag^+ by oxidation of the Ag nanowires and the synergistic action of Zn^{2+} ions and the Ag core in ZIF-8 shell.

Another study has showed the complete inhibition of *S. aureus, S. epidermidis*, and *E. coli* (1×10^{10} CFU/mL) growth in PBS solution (pH = 6) by iodine-loaded ZIF-8 [I_2@ZIF-8] at a concentration of 200 μg/mL in 3 min (Au-Duong and Lee 2017). The bactericidal effects of [I_2@ZIF-8] are attributable to the irreversible

damage to the bacterial membrane triggered by the release of iodine and Zn^{2+} from the framework hydrolysis.

10.7 MOFs for Antibiotic Delivery

Drug-resistant bacteria have continuously evolved and beaten the efficiency of several common antibiotics in use. Despite the extensive use of broad-spectrum antibiotics, bacterial infections continue to be a worrying cause of morbidity and deaths worldwide. This is because the lipid and protein compositions of the bacterial plasma membrane have a strong impact on the sensitivity of bacteria to a wide range of antibiotics, and drug resistance generally involves modifications of these macromolecules in the plasma membrane. Therefore, it is always challenging to treat intracellular bacterial infections. Along this line, biocompatible nanocarriers can be advantageous in delivering the antibiotic within the cells, which is considered a promising method to improve the treatment of intracellular bacterial infections. Microporous and mesoporous frameworks, owing to their porous structures, can be efficiently used for drug encapsulation and delivery. Several nanoscale MOFs loaded with common antibiotics may be regarded as a simplified and efficient platform for drug release in the treatment of infectious bacteria borne diseases. Gradual release of antibiotics at the designated infection site can possibly be achieved using MOFs as nanocarriers for antibiotics and facilitating drug delivery into the cells. MOFs can encapsulate both lipophilic and lipophobic drugs which is attributable to the presence of both hydrophilic (metal ion nodes) and hydrophobic (organic ligands) internal microenvironments. The porosity of MOFs enables drug loading and protection of drugs from permeation and degradation, justifying their potential applicability as drug delivery materials.

Zeolitic imidazolate frameworks (ZIFs), a kind of porous MOFs (Chen et al. 2014), are considered to be ideal hosts for the encapsulation and release of antibiotic molecules (Fig. 10.3), where MOFs could be grown around the large bioactive drug molecule, while preserving its bactericidal functions. ZIFs have been successfully employed to encapsulate antibiotics such as ciprofloxacin, ceftazidime, tetracycline, gentamicin, vancomycin, and others, demonstrating their efficacy as carrier materials for antibacterial treatment. ZIF-8 has been confirmed as a safer drug delivery material for efficient chemo-photothermal therapy with favourable biocompatibility and negligible cytotoxicity within a certain dose limit. ZIF-8 is formed by coordination between the nitrogen atoms of 2-methylimidazole (MIm) and Zn^{2+} in aqueous media under ambient conditions. ZIF-8 is decomposable under mild acidic conditions, and thus, it has been used to develop pH-sensitive drug delivery materials. Unlike copper- and silver-based MOFs, the antibacterial action of ZIF-8 has been proposed to be the formation of ROS which accelerates the antibacterial response.

Ceftazidime is a broad-spectrum penicillin antibiotic effective against Gram-negative bacteria. ZIF-8 has been reported to prevent the growth of *E. coli* after 1 day of incubation. Ceftazidime encapsulated in ZIF-8 [Ceftazidime@ZIF-8]

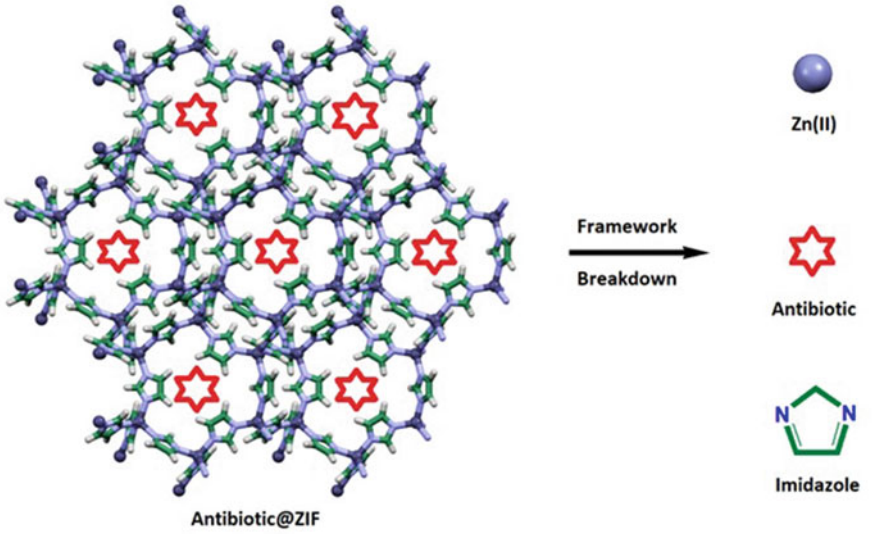

Fig. 10.3 Release of bioactive agents by framework hydrolysis of antibiotic encapsulated in zeolitic imidazolate frameworks [Antibiotic@ZIFs]

showed similar bactericidal action against *E. coli* as that of pure ZIF-8 in mildly acidic Mueller−Hinton (MH) medium at pH 5.0 after 1 day of incubation (Gallis et al. 2019). However, after 3 days of incubation, the [Ceftazidime@ZIF-8] showed complete inhibition of *E. coli* growth at MICs of 100 μg/mL. Interestingly, the antibacterial efficacy of pure ZIF-8 was no longer present with longer incubation time. This suggests that [Ceftazidime@ZIF-8] can be used as a long-term drug release carrier material for antibacterial remediation. Ceftazidime accounts for 10.8% of the total weight of [Ceftazidime@ZIF-8] as assessed by elemental analysis and thermogravimetric analysis.

ZIF-8 can also encapsulate ciprofloxacin, a broad-spectrum fluoroquinolone antibiotic effective against several bacterial strains. In the nano-precipitation methodology, ZIF-8 powder was added to a ciprofloxacin solution and stirred continuously for 5 days to obtain a [Ciprofloxacin@ZIF-8] nanocomposite (Nabipour et al. 2017a). A high drug loading efficiency of 21% was achieved in [Ciprofloxacin@ZIF-8] by following this simple method. [Ciprofloxacin@ZIF-8] releases the encapsulated drug slowly under physiological pH 7.4 (phosphate buffer saline) and much faster in mild acidic media at pH 5.0 (acetate buffer media) during a period of 2–3 days. [Ciprofloxacin@ZIF8] showed about 22% of drug release during the first 2 h, followed by a relatively faster release of the drug during the next 72 h in physiological pH. The antibiotic release profiles of [Ciprofloxacin@ZIF-8] in acetate buffer media showed a faster release of 33% during the first 2 h and a relatively slower release during the next 72 h. [Ciprofloxacin@ZIF-8] showed higher antibacterial efficacy against *S. aureus* and *E. coli* as compared to pure ciprofloxacin and ZIF-8 NPs, confirmed by the diffusion disc assays. Similarly,

[Gentamicin@ZIF-8] with a drug loading capacity of 19% has been synthesized by the nano-precipitation method, which showed better antibacterial efficacy against *S. aureus* than *E. coli* (Soltani et al. 2017); however, this is not so promising in comparison to the efficacy of [Ciprofloxacin@ZIF-8] on the *S. aureus* and *E. coli* strains.

Gentamicin is a broad-spectrum aminoglycoside antibiotic, with low bioavailability after oral administration and poor cellular penetration. Consequently, the repeated intake and high prescribed doses of this drug eventually demand its encapsulation within a biocompatible nanocarrier for sustained drug delivery. Gentamicin-loaded MIL-100(Fe) NPs exhibited similar bactericidal action as free gentamicin, against *S. epidermidis*, *S. aureus*, and *P. aeruginosa*, suggesting that the antibiotic effect of the encapsulated gentamicin was conserved. However, the controlled and sustained delivery of the drug under intestinal conditions (pancreatin supplemented SIF) and at physiological pH (PBS and BSA) makes this MOF nanocomposite highly desirable for the oral ingestion of gentamicin (Unamuno et al. 2018).

ZIF-8 can also be used to co-encapsulate vancomycin and folic acid (FA) in a one pot reaction, and the [Vancomycin@ZIF-8-FA] nanocomposite was applied to boost the therapeutic effectiveness against multidrug-resistant (MDR) Gram-positive *S. aureus* (Chowdhuri et al. 2017). Vancomycin is a glycopeptide antibiotic used generally for the treatment of bacterial diseases caused by Gram-positive strains, and folic acid is a vital nutrient required for nucleotide synthesis in bacteria. Experimental evidences (TEM and DLS) showed encapsulation of vancomycin within the ZIF-8 structure, and folic acid, which can act as a target-specific ligand for bacterial cells, was anchored on the surface of ZIF-8 in [Vancomycin@ZIF-8-FA]. Vancomycin alone and folic acid-conjugated ZIF-8 [ZIF-8-FA] showed negligible antibacterial efficacy against *S. aureus*. However, [Vancomycin@ZIF-8-FA] showed high antibacterial efficacy and effectively killed *S. aureus* strains at MIC of 8 μg/mL. No antibacterial efficacy of [Vancomycin@ZIF-8-FA] has been detected for *E. coli* strains up to MIC of 512 μg/mL, possibly because the nanocomposite cannot penetrate the plasma membrane of Gram-negative *E. coli* cells. Gram-negative bacteria have an outer membrane outside the peptidoglycan layer which is usually not present in Gram-positive strains. Thus, folic acid anchored on the surface of ZIF-8 in [Vancomycin@ZIF-8-FA] enables the facile uptake of the nanocomposite by *S. aureus* cells, and the generation of intracellular ROS (alongside vancomycin release from ZIF-8) resulted in bacterial death. In contrast, vancomycin and ZIF-8-FA showed an insignificant ROS generation.

The antibacterial efficacy of tetracycline-encapsulated MOFs, namely, [Tetracycline@ZIF-8], [Tetracycline@ZIF-67], [Tetracycline@MOF-5], and [Tetracycline@HKUST-1], was evaluated against *S. aureus*, and it was found that [Tetracycline@ZIF-8] has relatively lower cytotoxicity and high antibacterial efficacy (MIC 16 μg/mL) for eliminating intracellular infections (Zhang et al. 2019). Although ZIF-67 has similar structural features to ZIF-8, the leaching of Co^{2+} ions caused a high cytotoxicity, and the MIC value (62.5 μg/mL) exceeded the safe dose range (10 μg/mL). [Tetracycline@MOF-5] and [Tetracycline@HKUST-1] showed

Table 10.6 Comparison of cytotoxicity (μg/mL) and MICs (μg/mL) of the four [Tetracycline@MOFs] and that of pure MOFs against *S. aureus*

MOFs	Metal ions	Safe dose limit	MICs of [Tetracycline@MOFs]	MICs of MOFs
ZIF-8	Zn^{2+}	50	16	125
ZIF-67	Co^{2+}	10	62.5	62.5
MOF-5	Zn^{2+}	30	250	500
HKUST-1	Cu^{2+}	60	>1000	>1000

MICs higher than the safe dose limit (Table 10.6) and no improvements in antibacterial efficacy were observed before and after antibiotic encapsulation. In order to achieve highly efficient inhibition of intracellular bacteria, hyaluronic acid (HA) molecules were immobilized on the surface of [Tetracycline@ZIF-8] particles via coordination of Zn^{2+} in ZIF-8 with the carboxylate groups of HA. HA is a linear polysaccharide from the natural extracellular matrix that could specifically bind to cell-surface CD44 antigen receptors and promote cellular uptake in bacteria. The drug loading efficiency of [Tetracycline@ZIF-8-HA] composite was found to be 59% according to the UV–vis absorption spectrum. [Tetracycline@ZIF-8-HA] displayed an efficient release of antibiotic well above 80% within 3 h at pH 5.5 (acetate buffer media) in comparison to 20% release of antibiotic at pH 7.4 (phosphate saline buffer). The efficient release of tetracycline at acidic pH occurred by the decomposition of the ZIF-8 structure through breakage of the coordination bonds between Zn^{2+} and the imidazolate ligand. [Tetracycline@ZIF-8-HA] promoted the cellular uptake due to the presence of HA and showed higher antibacterial efficacy than pure tetracycline or ZIF-8 due to synergistic effect triggered by the structural breakdown of ZIF-8, releasing both the antibiotic and Zn^{2+} ions. [Tetracycline@ZIF-8-HA] could attain above 98% intracellular bacterial clearance rate at MIC of 50 μg/mL which is within the safe dose limit.

A novel strategy for bacterial infection therapy has been developed by post-modification of ZIF-8 NPs into a light-responsive material (Song et al. 2018). *Ortho*-nitro benzaldehyde (ONBA), a light receptive pH-jump reagent, was incorporated into the mesoporous structure of ZIF-8 NPs that allows the in situ production of H^+ upon irradiation with UV light (365 nm) and, thereby, promotes the pH-dependent framework breakdown of ZIF-8. Thus, ONBA-encapsulated ZIF-8 has further been modified with an antibiotic, [Antibiotic@ZIF8-ONBA], which upon UV light treatment resulted in hydrolysis of ZIF-8, and the antibiotic in the mesopores of ZIF-8 was released slowly in a controllable fashion. The pH-triggered release of antibiotic and bioactive Zn^{2+} ions in the presence of UV light enables the [Antibiotic@ZIF8-ONBA] nanocomposite to inhibit bacterial infections and encourage wound healing, indicating a synergistic and switchable bactericidal effect. Using methicillin-resistant *S. aureus* (MRSA) and ampicillin-resistant *E. coli*, it was shown that the light irradiated controlled hydrolysis of ZIF-8 and the release of antibiotic produced a synergistic bactericidal effect.

The antibacterial efficacy of kanamycin and ampicillin encapsulated in Zn^{2+} MOFs, namely, IRMOF-1 (also known as MOF-5), IRMOF-3, and [Zn-BTC]

were evaluated against *S. aureus, E. coli, S. lentus,* and *L. monocytogenes* (Bhardwaj et al. 2018). Each of these [Antibiotic@Zn-MOF] nanocomposites showed better antibacterial efficacy than the pure MOFs or drugs alone, indicated by the lower MICs of [Antibiotic@Zn-MOF] against both Gram-negative and Gram-positive strains. MICs of kanamycin and ampicillin against the four tested strains were reduced by twofold to fourfold when encapsulated within Zn^{2+} MOFs. [ampicillin@Zn-MOF] and [Kanamycin@Zn-MOF] exceeded the antibacterial efficacy of these antibiotics, but Zn^{2+} MOFs alone showed no significant antibacterial activity (MICs range 100–250 μg/mL). [Ampicillin@IRMOF-3] and [kanamycin@IRMOF-3] were the most potential combinations for the inhibition of bacterial growth. FE-SEM studies showed plasma membrane disruption of *S. aureus* and *E. coli* after 4 h of incubation with [ampicillin@IRMOF-3] and [kanamycin@IRMOF-3] due to synergistic and additive effects.

Nalidixic acid is an effective antibiotic against *S. aureus*, which is however largely resistant to ciprofloxacin and tetracycline. Nalidixic acid encapsulated within a mixed ligand-based Zn^{2+} MOF, [Nalidixic@$Zn_2(BDC)_2(DABCO)$] (DABCO = Diazabicycloctane), showed much better antibacterial efficacy against *S. aureus* and *E. coli* (MIC 0.05 μg/mL/), in comparison to nalidixic acid (MIC 1 & 5 μg/mL, respectively) and pure [$Zn_2(BDC)_2(DABCO)$] (MIC 5 μg/mL). Nalidixic acid release from the MOF takes place gradually in a sustained manner due to structural breakdown, showing 96% and 62% of antibiotic release after 120 h at pH 5.0 and 7.4, respectively (Nabipour et al. 2017b).

10.8 MOFs for Nitric Oxide Delivery

Nitric oxide (NO) is a key biological agent in regulating the cardiovascular, nervous, and immune systems. NO synthesized by the endothelial cells of blood vessels contributes in suppressing the cytotoxic effects of inflammatory cells produced by invading pathogens. The delivery of exogenous NO is an attractive therapy for several ailments. Controlled delivery of NO from a biocompatible material is highly desirable for many antibacterial and wound curing applications (Wheatley et al. 2006). MOFs having coordinatively unsaturated metal sites (CUSs) have been established as efficient NO storage materials where NO is coordinated to the metal centre rather strongly. Efficient NO storage MOFs should have strong interaction of the gas molecules with the porous material to avoid unwanted loss of NO upon storage. Simultaneously, the entire reservoir of NO in MOFs might also be released for therapeutic applications with a simple trigger (like moisture/water) under suitable conditions. Besides high storage capacity, NO release from MOFs should also proceed at the appropriate rate for the desired application, and having control over the kinetics of NO release is an additional advantage. Dihydroxy terephthalic acid (DHTP)-based MOFs, CPO-27-M (M = Co^{2+}/Ni^{2+}) [$M_2(DHTP)(H_2O)_2$]$(H_2O)_8$, upon thermal activation at 110 °C remove the coordinated water molecules together with the guest water molecules present in the pores leaving ~6.4 mmol of CUSs/g of

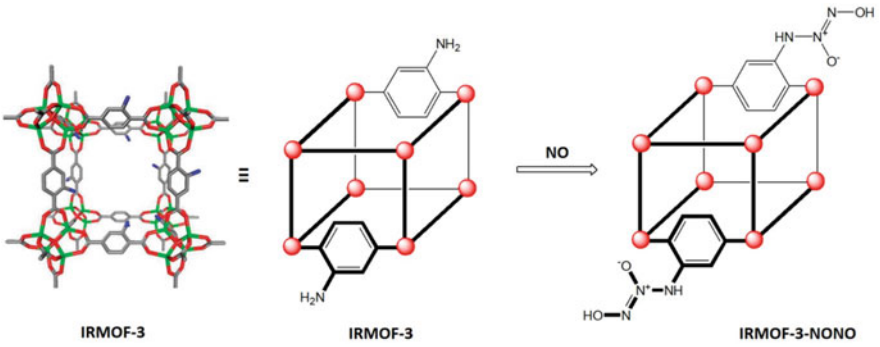

Fig. 10.4 Post-synthetic modification of an amine-functionalized MOF, IRMOF-3, into N-diazeniumdiolate-functionalized IRMOF-3-NONO

activated materials (McKinlay et al. 2008). The activated MOFs showed high uptake of NO by coordination with the CUSs (~7 mmol NO/g of CPO-27 at 25 °C) and water-triggered delivery of chemisorbed NO that is 7000 times more than the HKUST-1 and 7 times more than the best zeolite in use (Xiao et al. 2007). HKUST-1 adsorbs up to ~3 mmol NO/g of activated material at 25 °C, but the control over the NO release rate is inadequate (releases only ~1 μmol NO/g of HKUST-1). However, the toxicologically much more suitable zinc analogue, CPO-27-Zn (also known as MOF-74), upon thermal activation, showed the adsorption of ~5 mmol NO/g of material, which is considerably higher than that of HKUST-1 and other porous materials. It should be noted that there will also be a small amount of physiosorbed (weakly adsorbed) NO in the MOFs along with the chemisorbed (strongly adsorbed) NO. Thus, NO-loaded CPO-27 MOFs are very promising materials for antimicrobial therapeutic applications and to limit inflammatory responses in human tissue. Controlled and sustained delivery of pure NO by biocompatible MOFs is therefore a requirement for antimicrobial therapy, as this significantly reduces the unwanted side reactions like codelivery of carcinogenic or pro-inflammatory side products which may limit their applicability.

Further, primary and secondary amine functionalities can react with NO to form N-diazeniumdiolates (NONOate) which decompose in the presence of moisture at physiological conditions, generating up to two molecules of NO. Thus, post-synthetic modification (PSM) of MOFs by introducing amine functionalities in the frameworks would enable them to form NONOate functional groups. NONOate groups in MOFs are attractive because of the controllable rate of NO release, which can induce antimicrobial action at specific sites of infection. The NONOate groups can be incorporated either into the amine-functionalized organic linker (ligand) of a MOF by PSM or by attaching an amine-functionalized ligand to the unsaturated metal centre followed by PSM with NO.

Amine-functionalized IRMOF-3 and UMCM-1 have been post-synthetically modified to form NONOate-functionalized MOFs (Nguyen et al. 2010). The amount of NO released by the NONOate functionalized IRMOF-3–NONO (Fig. 10.4) and

UMCM-1–NONO in a phosphate buffer can easily be monitored by calculating the nitrite concentration with the Griess assay. In another study, the unsaturated Cu^{2+} centres in HKUST-1 have been functionalized with 4-(methylamino)pyridine, where the pyridine-nitrogen is coordinated to the Cu^{2+} centres and the free amino ($-NH_2$) groups were reacted with NO to form N-diazeniumdiolates (Ingleson et al. 2009). The advancement of NO-releasing MOFs and other similar materials is crucial from the future perspective of antimicrobial therapies.

10.9 Mechanisms of Antibacterial Action

MOFs and their nanocomposites have emerged as the new-generation antibacterial materials, but the systematic mechanistic investigations of their antibacterial effects have not been explored in detail. Nevertheless, several researchers have proposed some possible mechanisms for the antibacterial action of MOFs, based on their experimental results. In order to comprehend the different mechanisms of different antibacterial MOFs and MOF nanocomposites, it is important to know the structural dissimilarities among bacteria. The fundamental difference lies in the cell wall structure of bacteria, known as the peptidoglycan layer. Based on the nature of the peptidoglycan layer, most bacteria can be categorized into two types, Gram-positive strains and Gram-negative strains. Gram-positive strains have a comparatively thicker peptidoglycan layer than Gram-negative strains, whereas Gram-negative bacteria have an inimitable outer membrane structure around the peptidoglycan layer (Schleifer and Kandler 1972). Such structural differences in bacterial cell walls result in varying degrees of tolerance towards MOFs and their nanocomposites. In general, it is observed that MOFs of bioactive metal cations have more pronounced antibacterial effects on Gram-negative strains than Gram-positive strains, or in other words, the Gram-negative strains show greater sensitivity (lesser tolerance) to the antibacterial properties of MOFs (Sheta et al. 2018). This has been reflected in the inherent bactericidal effects of Ag^{+}-, Cu^{2+}-, and Zn^{2+}-based MOFs, which are more potent against Gram-negative strains showing lower MICs and MBCs in comparison to Gram-positive strains, in the majority of the examples discussed in this chapter. Nevertheless, it is interesting to note that the mixed-ligand MOFs of bioactive metal ions like $[Cu_2(Glu)_2(\mathbf{L})]\cdot(H_2O)_x]$ and $[Ag_2(PTA)_2\cdot(\mathbf{L})]$ ($\mathbf{L}$ = bipyridyl and dicarboxylate ligands, respectively) have shown strong antibacterial activities against both Gram-positive and Gram-negative strains. The use of bioactive organic linkers such as azelaic acid and hydrazine benzoic acid in Zn^{2+} MOFs has resulted in superior antibacterial efficacy against Gram-positive strains though.

The antibacterial activities of MOF nanocomposites largely depend on the correct combination of metal/metal oxide NPs and MOFs, which are judiciously used for the encapsulation or immobilization of different NPs. A combination of bioactive Ag NPs encapsulated in a Cu^{2+} MOF [Ag@Cu(TCPP)] was observed to show superior bactericidal effects against Gram-positive strains, while a combination of bioactive

Ag NPs immobilized on the exterior surface of a Ni^{2+} MOF $[Ag@Ni_3(BDC)_2(OH)_2(H_2O)_4]$ was observed to be more effective against Gram-negative strains. In contrast, Ag NPs in combination with ZIF-8 showed a superior bactericidal action against Gram-positive *B. subtilis* in comparison to Gram-negative *E. coli*, but the effective bactericidal concentrations were determined to be significantly much higher as compared to the other two nanocomposites. Further, Fe_3O_4-loaded Cu^{2+} MOF $[Fe_3O_4@Cu(BTC)]$ showed much superior antibacterial action against both Gram-negative *E. coli* and Gram-positive *S. aureus* (> 90% inhibition in 2 h using 30 μg/mL), as compared to ZnO-loaded ZIF-8 and mixed ZnO and TiO_2-loaded zeolite-A tested against the same strains. This has been attributed to the facile generation of ROS by $[Fe_3O_4@Cu(BTC)]$ in comparison to other metal oxides in MOF nanocomposites.

It has previously been proposed that the thick peptidoglycan layer around Gram-positive bacteria prevents the diffusion of most metal cations and metal/metal oxide NPs or any other bioactive substances inside their cell structure (Slavin et al. 2017), and thus, several MOFs and their nanocomposites are less effective against Gram-positive strains such as *S. aureus* and *B. subtilis* among others. However, from the above discussions, it is also evident that the judicious choice of organic linker(s) and metal cations in the synthesis of MOFs and the right combination of bioactive NPs and MOFs in the synthesis of nanocomposites play a crucial role in deciding the preferential antibacterial activities. Thus, it is important to understand the lipid and protein compositions of the peptidoglycan layer of different bacterial strains, which respond differently to different MOFs and their nanocomposites. The general statement quoting the lesser effectiveness of MOFs against Gram-positive strains attributable to the thickness of their peptidoglycan layer may not be the only limiting factor, as discussed above. The more negatively charged cell walls of Gram-positive strains (with respect to Gram-negative strains) have often been attributed to the observed bactericidal effects of some MOFs, but without any proper justification.

Finally, we also need to look at some important points which decide the antibacterial activities of MOFs and their nanocomposites, in general. Along this line, the structure, stability, surface area, pore volume, and loading capacity of MOFs play decisive roles in antibacterial activity. The antibacterial activities of MOFs largely depend on the following factors (Shen et al. 2019; Kaur et al. 2020), if not all together.

1. Ease of release of bioactive ions (Ag^+, Cu^{2+}, and Zn^{2+}) and/or bioactive linkers (ligands) by framework hydrolysis of MOFs (Fig. 10.5).
2. Sustained and controlled release of bioactive ions (Ag^+, Cu^{2+}, and Zn^{2+}) and/or bioactive linkers (ligands) depends on the hydrolytic stability of MOFs operating at physiological conditions.
3. Specific surface area, particle size and morphology, and nature of pores (micropores/mesopores/micro-mesopores) in MOFs are also established to be some important factors.
4. Loading capacity of active pharmaceutical molecules, metal/metal oxide NPs, or any other antibacterial agents is a crucial factor for obtaining effective

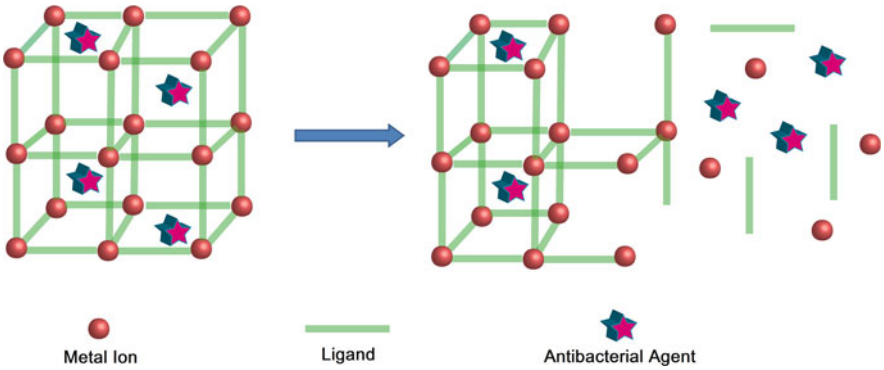

Fig. 10.5 Degradation of MOF composite and subsequent release of metal ions, ligands, and antibacterial agents at the site of action

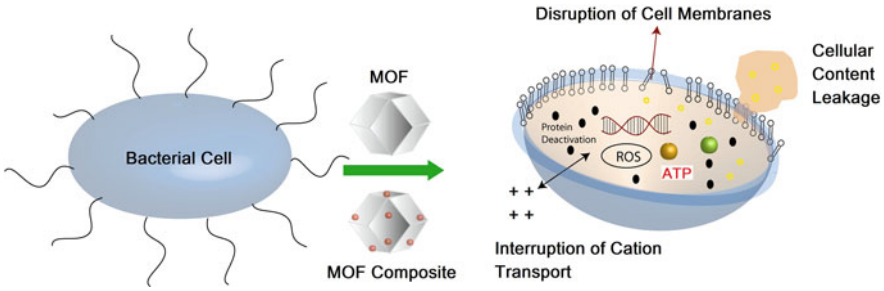

Fig. 10.6 The probable modes of antibacterial actions of metal-organic frameworks and their composites

antibacterial MOF nanocomposites, along with good stability of MOFs for sustained release.

5. Release of metal/metal oxide NPs or antibacterial agents at the infected site of action over a specific time period under the influence of external stimuli such as pH change and light irradiation.
6. Ease of ROS generation by MOFs and MOF nanocomposites at physiological conditions.

The structural degradation of MOF nanocomposite at the site of antibacterial action is shown in Fig. 10.5. It has been observed that the gradual release of metal cations, organic linkers, and antibacterial agents loaded in MOFs (MOF composites) plays some complex synergetic effect (Wyszogrodzka et al. 2016) in damaging the cell membrane by various possible mechanisms such as diffusion-controlled lipid oxidation, direct damage of cell membranes, formation of reactive oxygen species (ROS), interruption of cation transport, chelation induced cell damage, and membrane depolarization (Fig. 10.6).

10.10 Future Prospects

It has been estimated that if no potential antibiotics are developed for the highly infectious bacterial strains, more annual deaths will be caused by drug-resistant bacteria than cancer by 2050, and this might lead to another global health catastrophe. The development of newer broad-spectrum antibiotics is slow at present, and only fewer pharmaceutical companies are investing significantly in the research and development of new antibiotics. Some pharmaceutical companies are continuously in the process of developing new generation of effective antibacterial agents, such as macrolides, cephalosporins, quinolones, and dihydrofolate reductase inhibitors (DHFRs), to overcome the challenges of multidrug-resistant bacterial infections. Bioactive biomaterials with superior antibacterial effects can also be developed in future from natural resources.

One of the major limitations of antibacterial therapy is the lack of an ideal carrier material for the controlled release of antibacterial agents to minimize the cytotoxicity and to maximize the antibacterial efficacy. Along this line, biocompatible and bioactive MOFs can serve the purpose of nanocarriers for active pharmaceuticals including antibiotics and nitric oxide (NO) for effective antibacterial therapy. Similarly, MOFs in combination with phytochemicals having antibacterial properties can also be considered for antibacterial therapy in future. However, a significant amount of research has to be carried out to establish the efficacy, biocompatibility, and cytotoxicity of bioactive MOF nanocomposites for real-life bactericidal applications (Table 10.7).

The demand for medical devices and implants with antimicrobial effects is increasingly growing in the health care market. The antibacterial materials are generally present on the surface or in the matrix of the devices. The use of silver as an antibacterial agent has rapidly increased in silver-containing dressings for infections and surgical care including long-term implants such as artificial joints, catheters, and drains. Unlike antibiotics, the development of bacterial resistance against antiseptics such as Ag and Zn is rare, but not impossible though. Along this line, it can be emphasized that the Ag^+ MOFs and Ag NP-loaded MOFs can be tested as viable options for use in surgical implants and medical devices for long-lasting bactericidal action. However, an ideal antibacterial material should possess the following properties and features for their practical applicability. They must have high antibacterial efficacy, cost efficiency, biocompatibility and biodegradability, high thermal stability, low cytotoxicity, and efficacy over a wide pH range.

10.11 Conclusion

A comprehensive review of the literature available on the antibacterial properties of MOFs revealed that several MOFs which are susceptible to hydrolysis (at neutral or mild acidic pH) have been directly used as antibacterial agents, since their

Table 10.7 List of bioactive MOFs discussed in this chapter of antibacterial MOFs

No	Bioactive MOFs	Ligand used	Co-ligand used	Metal salt
1	$[Ag_3(PBA)]$	3-Phosphonobenzoic acid (PBA)	None	$AgNO_3$
2	$[Ag_2(HIP)(H_2O)]$	5-Hydroxyisophthalic acid (HIP)	None	$AgNO_3$
3	$[Ag_5(PDC)_2(OH)]$	Pyridine-3,5-dicarboxylic acid (PDC)	None	$AgNO_3$
4	$[Ag(PTA = O)]NO_3$	Triaza-7-phospha adamantane-7-oxide (PTA = O)	None	$AgNO_3$
5	$[Ag_2(PTA = O)]SO_4$	Triaza-7-phospha adamantane-7-oxide (PTA = O)	None	Ag_2SO_4
6	$[Ag(PTA = S)]NO_3$	Triaza-7-phospha adamantane-7-sulfide (PTA = S)	None	$AgNO_3$
7	$[Ag_4(PTA = S)](SO_4)_2$	Triaza-7-phospha adamantane-7-sulfide (PTA = S)	None	Ag_2SO_4
8	$[Ag_2(PTA)_2{\cdot}2]$	Triaza-7-phospha adamantane (PTA)	Succinic acid (2)	Ag_2O
9	$[Ag_2(PTA){\cdot}3]$	Triaza-7-phospha adamantane (PTA)	Malonic acid (3)	Ag_2O
10	$[Ag_2(PTA)_2{\cdot}(4)_2]$	Triaza-7-phospha adamantane (PTA)	Phenylmalonic acid (4)	Ag_2O
11	$[Ag(PTA){\cdot}5]$	Triaza-7-phospha adamantane (PTA)	Dimethylglutaric acid (5)	Ag_2O
12	$[Cu(DCBP)(H_2O)_4]$	4,4′-Dicarboxy-2,2-′-bipyridine (DCBP)	None	$Cu(CH_3CO_2)_2$
13	$[Cu_2(Glu)_2(6)](H_2O)_x$	Glutaric acid (Glu)	4,4′-Bipyridyl (6)	$Cu(NO_3)_2$
14	$[Cu_2(Glu)_2(7)](H_2O)_x$	Glutaric acid (Glu)	1,2-Bis(4-pyridyl) ethane (7)	$Cu(NO_3)_2$
15	$[Cu_2(Glu)_2(8)](H_2O)_x$	Glutaric acid (Glu)	1,2-Bis(4-pyridyl) ethylene (8)	$Cu(NO_3)_2$
16	$[Cu_2(Glu)_2(9)](H_2O)_x$	Glutaric acid (Glu)	1,2-Bis(4-pyridyl) propane (9)	$Cu(NO_3)_2$
17	$[Cu_3(BTC)_2(H_2O)_3]$ (HKUST-1)	1,3,5-benzene tricarboxylic acid (BTC)	None	$Cu(NO_3)_2$
18	BioMIL-5	Azelaic acid	None	$Zn(NO_3)_2$
19	$[Zn{\cdot}(DCBP)(H_2O)_6]$	4,4′-Dicarboxy-2,2-′-bipyridine (DCBP)	None	$Zn(CH_3CO_2)_2$
20	$[Zn(HzBA)_2]_2{\cdot}(H_2O)_4$	4-hydrazine benzoic acid (HzBA)	None	$Zn(CH_3CO_2)_2$
21	$[Cu{\cdot}(TCPP)]$	Tetrakis(carboxyphenyl) porphyrin (TCPP)	None	$Cu(NO_3)_2$
22	$[Ni_3(BDC)_2(OH)_2(H_2O)_4]$	1,4-Benzenedicarboxylic acid (BDC)	None	$Ni(NO_3)_2$

(continued)

Table 10.7 (continued)

No	Bioactive MOFs	Ligand used	Co-ligand used	Metal salt
23	[Cu·(BTC)]	1,3,5-benzene tricarboxylic acid (BTC)	None	$Cu(CH_3CO_2)_2$
24	ZIF-8	2-Methylimidazole	None	$Zn(NO_3)_2$
25	ZIF-67	2-Methylimidazole	None	$Co(NO_3)_2$
26	IRMOF-1 (MOF-5)	1,4-Benzenedicarboxylic acid (BDC)	None	$Zn(NO_3)_2$
27	IRMOF-3	2-amino-benzene dicarboxylic acid	None	$Zn(NO_3)_2$
28	[Zn·(BTC)]	1,3,5-benzene tricarboxylic acid (BTC)	None	$Zn(NO_3)_2$
29	$[Zn_2(BDC)_2(DABCO)]$	1,4-Benzenedicarboxylic acid (BDC)	Diazabicyclooctane (DABCO)	$Zn(NO_3)_2$
30	CPO-27-Co	Dihydroxy terephthalic acid (DHTP)	None	$Co(NO_3)_2$
31	CPO-27-Ni	Dihydroxy terephthalic acid (DHTP)	None	$Ni(NO_3)_2$
32	CPO-27-Zn	Dihydroxy terephthalic acid (DHTP)	None	$Zn(NO_3)_2$
33	UMCM-1-NH_2	Tris(4-carboxyphenyl) benzene	2-Amino-terephthalic acid	$Zn(NO_3)_2$

antibacterial efficacy depends on the progressive release of metal ions (and organic ligands) by framework breakdown. Because of the gradual release of metal cations, it has often been thought that antibacterial actions of MOFs are quite similar to the antibacterial actions of bioactive metal ions (salts) or metal NPs. However, several MOFs are known to exhibit better antibacterial activities when compared to metal ions (salts) and metal NPs, due to the controlled release of metal ions by gradual hydrolysis of the metal-ligand coordination bonds in the frameworks. Further advancement in the field of antibacterial MOFs is possible by tailored designing of MOFs through judicious selection of known bioactive ligands and metal salts having pronounced antibacterial properties. BioMIL-5 and $[Zn(HzBA)_2]_2$ are examples of MOFs, where both the ligand and metal cations have antibacterial properties (see bioactive zinc-organic frameworks). The combined effects of bioactive ligands and metal cations of the synthesized bio-MOFs are expected to show superior bactericidal properties. More research has to be directed towards finding the most effective combinations of bioactive ligands and metal cations to obtain bio-MOFs, which will have MICs and MBCs well below the safe dose limit and negligible cytotoxicity to human cells.

Several MOFs can be loaded with metal and metal oxide NPs having antibacterial properties like Ag and ZnO. The bioactive NPs can either be encapsulated within the porosity of MOFs or can evenly be dispersed on the surface of MOFs, depending on the synthesis procedures employed to obtain the MOF nanocomposites. This concept has been widely acknowledged, and many researchers are devoted to finding biocompatible MOF nanocomposites which will be highly effective against

drug-resistant bacteria with little or no side effects. Microporous and mesoporous MOFs having large surface areas can be advantageous for the preparation of nanocomposites due to their ability to encapsulate substantial amounts of bioactive NPs in the pores, which can then be released at the infected site with a simple physiochemical trigger. Thus, the overall synergistic effects of bio-MOF nanocomposites consisting of antibacterial ligands, metal cations, and metal/metal oxide NPs will be larger than the bio-MOF alone for a selected bacterium. Slow degradability of bio-MOFs at physiological pH will be an added advantage for long-term antibacterial action.

MOFs have also been tested as nanocarriers for different types of antibiotics, therapeutic agents, and phytochemicals. MOFs having a high surface area and a large pore volume usually help in the effective loading of larger drug molecules within the pores. Slow and sustained release of such antibacterial drugs from MOFs can then be achieved for therapeutic action under physiological conditions. In most cases, an antibiotic-loaded MOF undergoes degradation in the presence of water, and the rate of release of drug molecules depends on the hydrolytic stability of the MOF in an aqueous medium. Thus, the key challenge in exploring MOFs as effective drug delivery materials lies in the development of bioactive and biocompatible MOFs having high drug loading capacity and good hydrolytic stability for sustained release of the encapsulated drugs at the infected site by slow framework degradation. As discussed earlier, the quest for bioactive and biocompatible MOFs could be overcome by using biologically active ligands and metal salts. Further, surface modification of MOFs with biomaterials or biomolecules can be seen as another alternative to make MOFs biocompatible, similar to folic acid (FA)- and hyaluronic acid (HA)-modified ZIF-8 loaded with antibiotics to counter intracellular bacterial infections (see MOFs for antibiotic delivery). It must be borne in mind that the purpose of the surface modification of MOF loaded with a specific antibiotic [Antibiotic@MOF] is to significantly improve the antibacterial efficacy of the antibiotic by controlled release of the drug via hydrolysis of the MOF and by synergistic effects. The sustained release of NO from biocompatible MOFs and other porous materials is also recognized as one of the most effective and benign strategies for countering different microbial infections in humans.

Finally, MOFs based on photoactive organic ligands and bioactive metal cations can be activated upon external light irradiation to generate reactive oxygen species (ROS) which would exert photodynamic inactivation against bacteria. The photoactive Bio-MOFs can carry more oxygen in the porous structure to accelerate the photodynamic inactivation efficiency. It is to be noted that the application of MOFs and their various composites for antibacterial effects is an emerging area of research (Shen et al. 2019). Thus, an extensive and systematic research focusing mainly on the development of bioactive MOFs is crucial to come to a definitive conclusion for their real-life bactericidal applications. Not to mention, widespread attention among researchers is required to unknot the many covered features of bioactive MOFs for better understanding of their antibacterial properties and bactericidal mechanism, for the advancement of this elusive field.

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Conflict of Interest None to declare.

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Chapter 11
Cationic Amphiphilic Molecules as Bactericidal Agents

Koyeli Das, Vickramjeet Singh, and Ramesh L. Gardas

Abstract Cationic amphiphiles belong to the large as well as assorted category of antimicrobial agents, which has emerged as a sizzling topic of discussion among scientists these days. These antibacterial molecules are being evaluated preclinically and clinically for the treatment of infection caused by drug-resistant bacteria. Due to the widespread application of the cationic amphiphilic molecule (CAM), it's vital to know the effects and detailed chemistry related to the solid surfaces, degrees of confinement on aggregation morphologies, plus chemical kinetics in the self-assemblies of cationic amphiphilic systems. This chapter has included the points described above and phase transitions exhibited by CAMs in the peptides. CAM offers a new tool designed for scientific research with various industrialized applications required for bacterial membrane permeabilizations by optimizing the goal of antibacterial activity, reaching the target drugs, and thereby compromising their structural integrity by cell rupture and death. These results revealed that the varied supramolecular morphologies of CAMs could be controlled by tuning ionic-hydrophobic, hydrophobic-hydrophobic, ionic-hydrophilic, and charge-transfer interactions.

Keywords Amphiphiles · Aggregation morphology · Antibacterial activity · Antimicrobial agent · Bacterial agents · Bacterial membrane permeabilizations · Cationic amphiphilic molecule · Cationic amphiphilic drugs · Cell rupture · Charge-transfer interactions · Diverse self-assemblies · Dispersion medium · Equilibrium · Food industries · Foodborne diseases · Gonorrhea · Hydrophilic and hydrophobic side moiety · Methicillin-resistant *Staphylococcus aureus*

K. Das · R. L. Gardas (✉)
Department of Chemistry, Indian Institute of Technology Madras, Chennai, Tamil Nadu, India
e-mail: gardas@iitm.ac.in

V. Singh
Department of Chemistry, Dr B R Ambedkar National Institute of Technology, Jalandhar, Punjab, India

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Abbreviations

CAD	Cationic amphiphilic drug
CAM	Cationic amphiphilic molecules
MRSA	Methicillin-resistant *Staphylococcus aureus*
SWNT	Single-walled nanotubes

11.1 Introduction

Amphiphilic molecules usually include surfactants, ionic liquids, block copolymers, and other essential bioactive molecules, composed of at least two discrete groups, hydrophilic and hydrophobic. Unique structural designs of the amphiphiles may cause different aggregation morphology at the interfaces as well as diverse self-assemblies in the solutions.

As a result of the unique performances of amphiphiles, they can be extensively utilized in the material, chemicals, food industries, and petroleum products (Ash and Ash 1993). Mainly, self-assemblies in the amphiphiles engage in a crucial role of a biological system, synthesis of varied functional materials. Surfactants or block copolymers are universal templates to direct synthesis of organized nanostructures, while lipids and proteins constitute the main components of the biological membrane. As a result of their structures, amphiphiles possess unique properties (Holmberg et al. 2003; Myers 1999). Strong adsorption in diverse interfaces and self-assemblies in the various solvent (Meleshyn 2009; Sammalkorpi et al. 2008; Ma et al. 2008; Heinz et al. 2008; Jodar-reyes et al. 2008; Gu et al. 2008; Xu et al. 2008; Rodriguez and Laria 2007; Li et al. 2007; Dominguez 2007; Zhang et al. 2007a; Heinz et al. 2007; Zheng et al. 2006; Shah et al. 2006; Israelachvili 1992; Israelachvili et al. 1976). Larson et al. (1985) suggested a lattice model of the self-assembly among amphiphiles in bulk solutions (Larson et al. 1985). Atkin et al. showed adsorption of the amphiphile molecules on silica, graphite, etc., by various experimental techniques (Atkin et al. 2003). Besides adsorption morphologies, surface phase transitions also have essential importance for aqueous surfactants. Surfactant phase transition at the air-water boundary was experimentally discovered (Patti et al. 2007; Ramirez et al. 2007; Hynninen and Panagiotopoulos 2006).

In addition to aggregation on solid surfaces, adsorption of the amphiphiles at various interfaces (Ma et al. 2008; Rodriguez and Laria 2007) in recent years is also studied. With the recent progress of nanotechnologies, studies on the micellization of surfactants in confined systems have turned out to be increasingly important (Chen et al. 2009; Wang 2009; Tummala and Striolo 2009; Angelikopoulos and Bock 2008; Arai et al. 2008; Zhang et al. 2007b; Koopal et al. 2005). Critical micelle concentration (CMC) of amphiphiles is affected through interaction in a surfactant

with a degree of incarceration (Zhang et al. 2007a). Experimentally Wu et al. (2004) compared chains of a sphere, straight cylinders, single helixes, double helices, stack doughnuts, and arrangement of concentric inner shells (Zheng et al. 2007; Yu et al. 2006; Wu et al. 2004). Surfactants have been used as outlines to stimulate orderly nanostructured objects using experimental techniques (Wan and Zhao 2007; Wan et al. 2006; Beck et al. 1992). Carbon nanotubes (SWNTs) are structurally unique, exhibiting mechanical, thermal, electrical, and optical properties, which may offer promises for several novel applications using amphiphiles that would act as a dispersion medium (Wan et al. 2007; Iijima and Ichihashi 1993). Besides adsorption equilibrium, kinetic aspects of amphiphiles at hydrophilic solid surfaces progress rapidly for broad experimental studies (O'Connell et al. 2002). A necessary kinetic process in the case of aqueous surfactant is the micelle/vesicle fission and fusion (Müller et al. 2006; Venturoli et al. 2006; Paria and Khilar 2004). Li et al., in recent studies, investigated the kinetics of collision-based solute exchanges in aqueous phases (Yamamoto and Hyodo 2003).

11.1.1 Bacterial Infection and the Need for Antibacterial Drugs

In this chapter, we have focused on the role of cationic amphiphiles in the environment. Cationic amphiphiles are large as well as various categories of antimicrobial mediators. Though its modes of action are not yet entirely determined, they have emerged as a sizzling topic of discussion among scientists these days as cationic amphiphilic drugs act as a potential candidate in cancer therapy (Geertje et al. 2020; Li et al. 2008). The resistance of antimicrobials (bacteria) against commercially available antibiotic drugs has encouraged scientists to develop alternative safe anti-infection agents (Salta et al. 2013). Emerging multidrug-resistant bacteria have forced the therapeutic community to search for alternative antimicrobial treatments (Omardien et al. 2018). Antimicrobials successfully treat and control different infectious diseases and save several lives (Gunasekaran et al. 2019). Treatment of infections by fighting against infection-causing microbial agents is becoming challenging as per WHO (Zhang et al. 2018), and these infections are tuberculosis, foodborne diseases, gonorrhea, pneumonia, and throat infections (Wahab et al. 2021).

Ever since the discovery of penicillin, various antibiotics have been used for the treatment of infections (Díaz et al. 2012). However, with the widespread use and misuse of antimicrobial agents, various multidrug-resistant bacteria have become a global threat (Santajit and Indrawattana 2016). These superbugs are emerging as a severe concern to pharma industries and pose a significant threat to the human population (Liscovitch and Laviey 1991). Many drug-resistant pathogenic bacteria, the so-called "ESKAPE" bacteria group, include *Staphylococcus aureus*, *Enterococcus faecium*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas*

aeruginosa, and *Enterobacter* (Indoria et al. 2020; Gunasekaran et al. 2019; Kim et al. 2017). Ever since the report of methicillin-resistant *S. aureus* (MRSA) in 1960 (Gunasekaran et al. 2019; Santajit and Indrawattana 2016), it has been identified commonly as a drug-resistant bacteria (Gunasekaran et al. 2019; Liscovitch and Laviey 1991). Various antimicrobial agents inspired by nature have been developed and are still being explored to overcome these resistant pathogens' challenges (Wahab et al. 2021; Omardien et al. 2018; Salta et al. 2013). These antimicrobial agents can be organic or inorganic compounds; nanoparticle-based formulations (Zhang et al. 2018; Anderson and Borlak 2006), antimicrobial coatings, fabrics, textiles, cationic amphiphilic peptides (Indoria et al. 2020), and composite materials have been tested as antimicrobial agents. The antimicrobial drugs, either derived from natural microbes or synthesized in a lab, either destroy the bacteria or hinder bacterial growth, and the bactericidal drugs can kill the bacteria (Kim et al. 2017; Penta 2015). These antimicrobial agents can work by targeting any one of these: (a) translational machinery, (b) cell wall, or (c) DNA replication (Zou et al. 2021; Montazer and Harifi 2020). Various interactions (may cause genetic or enzymatic interference) can occur between the antimicrobial drug molecules and targeted bacteria; among these, hydrophobic and electrostatic interactions occur when cationic amphiphilic (surfactant) disintegrates the membrane of bacteria (Zhou and Wang 2020; Ciumac et al. 2019); however, excessive use of such agents can cause bacterial defiance (Zhou and Wang 2020; Ciumac et al. 2019). Thus, there is a need to improve the current treatment method or develop new biocides and antimicrobial drugs (Zhou and Wang 2020; Ciumac et al. 2019).

11.1.2 Multidrug-Resistant Bacteria and the Search for New Therapeutic Antibacterial Drugs Based on Cationic Amphiphilic Molecules

Novel categories of macrocyclic amphiphilic molecules are achieving progressive interest in the field of nanomedicine because of their basic aspects of molecular identification and robust assemblies. Cationic amphiphilic drugs (CADs) have common physicochemical properties such as a hydrophilic side moiety with a cationic group attached to a hydrophobic part (hydrophobic ring structure or alkyl group) (Vater et al. 2017; Halliwell 1997). Thus, an amphiphilic character is due to a combination of hydrophilicity and hydrophobicity. The former arises due to ionizable amine groups, which are hydrophobic alkyl chains or aromatic ring structures (Vater et al. 2017; Santajit and Indrawattana 2016). The physicochemical properties of CAD molecules are responsible for the distribution pattern of CAD drugs within interacting biological systems; thus, their clinical efficacy involves a complicated interplay of pharmacokinetics and pharmacodynamics (Vater et al. 2017; Halliwell 1997). The CAD-based drugs include different classes such as antipsychotics, tranquilizers, antidepressants, and antiarrhythmics (Vater et al. 2017; Halliwell

1997). CAD led to a morphological change to cells as CAD can accumulate into intracellular compartments (Vater et al. 2017; Halliwell 1997). The intracellular distribution behavior of CAD can be monitored by radio-labeling but may have associated adverse effects due to the random distribution of radioactive tracers into the neighboring sections (Vater et al. 2017). CAD molecules showing ability to act as antidepressants, local anesthetics, neuroleptics, or antiarrhythmics can occur due to their tendency to cause lipidosis (Halliwell 1997).

Owing to various interactions, CAD can accumulate into acidic intracellular components such as lysosomes or endosomes (Salata et al. 2017). The physicochemical properties and structure of CAD, dose, duration of dose, inter- and intraspecies susceptibility, and the mechanism by which CAD shows its action can influence their accumulation in lysosomes, which may take a few minutes or hours (Halliwell 1997; Salata et al. 2017). The intracellular accumulation of phospholipids induced by drugs shows adverse side effects, but the association between drug-based phospholipid accumulation and adverse effects cannot be explained (Liscovitch and Laviey 1991). Mazzaglia et al. (2003), studied an amino-group customized and amphiphilic cyclodextrin complex, which produced aggregation with porphine ligand, a widely used in water-soluble photosensitization (Mazzaglia et al. 2003). Consoli et al. (2018), reported polycationic calix(4) are new amphiphiles, forming assemblies in aqueous solutions (Granata et al. 2017; Bari et al. 2016). Cationic amphiphiles and supramolecules are speedily cleared by circulation and show a greater ability to uptake cells (Blanco et al. 2015). There are specific challenges for the development of these amphiphilic drug delivery systems. Firstly, rare models tested stabilities in addition to target abilities in vivo. Secondly, long-term toxicities of macrocyclic cationic amphiphiles are still now not discovered. On the way to deal with these above concerning facts, interdisciplinary research in all scientific disciplines is needed.

11.2 Cationic Amphiphilic Molecules (CAMs)

CAM is designed to conflict speed rise in drug-resistant bacteria. The design targets the structural integrities of the bacterial membrane, leading towards cell rupture and death. The discrete characteristics of CAMs were wide-ranging; structural activity relations were executed to direct rational designs on potential antimicrobials by desired selectivity and cytocompatibility. Mainly, the effects of CAMs show conformational flexibilities, hydrophobic domain flexibilities, as well as hydrophobic domain architectures. So, CAMs' influences on the antimicrobial efficiencies of Gram-positive and Gram-negative bacteria were determined; the safety profile was created via their impact on the mammalian cells. Every CAM has various potential activities against bacteria, hydrophobic sphere rigidity, and structural designs that contribute to their specificities (Kobisy et al. 2021; Dahlin et al. 2021).

Several therapeutically effective as well as clinically valuable drugs are possibly categorized as CAM drugs**.** Their classifications are based on physicochemical

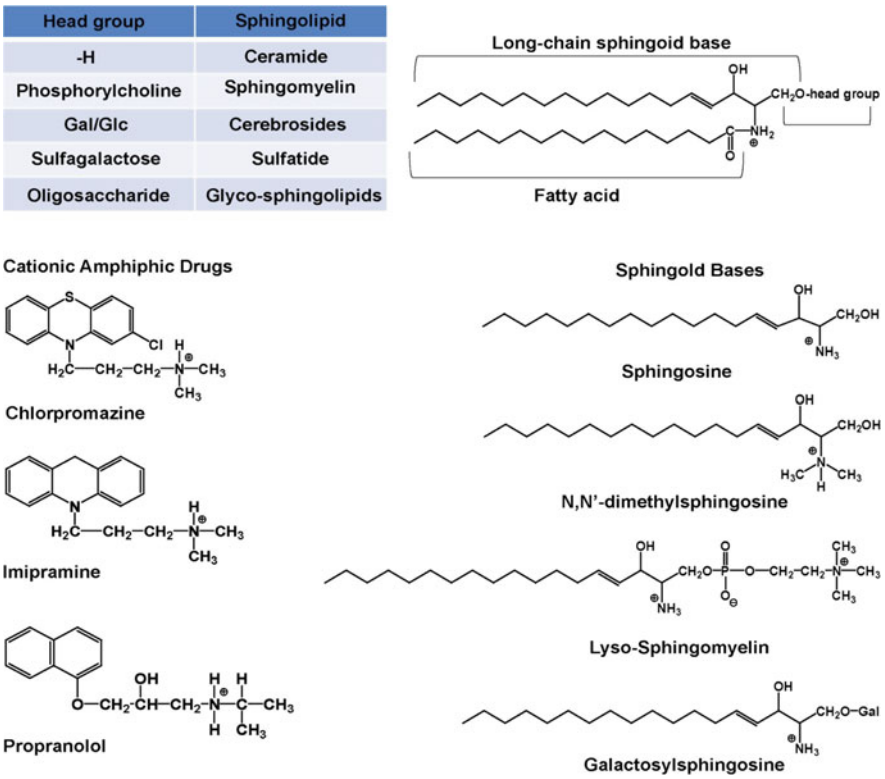

Fig. 11.1 Structures of innovative cationic amphiphilic drugs (CADs). (Liscovitch and Laviey 1991)

properties. In a group, CAM drugs are divided into two specific domains: hydrophobic (i.e., aromatic) spheres with ionizable nitrogen, which is capable of being converted into positive charge atoms (Fig. 11.1).

11.2.1 Natural Cationic Amphiphilic Molecules

Naturally occurring CAM and associated ionic liquids form diverse antibacterial agents, currently validated for preclinical and clinical treatment via antimicrobial resistive bacteria (Kundu 2020). Several studies with cyclic, diastereomers, linear CAM maintained hypotheses with physicochemical properties that are dependable for microbiological activities. It is assumed topologies of CAM are vital for insertion as well as disruption of cytoplasmic membranes. Particularly, the ability to kill bacteria and the difficulties in which bacteria develop resistance make CAMs an attractive target in drug development. However, therapeutic uses in CAMs are hampered due to high manufacturing rates, poor pharmacokinetics, and low bacterial

effectiveness in animal studies. Sequentially, to surmount the problems, various novel and structurally varied CAMs which mimic amphiphilic topologies have in recent times developed. Cationic peptide amphiphiles are a promising stage for the development of novel antimicrobials which can toil as the nanocarriers can be used in synergistic antibacterial therapy (Tague et al. 2019; Almeida et al. 2019; Weeks et al. 2019). In 2014, Kabir-Ud-Din et al. (Yaseen et al. 2014) studied the binding interaction of CAM, which acts as a drug with DNA; absorption studies proved the stabilization of energy levels. In 2018, Joris et al. (2018) demonstrated that cationic amphiphiles (CAM) acting as drugs could be repurposed for the stimulation of lysosomes for siRNA in cancer cells (Joris et al. 2018). Many compounds display finer pharmacokinetics and lower in vitro toxicities by preserving strong antibacterial activities that are hostile to resistant and nonresistant bacteria.

In conclusion, CAM promises soon to provide a novel source of diverse antibacterial compositions. The sphingoid base with amino bases constitutes strength in every sphingolipid (shown in Fig. 11.1). Natural CAMs, also known as sphingoid bases, vary in isomeric configurations, the occurrence of a double bond, the extent of aliphatic chains, and N-methylation group existence. Hopefully, this application can stimulate more research by naturally and synthetically exploring further biochemical processes affected by CAM drugs.

The activity of sphingoid bases in biological modifiers was adequately demonstrated in the latest researches, to appreciate the metabolism of sphingoid bases and their task in cell physiology along with cell pathology (Santajit and Indrawattana 2016).

11.2.2 Synthetic Cationic Amphiphilic Molecules

We present here in this chapter new versatile synthetic strategies for CAM which show tunable amphiphilicity. It is derived from reactive crosslinked precursor molecules, which provide a stage for secondary functionalization by hydrophilic and hydrophobic particles. Since hydrophilic moiety with changeable amphiphilicity instigates from the same precursor, it, therefore, shows related particle size, size distribution, and homogeneous morphology. Consequently, our explanation represents an innovative type of CAM nanocarrier that combines with biocompatible hydrophilic moieties to transport the hydrophobic cargoes (Charrueau and Zandanel 2016; Mura et al. 2013). In 2020, Anje Dong et al. (Zhao et al. 2020) showed how CAM polymers that mimic antimicrobial peptides show excellent antibacterial activity. In 2017, Mark W. Grinstaff et al. (Prata et al. 2018) highlighted the role of size, charge, hydrophobicity, and compaction in the binding of DNA-CAM polyester dendrimer complexes, resulting in improved transfection efficiency. In 2021, Herve Javelot et al. (Xu et al. 2021) proposed that antihistamines and CAM together show varied protective effects against SARS-CoV-2 in patients with mental health disorders. In 2021, Fangong Kong et al. (Yuan et al. 2021) studied lignin-based CAM surfactant properties by amine methylation, ketamine condensation with

alkali lignin acting as raw material. Concluding, alkali lignin can be used as a CAM surfactant in W/O emulsifiers. Therefore, lignin-based CAM surfactants will show immense application prospects shortly soon. Further, Jingcheng Hao et al. (Sarkar et al. 2021) showed the amphiphilicity of the copper nanocluster by tuning the electrostatic interactions with CAM will find applications in light-emitting diodes (LEDs).

Presently, much attention is paid to the organic synthesis catalyzed by ionic liquids (ILs) (Pajuste et al. 2011; Mantarosie et al. 2008; Navajas et al. 2008). According to literature, numerous pyridinium ILs are successfully used as catalysts in various reactions (Wei et al. 2015; Hyvönen et al. 2002). Physicochemical properties of pyrimidine-based CAM were analyzed for their antiproliferative plus antitubercular behavior (Samarkina et al. 2017; Liu et al. 2007; Haldar et al. 2005). D. H. Dagade et al. (Luczak et al. 2010) studied the influence of protic ionic liquids on peptide solvation based on H-bonding, hydrophobicity, etc. and observed the superior ionic-hydrophobic and hydrophobic-hydrophobic interactions. Gabdrakhmanov et al. (Palermo et al. 2012) studied the cationic amphiphiles' behavior with imidazole-based ionic liquids, showed their aggregation properties, and proved their potential in the field of biotechnologies. Fridman (Grenier et al. 2012) reported cationic amphiphiles in the presence of light-induced isomerization could act as antimicrobial drugs. Among various CAMs, surfactants from the imidazolium group deserve special attention. Adjacent to that, imidazolium-based CAM is appropriate for various biotechnological uses: their antibacterial property is reported in the literature (Kunal et al. 2021; Mohammadi et al. 2015) in addition to successful attempts at production of sustainable nanocontainers. CAM drugs engaged clinically to treat a range of disorders; the prospect arises of exogenously controlled sphingoid bases (as well as their synthetic derivatives), which also show comparable therapeutic results. In support of evident reasons, research in pharmacokinetics in vivo is still below structural modifications (Santajit and Indrawattana 2016). CAM drugs that target the enzymes involving whichever sphingolipid hydrolysis or else sphingoid base utilize possibilities towards clinical benefits.

The development of bacteria-resistant strains is of global concern regarding health issues. Scheming antibiotics limiting the rise of pathogen resistance is therefore necessary to treat pathogenic infections. Self-assembling CAMs are a fascinating platform to treat pathogens owing to their capacity to interrupt bacterial membranes and function like drug nanocarriers. Specially designed peptides (CAMs) that form micelles, twisted ribbons, nanofibers, etc., aim to perceptive antimicrobial activities at the supramolecular level. It has been studied by scientists that micelle-forming CAM peptides possess brilliant antimicrobial activities against a variety of Gram-positive and Gram-negative pathogens, for example, MRSA, multidrug-resistant *Klebsiella pneumonia*, and *Pseudomonas aeruginosa* with (MICs) range 1–8 μg/mL, in contrast to the nanofiber which has (MIC>32 μg/mL). All reported records suggest that antimicrobial activities in CAM peptides depend on morphologies, length of alkyl chains, amino acid sequence, and overall hydrophobicity. Various experimental and spectroscopic techniques using MRSA and *E. coli* showed CAM increases cell membrane permeability plus dislocates

integrity of the pathogen membrane, which leads to cellular lysis and, finally, death. (CAMs) peptides are the most promising platforms to build novel antimicrobials that might act like nanocarriers to develop synergistic antibacterial therapies.

11.3 Antibacterial Actions of the CAM Drug Molecules and Mechanism

CAM is generally evaluated for its antibacterial efficacy adjacent to both Gram-positive and Gram-negative bacteria. MIC determines antibacterial activities of the test agent against a specific bacterium (Darya et al. 2017). Assays are carried out by the micro broth dilution technique, and the lowest concentration of the subjected CAM results is noted by seeing the bacterial growth. According to the literature, most CAMs show MICs in proper therapeutic ranges between 15 and 50 μg/mL (Darya et al. 2017; Lind et al. 2015; La Dow et al. 2011; Rotem and Mor 2009; Delcour 2009; Brogden 2005). Conformational flexibilities of cationic head group have less influence on the antibacterial efficacy in experimented pathogens. The increased flexibility of CAM may result in electrostatic repulsions, thereby avoiding simultaneous interactions with a negative charge constituent of the bacterial membranes. So, these results indicate CAM membrane disruption is ruled by electrostatic interaction at the short-charged linker length while at the long linker length; hydrophobic interaction controls hydrophobic domain flexibility (Palermo et al. 2012; Shai 2002; Dagan et al. 2002; Wieprecht et al. 1997; Chikindas et al. 1993). Vemula et al. (Sunnapu et al. 2020) suggested a simple model membrane with a high concentration of antibacterial headed for membrane damages. It provided a preliminary estimation of the potential effectiveness of the studied CAM. The authors (Petaccia et al. 2016) mentioned that liposome-based forms could be used for future studies to improve understanding of the interaction between membranes and CAM (Zana and Xia 2003); one of the leading emergent parts of CAM is the Gemini surfactant in pharmaceutical applications. Interactions of CAM with oppositely charged cell membranes have been acknowledged for several years (Kronberg et al. 2014). However, CAM Gemini surfactants curved to be incredibly capable as bacterial and antimicrobial agents (Mittal and Bothorel 1986). Furthermore, current research in this area indicates the cancerostatic phenomenon of CAM Gemini, during selective interactions in CAM Gemini surfactant by cancer cells (Sharma and Ilies 2014; Misra et al. 2013; Moroi 1992; Porter 1991). An additional revolutionary area in research of CAM Gemini interaction with oppositely charged electrolytes like DNA has importance with respect to gene transmissions through the cell membrane to attain therapeutics within the nucleus (Tague et al. 2019; Pietralik et al. 2015; Hoque et al. 2014; Paniak et al. 2014; Silva et al. 2014; Badr et al. 2010; Moroi 1992; Taft 1952).

(CAM) drugs, also known as cationic amphiphilic drugs (CAD), interrelate with the cell membrane and gather inside the acidic medium intracellular compartments,

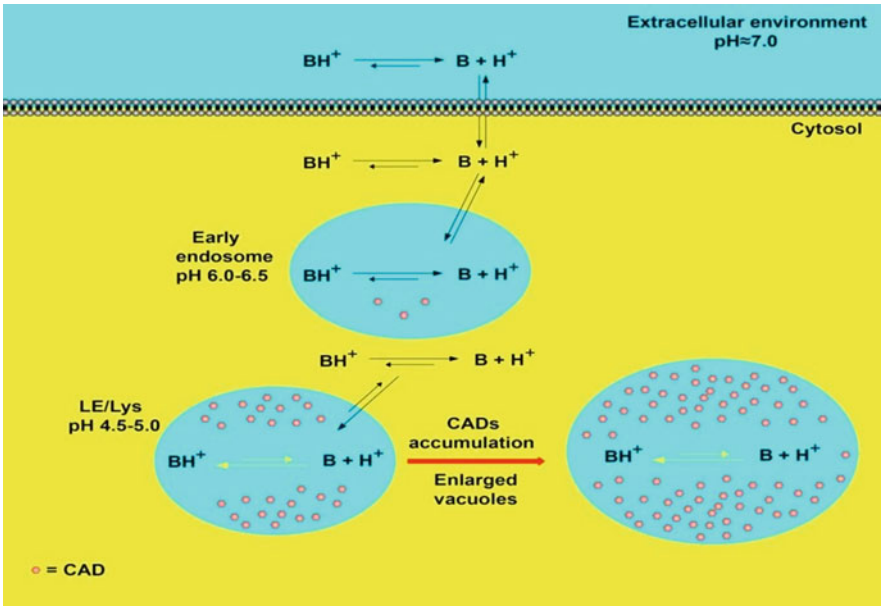

Fig. 11.2 Lysosomal trapping of CADs acting as weak bases (B) as well as cumulates in the intracellular acidic section since lysosomal membranes are to a large extent lesser permeable to protonated base (BH+) in comparison to the uncharged structure. Growths in CADs within late endosomes/lysosomes (LE/Lys)) induce arise in organelles, thereby creating large vacuoles. (Salata et al. 2017)

such as late endosomes or late lysosomes (LE/Lys). So, cellular uptake methods vary among different CADs. Indeed, they collect in (Lys) specific time intervals following in vitro cell exposition, resulting in more diverse kinetics from chemical to physical distinctiveness of the particles. The amine group of CAD is mostly unprotonated at physiological pH. The molecules turn out to be protonated inside the acidic medium of (LE/Lys), since CAD can't further permeate the membrane; they are rapt within the organelle (shown in Fig. 11.2). Many CADs illustrated stimulating phospholipidosis therapeutically, on significant concentrations following chronic treatment (Salata et al. 2017). Though CADs in clinical use are endured, changes in cells are held as a result of the interaction among CADs by the membrane phospholipids. Abilities of precise CAD induced phospholipidosis erstwhile connected with the potency of (drug/phospholipid) interaction. Thus CADs also cross multiple cell membranes to arrive at their target site via catalytic reaction by degrading phospholipid. So, we can find CADs screen to the polar–apolar section of the membrane. At a definite pH, the positive group on the CAD is catalyzed by acid hydrolysis. So, the entire processes, from CAD adsorption to the controlled drug release inside the micelles, occur lying on the particular time-balance by in vivo diffusion rate. So, this process may act significantly on CAD transport (Baciu et al. 2006).

Many researchers have already reported in vitro special antimicrobials (shown in Table 11.1), but less information is known regarding in vivo toxicities. So, further in vivo studies are required sequentially towards understanding (CADs) therapeutic efficacies. Another concerning point in the development of antimicrobial studies is the deficiency of standard experimental procedures. This permits consistent clinical studies to provide information on coating stability and its efficacies, which will cause myriads of innovations (Li et al. 2018; Huang et al. 2016; Hsu and klibanov 2011).

11.4 Synthetic Cationic Amphiphiles in Combination Therapy

Antibiotic resistance is a serious global issue which, without delay, needs efficient solutions. Though small molecules (CADs) are protecting us for almost a century, the emergence of a novel class of antimicrobial drugs also known as synthetic antimicrobial polymers has driven advances in the polymerization as well as the ability to mimic the natural occurrence of antimicrobial peptides which could play a vital role in fighting multidrug-resistant bacteria at future. In exploiting the abilities by controlling chemical as well as structural properties of polymers, the synthetic antimicrobial polymeric materials formed initially from (CADs) could be strategically used in the combination therapies of diverse antimicrobial co-agents with the diverse format to capitulate extra powerful (synergistic) results (Judzewitsch et al. 2018, 2020; Namivandi-Zangeneh et al. 2020; Chandna et al. 2020; O'Neill 2020; Song et al. 2012). Acceptance of the combination therapies in other settings suggests recital efforts by academicians, research funding bodies, international health agencies, governments, regulators, and pharmaceutical manufacturers makes it available at affordable prices worldwide. It is expected that widespread use of the combination pills with routine modifications can bring about substantial risk reductions in several diseases (mainly heart problems). Healthcare systems require deploying strategies efficiently. If implemented, these combination therapy strategies could thereby avoid millions of fatal as well as non-fatal events.

11.5 Challenges and Future Perspectives

Synthetic small-molecule antibacterial peptidomimetics (AMPs) represent a promise in the innovative field of potent antibiotics. AMPs are found in a broad assembly of organisms that protect against pathogens. They are naturally CAMs, which have essential amino acids and hydrophobic side chains. The cationic group shows electrostatic attraction with anionic bacterial membranes, whereas the hydrophobic group gets inserted into the lipophilic core; this eventually leads to the disruption of the bacterial membrane and cell death (Chen et al. 2021; Tague et al. 2021; Mauceri

Table 11.1 Categories of cationic surfactants (CAM) identified in the literature and bacteria involved show various applications presently under preclinical and clinical trial phase

Sl no	Cationic surfactants	Microorganisms involved
1	Rhamno lipids, Vicosin (Araújo et al. 2018; Yazdany and Kazi 2016)	*Pseudomonas aeruginosa, Pseudomonas fluoroscens, Pseudomonas* sp *DSM 2874, Pseudomonas aeruginosa Strain BS2, Pseudomonas aeruginosa DS 10-129 4.31*
2	Trehalose lipids (Ratnikova and Titok 2020; Varjani et al. 2020; Nikolova et al. 2020; Mawgoud and Stephanopoulos 2017)	*Arthrobacter* sp., *Mycobacterium, Corynebacterium, Rhodococcus erythropolis*
3	Glucose lipids (Li et al. 2021)	*Bacillus subtilis*
4	Glycolipids Glycolipids (Pentasaccharide lipids) Sucrose and Fructose Glycolipids (Jana and Kulkarni 2020; Vacca 2017; Brundish et al. 1966)	*Alcanivorax borkumensis, Pseudomonas cepacia, Streptococcus thermophilus, B, Rhodococcus aurntiacus, Rhodococcus* sp. *Strain H13A, Rhodococcus aurantiacus (or R. aurantiacus), Nocardia coryne bacteroides, Arthrobacter paraffineus*
5	Triacylglycerols, steryl esters and wax esters: Neutral lipids, fatty acid + neutral lipids Fatty acids (Holert et al. 2020; Kalscheuer et al. 2007)	*Clostridium pasteurization Corynebacterium salvonicum SFC Nocardia erythropolis Corynebacterium lepus*
6	Acyl glucoses (Haozhe et al. 2020)	*Corynebacterium diphtheriae*
7	Surfactin peptides (Wu et al. 2019)	*Bacillus subtilis*
8	Iturin peptides (Zhao et al. 2021)	*Bacillus subtilis*
9	Fengycin peptides (Yaseen et al. 2018)	*Bacillus subtilis*
10	Viscosin peptides (Bonnichsen et al. 2015)	*Pseudomonas fluorescens*
11	Lichenysin peptides (Coronel et al. 2017)	*Bacillus licheniformis*
12	Serrawettin peptides (Zhang et al. 2021)	*Serratia marcescens*
13	Streptofactin peptides (Crnovčić et al. 2018)	*Streptomyces tandae*
14	Lipo peptides (Sardar et al. 2021)	*Bacillus subtilis, Bacillus licheniformis JF2, Bacillus licheniformis 86, Serratia marcescens, Bacillus subtilis 2.7, Bacillus subtilis ATCC 21332, Bacillus subtilis LB5a*
15	Phospholipids Protein phospholipids (Noba et al. 2019)	*Corynebacterium lepus, Acinetobacter* sp., *Corynebacterium insidiosum*
16	Gramicidins Gramicidin S (deca peptide) (Wenzel et al. 2018)	*Brevibacillus brevis*
17	Polymixins Polymyxin D (deca peptide) (Galea et al. 2017)	*Bacillus polymyxa*
18	Antibiotic TA (Heil et al. 2021)	*Myxococcus xanthus*
19	Corynomicolic acids (Aisaka et al. 2007)	*Corynebacterium insidibasseosum*
20	Emulsan based CAM Lipoteteropolysaccharide (Amani and Kariminezhad 2016)	*Acinetobacter calcoaceticus Acinetobacter calcoaceticus RAG – 1*

(continued)

Table 11.1 (continued)

21	Alasan-based CAM (Navon-Venezia et al. 1995)	*Acinetobacter radioresistens*
22	Liposan-based CAM (Steinmassl et al. 2018)	*Acinetobacter calcoacetices*
23	Lipomanan-based CAM (Steinmassl et al. 2018)	*Acinetobacter calcoacetices*
24	Vesicle-based CAM (Villalón et al. 2019)	*Acinetobacter calcoaceticus*
25	Microbial whole-cell biosensors (MWCBs) (Michael Moraskie et al. 2021)	*Cyanobacter*
26	Phosphatidyl ethanolamine CAM (Tsubaki et al. 2021)	*Acinetobacter* sp.
27	Lipopolysaccharides (Nikolay et al. 2010)	*Acinetobacter* sp.
28	Polysaccharide-protein-based CAM, Protein-lipid-carboxy-based CAM, Sucrose ester -based CAM (Li et al. 2019)	*Corynebacterium hydrocarboclastus*
29	Corynomycolic acid, fatty acid (Cooper et al. 1979)	*Corynebacterium lepus*
30	Ornithin-based CAM (Nigro Di Gregorio et al. 2017)	*Pseudomonas rubescens, Thiobacillus thiooxidans*
31	Trehalosedimycolates (Zhang and DeBosch 2020), Trehalose (mono and di) corynomycolate, Phosphatidyl ethanolamine	*Rhodococcus erythropolis*
32	Rubiwettins R1 and RG1 (Matsuyama et al. 1990)	*Serratiarubidae, Serratiarubidoea*
33	Protein Carbohydrate complex (Ghosh et al. 2019)	*Pseudomonas fluorescens*
34	Methyl mannosylerythritol lipid (Okuhira et al. 2020; Mohamed et al. 2018)	*Streptococcus bovis*, *Fibrobacter succinogenes*, *Ruminococci, Megasphaera elsdenii*, *Selenomonas ruminantium, Succinivibrio dextrinosolvens*
35	Gemini surfactant Gemini pyrimidine CAM (SPYRIT 68, SPYRIT 7) (Koziróg et al. 2017; Zhao and Wang 2017)	*Gram-positive* sp *(S. aureus)*, *Asaia* sp.
36	Methyl imidazolium-based CAM (mim-based IL), Dicationic imidazolium surfactant (Daniel et al. 2021; Liu et al. 2016)	*Escherichia coli, Staphylococcus aureus, P. aeruginosa, S. epidermidis*
37	Lauryl isoquinolinium-based CAM (Yu et al. 2021)	*Escherichia coli*, *B. cereus*
38	Quaternary ammonium-based CAM (QASs) (Borkowski et al. 2018)	*Escherichia coli*
39	Nucleotides, nucleosides, nucleolipids, amino acids, lipo–amino acids, diterpenoids (i.e., natural CAM) (Borkowskia et al. 2018)	*Bifidus* sp., *E. coli, P. gingivalis, Streptomyces Iysosuperficus, Pseudopedobactersaltans, Cyanobacteria*
40	Pyrimidinophanes, pyrimidinocyclophane, multiuracilophane, cryptand-like uracilophane, pyrimidinicamphiphiles (Kumar et al. 2021)	*Escherichia coli, Bacillus subtilis, Mycobacterium tuberculosis (MTB), Neisseria meningitides*

(continued)

Table 11.1 (continued)

41	Gemini lipoaminoacids alkylated lauryl arginine-based liposomes (Gemini analogs) Lysine-based lipoaminoacids Serine-based lipoaminoacids (Azimullah et al. 2020; Pavlov et al. 2020)	*Escherichia coli, Salmonella typhimurium, Azospirillum lipoferum 4B, Bacillus subtilis*
42	Cholesterol (Chol)-based CAM (Araya et al. 2019; Jameson and Wilkinson 2017)	*Escherichia coli, Bacillus subtilis*
43	Arginine-based QACs (Cairns 1980), Alkylated arginine (LAM), Gemini alkylated arginine (C6(LA2) micelles, C9(LA2), C12 (LA2) vesicles, diacylglycerol Arg vesicles (Elwakeel et al. 2018; Savoini et al. 1984)	*Enterobacter lignolyticus, Escherichia coli*
44	Alanine (Gemini-ester-QAC-surfactant) (de Camargo et al. 2017)	*Pseudomonas striata 63, Salmonella typhimurium, Streptococcus faecalis, Staphylococcus aureus, Bacillus stearothermophilus*
45	Lysine-CAM in liposomes (Uyeda et al. 2016)	*E. coli*
46	Serine-gene delivery CAM systems (Mukherjee et al. 2019)	*E. coli, Aeromonas salmonicida, Vibrio alginolyticus*
47	CTAB for insulin delivery (Robeson et al. 1983)	*Escherichia coli, Streptococcus mutans*
48	Di-oleoyl-phosphatidyl-ethanol-amine (DOPE) (Wu et al. 2016)	*Escherichia coli*
49	Di-acyl-glycerol-arginine (Brunello and Marshall 2018)	*Staphylococcus aureus*
50	Di-palmitoyl-phosphatidyl-choline (DPPC) (Thanh et al. 2018; Apisarnthanarak et al. 2017)	*Acinetobacter baumannii, Staphylococcus aureus, Bacillus cereus, Brochothrix thermosphacta*
51	Di-dodecyl-dimethyl- ammonium-bromide (DDAB) (Laalami et al. 2021; Zhou et al. 2020)	*E. coli, Bacillus subtilis*
52	Cetylpyridinium chloride (Pardini et al. 2005)	*Staphylococcus aureus*
53	Diamidequat-based CAM (Im et al. 2019)	*Bdellovibrio bacteriovorus HD100*
54	4-Vinyl-benzyl-phospholipids, Dexamethasone-21-di-sodium-phospholipids (Konuray and Erginkaya 2018)	*Bacillus coagulans*
55	Benzalkonium chloride, Cetalkonium chloride (Forbes et al. 2019)	*Enterococcus faecalis, Escherichia coli*
56	Dopamine hydrochloride acetylcholine chloride, 1-Tetradecyl-3-methylimidazolium bromide (Mahajan et al. 2012)	*K. pneumonia, Bdellovibrio bacteriovorus HD100, Vibrio fischeri*
57	Tryptophan-213-based CAM (Huang et al. 2012)	*E. coli*, *B. cereus*
58	Di-myristoyl-phosphatidyl-choline (DMPC) (Taleb et al. 2016)	*E. coli, S. aureus*

(continued)

Table 11.1 (continued)

59	Bola-form analogue CAM (Orellana et al. 2017)	*Enterobacter lignolyticus*
60	1,4-Di-aza-bicyclo(2.2.2)octane-based CAM (DABCO-n series) Quinuclidine (Q-Nuc-n) (Burilova et al. 2018)	*E. coli, B. aureus*
61	Di-alkyl-amino and nitrogen analogue of hexa-decyl- phospho-choline (Amino and Suzuki 2017; Kurosawa et al. 2017)	*Enterobacter lignolyticus*
62	Di-butyl-amino-based analogue of CTAB (Taleb et al. 2016)	*E. coli, S. aureus*

et al. 2020). There are certain limitations in AMPs because of their in vivo toxicity, high industrialized costs, and susceptibility to the metabolism of proteases. Latest developments in the small molecular AMPs eliminate all limitations; for example, LTX-109, via Lytix Biopharma has already finished (Phase 2) clinical trials of MRSA. Small molecular AMPs show a positive correlation between antibacterial activities and cytotoxicity for on-selective disrupting membrane abilities, suitable for antimicrobial drugs. Further elucidation of vital constituents can cover a broad spectrum of antibacterial efficacy and membrane selectivity, thereby designing potent, selective small AMP mimics in chemotherapeutic agents.

Among the reported antimicrobial (CAM) compounds with high drug-resistant pathogenic activity, some can cause minor damage to the membranes of mammalian cells. The cases presented in this assay suggest synthetic antimicrobial (CAM) may ultimately be highly effective and safer for treating topical, systemic infections; however additional studies are required to attain this goal.

11.6 Conclusions

Bacterial infections can cause various life-threatening diseases and have been developed into severe public health problems by drug-resistant strains. As a result, novel antibiotics with brilliant antibacterial activity and low cytotoxicity are urgently required. Electrostatic interactions caused by the cell membrane of bacteria interference are trailed by cellular component leakage and cell death. Due to bacterial remarkable cell damage, AMPs emerged as valuable against drug-resistant bacteria proved more effective than other classical antibiotics in definite cases. Moreover, structural complexity deprived pharmacokinetic property; low antibacterial activity of AMPs hinders progress in their development. So, researchers took more interest in the modification of it and synthetic AMPs.

Nevertheless, it is crucial to build up complex carriers which are tunable and simpler for industrial scale. To expand application in a domain for specified delivery, two essential factors are required: (1) synthetic peptides to influence pathogens and (2) through design prevention of the production of toxic synthetic peptides.

Together, these two factors can develop novel technologies for synthesis and innovative design strategies at a small price. Therefore, it is necessary to enhance the discovery of potent antimicrobial therapeutic peptides for target bacteria, fungi, viruses, helminths, and protozoa.

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Part IV
Nanomaterial Based Alternative Approaches

Chapter 12
Polymeric Nanoparticles and Nanocomposites as Antibacterial Agents

Akriti Tirkey, Rina Ningthoujam, Bidya Leima Chanu, Yengkhom Disco Singh, Punabati Heisnam, and Punuri Jayasekhar Babu

Abstract The concern of antibiotic resistance is alarming as more bacterial strains acquire resistance against multiple drugs and therefore, infections caused by them are becoming further challenging to treat. According to the Centers for Disease Control and Prevention (CDCP) reports, more than 2.8 million cases of infections due to antibiotic resistance are recorded each year in the United States alone resulting in deaths of about 35,000 people (CDCP 2019). These resistant pathogens besides being a menace in hospital settings, causing nosocomial infections and failure of medical equipment, also pose a threat in food and water industries. Thus, there is an immediate need to address this problem and come up with innovative solutions like fabrication of novel materials or antibacterial agents against which acquiring of resistance can be eradicated. The use of polymeric nanoparticles and creation of polymeric nanocomposites (PNCs) to tackle the problem of antibacterial drug resistance seems like a promising countermeasure which can reduce adhesion and prevent the colonization of bacteria, impede formation of biofilm and kill them. Polymeric nanocomposites are multiphasic composites formed by the amalgamation of two different materials—nanoparticles (NPs) and polymers which results in the creation of a novel substance whose at least one dimension falls in the nanoscale,

A. Tirkey · P. J. Babu (✉)
Biomaterials and Bioengineering Research Laboratory, Department of Biotechnology, Pachhunga University College Campus, Mizoram University (A Central University), Aizawl, Mizoram, India

R. Ningthoujam · B. L. Chanu
Department of Vegetable Science, College of Horticulture and Forestry, Central Agricultural University, Pasighat, Arunachal Pradesh, India

Y. D. Singh
Biomaterials and Bioprocessing Research Laboratory, Department of Post-Harvest Technology, College of Horticulture and Forestry, Central Agricultural University, Pasighat, Arunachal Pradesh, India

P. Heisnam
Department of Natural Resource Management, College of Horticulture and Forestry, Central Agricultural University, Pasighat, Arunachal Pradesh, India

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possessing the intrinsic properties of both the substances along with some new characteristics acquired owing to their synergistic affect (Tamayo et al., Mater Sci Eng C 69:1391–1409, 2016). In this chapter we have discussed about the various polymeric nanoparticles and polymeric composites which have been successfully tested against the different bacterial strains. Also, we have explained the different polymeric nanoparticles and nanocomposites' synthesis methods and their mechanism of action against the microbes.

Keywords Aerosol-based synthesis · Antibacterial · Antibiotic resistance · Bottom-up approach · Coprecipitation · Green synthesis · Inert gas condensation · Liposomes · Matrix nanocomposite · Metallic nanomaterials · Micelles · Nanocomposites · Nanoparticles · Plasma-based synthesis · Polymers · Sol-gel method · Star polymeric nanoparticles · Synthesis · Top-down approach · Vesicles

Abbreviations

AFM	Atomic force microscopy
CMNC	Ceramic matrix nanocomposites
CNT	Carbon nanotube
DLS	Dynamic light scattering
PEG	Polyethylene glycol
PGA	Polyglycol acid
PMMA	Polymethyl methacrylate
PMNC	Polymer matrix nanocomposites
XPS	X-ray photoelectron spectroscopy

12.1 Introduction

Bacterial infections pose a great peril to the entire global health especially now when the efficacy of antibiotics on them has significantly reduced due to their potential to develop resistance against them. Bacteria owing to their fascinating skills are able to develop or acquire resistance against antibiotics and other chemical-based antibacterial agents over the course of their exposure due to problems like inactivation of the agent intracellularly or its limited diffusion. The concern of antibiotic resistance is alarming as more bacterial strains become resistant to multiple drugs, and therefore, infections caused by them are becoming further challenging to treat. According to the CDC reports, more than 2.8 million cases of infections due to antibiotic resistance are recorded each year in the United States alone resulting in deaths of about 35,000 people (CDC 2019). These resistant pathogens, besides being a menace in hospital settings, causing nosocomial infections and failure of medical equipment, also pose threat in food and water industries. Thus, there is an immediate

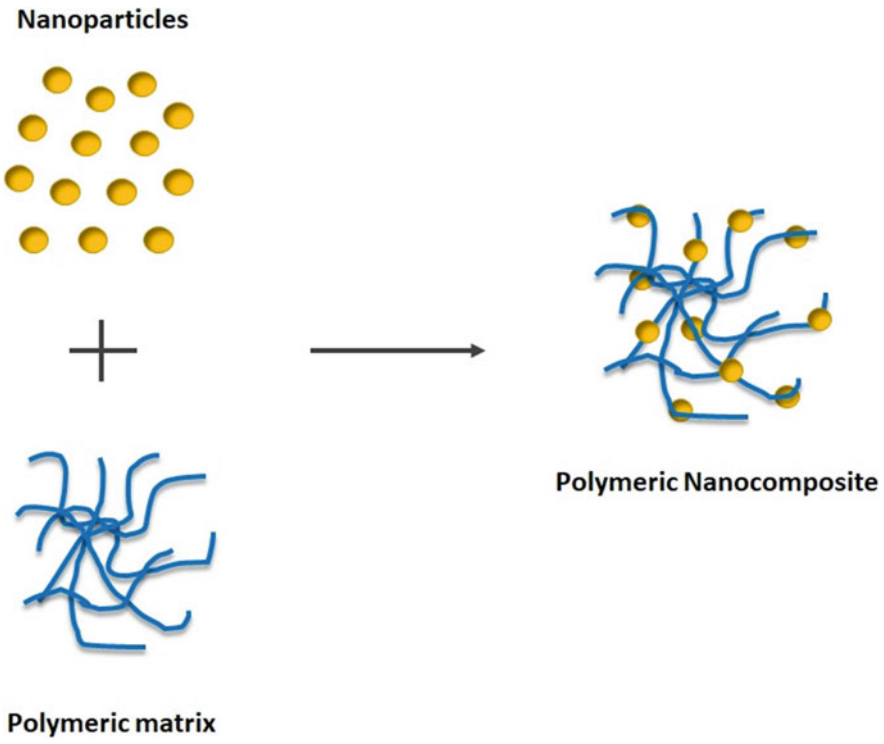

Fig. 12.1 Pictorial representation of a polymeric nanocomposite

need to address this problem and come up with innovative solutions like fabrication of novel materials or antibacterial agents against which acquiring of resistance can be eradicated.

The use of polymeric nanoparticles and fabrication of polymeric nanocomposites (PNCs) to tackle the problem of antibacterial drug resistance seems like a promising countermeasure which can reduce adhesion and prevent the colonization of bacteria, impede formation of biofilm and kill them. PNCs are multiphasic composites formed by the amalgamation of two different materials—nanoparticles (NPs) and polymers which give rise to a novel substance whose at least one dimension falls in the nanoscale, possessing the intrinsic properties of both the substances along with some new characteristics acquired due to their synergistic affect (Tamayo et al. 2016). Figure 12.1 shows a pictorial representation of nanocomposites. NPs/nanocomposites are particles having dimensions in the nano-range; between 1 and 100 nm can be synthesized using various methods (Babu et al. 2010, 2011a, b, 2012a, b, 2013a, b) and can be used for many biomedical applications (Babu et al. 2018a, b, 2020, 2021). These nanomaterials give an increased surface area to volume ratio and therefore, higher reactivity than their macro or bulk counterparts (Bardajee et al. 2012). Figure 12.2 gives a basic concept about the size of different substances

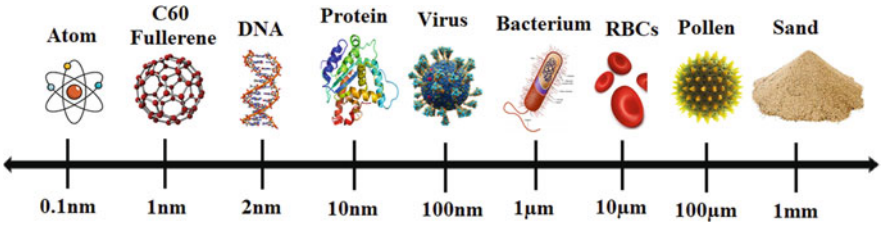

Fig. 12.2 Length scale showing the different substances that have size in the nano-range

whose size lies in the nano range. Polymers are materials formed by several repeating units or monomers and are known to have unique physical and mechanical characteristics like toughness, strength, thermal stability and are also eco-friendly. Owing to these properties, PNCs are advanced materials wherein the NPs are either incorporated into the polymeric matrix or are immobilized onto the polymeric surface to achieve improved features and novel and specific applications (Ahmad et al. 2011). Some commonly employed nanofillers include nanosilver (AgNPs) (Mei et al. 2014), gold nanoparticles (AuNPs) (Regiel-Futyra et al. 2015), copper nanoparticles (CuNPs) (Tamayo et al. 2016), alumina (Al_2O_3) (Viswanath and Ravishankar 2006), titanium dioxide (TiO_2) (Su and Kuramoto 2000), zinc oxide (ZnO) (Chae and Kim 2005), iron oxides (Fe_3O_4, Fe_2O_3) (Peymanfar et al. 2019), carbon nanotubes (CNTs) (Yazdani et al. 2015), carbon nanofibers (CNFs) (Shimoda et al. 2010), graphene (Yazdani et al. 2015). Few polymers utilized for the fashioning of PNCs include polyvinyl alcohol (PVA), polyglycolic acid (PGA), polyethylene glycol (PEG), polyvinyl pyrrolidone (PVP), peroxyacetic acid (PAA) (Jovanović et al. 2011; Shin et al. 2008), polyaniline (PANI) (Barkade et al. 2011), chitosan (Mallick et al. 2012), cellulose (Cady et al. 2011), carboxymethyl cellulose (CMC) (Zhong et al. 2013), silica (Cioffi et al. 2005), polypropylene (Palza et al. 2010), polyvinyl chloride (PVC) (Becerra et al. 2013), polyvinylidene fluoride (PVDF) (Cioffi et al. 2005), high-density polyethylene (HDPE) (Bikiaris and Triantafyllidis 2013), and polymethyl methacrylate (PMMA) (Weickmann et al. 2005).

Studies have already witnessed the bactericidal properties of both NPs and polymers individually, and now the focus has shifted to PNCs, which because of the mixing of two materials produce a highly effective antibacterial agent with improved efficacy and broad target spectrum. Also the presence of two or multiple components significantly reduces the likelihood of resistance. Nanomaterials have exhibited antibacterial activity through different mechanisms such as direct cellular interaction like interference with the electron transfer across their membrane, destruction of the cellular membranes or oxidation of cellular components like proteins, and damaging of DNA by production of secondary substances like ions and ROS (reactive oxygen species) (Li et al. 2008). Cationic polymers utilized in nanocomposites possess high concentration of positive charge which shows affinity for adsorption to the phospholipids which possess negative charge on the bacterial membrane,

through electrostatic forces, leading to the destruction of the membrane and resulting in the leakage of the cellular components (Mei et al. 2014; Murata et al. 2007). The coating or covering of NPs with polymers confers stability to them in aqueous solutions (Mei et al. 2014). The immobilization of NPs on the porous matrices of the polymers allows the delaying of the releasing time of the NPs, thus permitting access over controlled delivery of NPs for longer activity (Akhavan 2009), and it also prevents aggregation of NPs (Chen et al. 2014). In some instances, immobilization of NPs prevents its release and impedes its crossing of biological membranes, thus inhibiting direct uptake by the cells (Chen et al. 2014). Few PNCs having antibacterial activity can also be used as the main component or a booster ingredient in drugs (Ghaffari-Moghaddam and Eslahi 2014). The use of PNCs also widens their scope of applications in several fields including biomedicine (Hule and Pochan 2007), drug delivery (Ali and Ahmed 2018), textiles (Gowri et al. 2010), food (Emamifar et al. 2010), cosmetics (Kaur et al. 2021), water treatment (Yin and Deng 2015), agriculture (Merino et al. 2018), environment (Kumar et al. 2017), and household necessities like disinfectants (Jiang et al. 2016) by attaining a set of desirable features which may be absent in one of its components. This study gives a brief introduction to the NPs and PNCs. It also describes the various types and synthetic methods employed for these nanomaterials and focuses in detail on the applications of various types of nanomaterials—metallic, polymeric, and nanocomposites as antibacterial agents.

12.2 Shape and Properties of Nanocomposites and Nanomaterials

NPs are minute particles whose nanosize and high surface area to volume ratio imparts them extraordinary physicochemical characteristics and distinguished optical properties. Their morphology such as structure, shape, and size determines their other characteristics like reactivity and strength. They are said to have three different layers—the surface, which may contain various functionalized molecules; the second layer is believed to be chemically distinct from the innermost layer and is called the shell layer; and the last or the innermost central component of the NP is termed as the core (Shin et al. 2016). These NPs can be grouped into different categories based on their composition and features such as carbon-based NPs, metal NPs, ceramic NPs, semiconductor NPs, polymeric NPs, and lipid-based NPs (Khan et al. 2019). Among carbon-based NPs, carbon nanotubes (CNTs) and fullerenes are two very popular forms where CNTs have an elongated and tube-like structure whose diameter ranges between 1 and 2 nm (Ibrahim 2013), while fullerenes are made up of carbon units which are arranged pentagonally or hexagonally and have more allotropic shapes, i.e., their shapes can vary such as globular hollow cages (Khan et al. 2019). These nanomaterials are recognized for their extraordinary structure, strength, electron affinity, electrical conductivity, and versatility (Astefanei et al. 2015). The

Fig. 12.3 Pictorial representation of some common shapes of nanoparticles

hollow center of the CNTs enables the filling of different materials like biomolecules in it and therefore, can act as a carrier (Marosföi et al. 2006). CNTs are composed of cylinders of graphene and can exist in two forms—single-walled CNT (SWCNT) which has a single layer of graphene cylinders and multiwalled CNT (MWCNT) which has two or multiple concentric sheets of graphene cylinders surrounding a hollow central core. They possess microcrystalline properties of solids and act either as metals or semiconductors (Camargo et al. 2009). Metal NPs, as the name sounds, consist of metallic units and are known to have unmatched optoelectrical features due to their localized surface plasmon resonance (Khan et al. 2019). Ceramic NPs are inorganic in nature, and they can be present in different forms such as polycrystalline, amorphous, hollow, porous, or dense (Sigmund et al. 2006). Semiconductor NPs are known to possess certain characteristics which are both metallic and nonmetallic in nature because of the presence of large bandgaps during tuning of which, the features tend to vary (Khan et al. 2019; Ali et al. 2017). Polymeric NPs are organic in nature and found commonly in nanocapsular shape, where the particle encapsulates the solid mass or nanospherical shape where sphere itself is completely solid and has molecules immobilized on its surface (Mansha et al. 2017; Rao and Geckeler 2011). Lipid-based NPs have a lipophilic matrix which encloses a lipid core and are generally in the form of spheres with diameter between 10 and 1000 nm (Rawat et al. 2011). NPs having anisotropic shapes like rods, cubes, discs, or prisms whose properties vary along different dimensions have become very popular due to their unique features like large surface plasmons that can be altered by modifying their shapes and sizes (Hao et al. 2004; Xia et al. 2003), have varied affinities for different chemicals which allows the possibility of end-to-end assembly (Dimitrijevic et al. 2005) and increased surface area to volume ratio which results in better catalytic activity (Cozzoli et al. 2003). Figure 12.3 represents some common shapes of nanoparticles.

Nanocomposites are high-performance hybrid materials created by the incorporating NPs (discontinuous reinforcing phase) into the polymeric matrices (continuous phase). They possess unique and enhanced combinations of characteristics and allow numerous designing opportunities (Camargo et al. 2009). Their properties depend on the properties of their individual components like their shape, size and composition. They also rely on the loading, dispersion, and mobility of the NPs and the degree of polymerization of the matrix along with the interactions between the two components. These interfacial interactions become more prominent at the nanoscale range and thus, have a greater effect on the properties of the material. The NPs or nanofillers can directly affect the mechanical, optical, thermal, electrical,

and rheological characteristics of the nanocomposites (Kutvonen et al. 2012). The interaction and bond formation between the matrix and NPs determines the mechanical characteristics of the nanocomposites (Liu et al. 2011a). Studies have shown that the nanofiller size significantly affects the mechanical toughness of the PNCs and the toughness increases with reduction in nanofiller size as the high surface area to volume ratio leads to stronger interaction between the NPs and polymer (Liu et al. 2011b). Mobility of the nanofillers inside the matrix of the polymer affects its resistance and durability against deformation as the mobile NPs exhibit better local tension release (Gersappe 2002). The nanofiller shape also determines the properties of the PNCs as demonstrated in a study by Kutvonen et al. (2012). According to their results, rod-shaped NPs displayed better reinforcement of the polymer and elastic modulus than the triangular NPs, and the least effective were the spherical NPs. The loading requirement of the NPs also varied depending on their shape to attain the same elastic modulus. At 15% loading, triangular NPs demonstrated the best elastic modulus followed by rods and then the spherical NPs. Another study described the impact of the arrangement of the NPs on the polymer as it demonstrated that immobilizing the NPs in a raspberry-like arrangement on the spherical substrate allowed the recycling of the NPs while sustaining their size effects (Liu et al. 2013).

Depending upon the type of material used as the matrix, PNCs can be classified into three types—ceramic matrix nanocomposites (CMNCs), metal matrix nanocomposites (MMNCs), and polymer matrix nanocomposites (PMNCs). CMNCs are created by incorporating nanofillers like particles, fibers, whiskers, or platelets into the ceramic matrix to overcome its brittle nature and confer mechanical toughness on top of the existing features of the ceramic like resistance against wear, high chemical and thermal stability (Harmer et al. 1992; Becher 1991; Lange 1973). Some of the usual materials used as ceramic matrices are silicon carbide (SiC), silicon nitride (SiN), and alumina (Al_2O_3). MMNCs consist of a matrix which is made up of metals or metallic alloys such as aluminum, magnesium, lead, tin, iron, etc. which are known for their strength, toughness, high modulus and ductility. PMNCs have a polymeric matrix which offers benefits like ductility and light weight. The incorporation of metallic or ceramic nanofillers in it further reinforces its mechanical strength, improves modulus, heat resistance, flame retardancy, imparts resistance, biodegradability and reduces electrical conductivity and gas permeability (Fischer 2003; Kickelbick 2003). A few types of polymers used as matrix include polyolefins, vinyl polymers, specialty polymers and condensation polymers (Camargo et al. 2009). Another type of nanocomposites are the ones in which the polymers filled with nanofillers are layered to achieve improved properties such as high strength, increased modulus, thermal stability, gas barrier capacity, biodegradability and reduced flammability. These features are attained only on account of the dispersion of nanofillers amidst the layers and the ion exchange between the inorganic and organic ions (Camargo et al. 2009).

12.3 Synthesis of Various Nanoparticles and Nanocomposites

Since twenty-first century, nanotechnology has played a very vital part in the advancement of science and technology which is largely due to the development achieved in the synthesis of NPs. The development of nano-field has led to an incredible evolution in numerous areas such as food, agriculture, pharmaceutical, material science, biotechnology, medicine, energy, and environment. Comparison of bulk materials of corresponding NPs with respect to their surface to volume ratio shows that NPs emerge as a new material possessing amended properties and qualities such as catalytic activity, hardness, mechanical strength, optical characteristics, and antimicrobial effects. NPs are known for their large, efficient surface area which is able to fix, absorb, and transfer the supplementary compounds where this surface area is extrachemically vigorous than the fine analogue (Abhilash 2010). The description of NPs is necessary to know and regulate their size during nanoparticle synthesis. This description can be achieved by the employment of some prevailing methods such as dynamic light scattering (DLS), atomic force microscopy (AFM), Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), X-ray photoelectron spectroscopy (XPS), interferometer and nuclear magnetic resonance (NMR), etc. Synthesis of NPs refers to the approaches for generating NPs which are mainly derived from larger molecules (top-down) or by "bottom-up" approaches by allowing the growth of particles from the molecules present in a vapor or liquid form (Rosa et al. 2017). Since the early advances of nanoscience, nanomaterial synthesis has turned into a great challenge with respect to obtaining high yield along with low cost. NP application in medical science is determined by the capability of synthesizing the particles with distinct shape, size, chemical composition, and monodiveristy.

NPs can be generated by employing various chemical, physical, and biological approaches. Although the chemical and physical approaches have a long-term advantage because of the uniform-sized NPs but they are not compatible with processes involved in food, cosmetics and medicine as they are non-eco-friendly, expensive and generate many hazardous and toxic byproducts. So, for the last few years, biosynthetic approaches have been recognized as an important method for the fabrication of metal-based NPs like gold, silver, copper and platinum (Robin and Anwarul 2020). Production of NPs by biosynthesis is an eco-friendly pathway where the agents for reduction and stabilization are either plant, bacteria, fungi or yeasts themselves. There are numerous plants that comprise of several biologically active compounds such as alkaloids, citric acid, terpenes, flavonoids, phenols, polyphenols and reductases acting as reducing agents of metal salts (Sharma et al. 2012; Augustinea and Hasana 2020).

12.3.1 Synthesis Approach

NP synthesis methods are divided into two approaches: top-down and bottom-up approach (Fig. 12.4).

12.3.1.1 Top-Down Approach

Top-down approaches mainly utilize macroscopic structures and begin the process by reducing the size of the larger particles to NPs after performing sequential operation milling, attrition process, and electro-explosion wire techniques. Though these are quick manufacturing processes, they involve large equipment installation and more energy, and their setup requires a huge capital, so they are not appropriate for large-scale production. Additional drawback of top-down approaches is the limitation of surface structure, and their defects have a substantial impact on the physical properties of NPs (Thakkar et al. 2010). Synthesis of Copper nanoparticles (CuNPs) is done by employing the technique of electro-exploding wire (EEW) of the top-down approaches (Siwach and Sen 2008). In this technique copper plate is kept inside an appropriate medium such as water, where approximately 1010 A/m^2 current is provided to the medium through copper wire leading to the melting and evaporation of copper metal plate. The evaporation of metal makes the plasma easily disseminated in the media followed by dispersion of the particles. Similar to their findings, Alqudami and Annapoorni (2007) reported the silver nanoparticle synthesis and structural analysis using techniques like XRD, SEM, UV-Vis spectroscopy, etc. The conventional milling method generates large amount of heat, and also there is occurrence of particle accumulation in order to evade the development of wet milling method (Koch 1997).

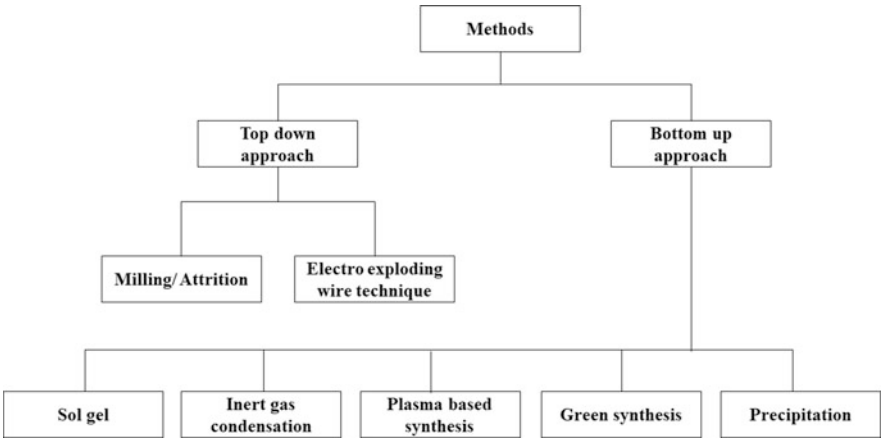

Fig. 12.4 Flow sheet for methods of nanoparticles synthesis

12.3.1.2 Bottom-Up Approach

The approaches of bottom-up are the invention or building of nanomaterial from molecule by molecule, atom by atom, or cluster by cluster. This approach requires the prereduction of elemental materials to the atomic level with the extra technique foremost to the progress of nanostructures. During the extra evolution process, there is an accumulation of simple units into larger stable one when physical forces act on the nanostructure. Nanotechnologists typically favor bottom-up approaches for the synthesis of complex nanostructures as they offer certain advantages in accurately controlling the size of the particle, thus ensuring good optical, electronic, and other properties (Doyle and Glass 2010). Bottom-up approaches involve different common methods such as the following:

12.3.1.2.1 Sol-Gel Method

Sol-get method is a well-recognized procedure for the synthesis of colloidal NPs from liquid phase permitting easy shaping with low temperature, flexible rheology, and versality. The process involved in sol-gel technique is considered suitable for the fabrication of oxide NPs and composite nanopowder. The development of TiO_2-based NPs is considered a well-established hot subject owing to its extensive application series in shape memory alloy, solar cells, and photocatalysis. Ramesh (2013) revealed the use of zinc nitrate, zinc chloride, and sodium hydroxide as precursors for the synthesis of zinc oxide particles.

12.3.1.2.2 Coprecipitation

Coprecipitation is an extensively used wet chemical process which is easy and economic and offers great reproducibility. NPs are formed by a quick dispersal of polymer solvent into a non-solvent polymer and finally giving the polymer solution a stir. Among these two phases, interfacial strain generates an extreme surface area leading to impulsive precipitation of NPs. Copper acetate and sodium hydroxide were used in the form of precursors and reducing agent, respectively, for the synthesis of copper oxide NPs, exhibiting biocidal activity against microbes by precipitation method (Kandasamy and Sorna 2015).

12.3.1.2.3 The Inert Gas Condensation

NPs were formed through the evaporation of a metallic base in an inert gas leading to the vaporization of the metal at a sensible rate and temperature. According to Perez-Tijerina et al. (2008), the synthesis of bimetallic Au/Pd NPs was through highly size-controlled inert gas condensation process. The synthesis of NPs was examined by

mass spectroscopy, electron microscopy, TEM, atomic force microscopy, etc. in order to prove the distribution and geometry of metals. An experiment was conducted on the development of Cu and Si group of NPs by inert gas accumulation (Banerjee et al. 2008).

12.3.1.2.4 Green Synthesis

Besides physical and chemical methods, the development of green synthesis in nanotechnology is a recent technique using plants and microorganisms which are eco-friendly without causing any harmful effect and avoiding extra cost of chemicals expected for a large scale of production (Babu et al. 2010, 2011b, 2012a, b, 2013a, b; Iravani 2011). Phenolic acid having carboxyl and hydroxyl groups are powerful antioxidants. The formation of metal NPs by reducing metallic iron was done by active hydrogen (Manjula et al. 2012). Both gold and silver NPs were synthesized from the fruits of *Averrhoa bilimbi* by utilizing chloroauric salt and silver nitrate as precursors. Their respective color changed to yellow and violet, signifying the generation of gold and silver NPs. Vidya et al. (2013) also reported the synthesis of ZnO nanoparticles from the leaf extract of *Calotropis Gigantea*, and particles were characterized by SEM and found to be 10–15 nm in size and spherical shape.

12.3.1.2.5 Plasma-Based Synthesis

This technique involves the creation of thin film NPs and is effectively employed for the synthesis of nanosized powders like borides, oxides, and carbides. The finding of Padmanabhan et al. (2006) reported TiO_2 nanoparticle synthesis by the method of thermal plasma which proceeded using the precursors titanium hydride powder and titanium metal powder. Typical functional parameters were considered like arc voltage, arc current, torch input power, feed rate for metal/hydride powders, and various gaseous flow rate. The description of the powder was finished by SEM, XRD, surface area analyzer, etc.

12.3.1.2.6 Aerosol-Based Synthesis

For the large-scale synthesis of NPs, aerosol-based methods are found to be broadly appropriate. In the gas phase, the aerosol size is around 100 nm and produces nanomaterials of distinct chemical composition and morphology. Laser pyrolysis and spray pyrolysis are two forms of aerosol-based methods. An investigation by Prakash et al. (2004) demonstrated the synthesis of nano-porous iron oxide particles by utilizing batch aerosol reactors by aerosol-gel technique.

12.4 Polymeric Nanoparticles as Antibacterial Agents

Application of nanotechnology in antibacterial studies is gaining quite a momentum in present-day research activities. It has been scientifically exploited as an alternative tool against antibiotic resistance catastrophe. Nanotechnological applications have extended usage as drug transporters, preventatives, and also as an analytical agent in antibacterial remedies. Nanomaterials are size efficient, thus making it very suitable for antibacterial assay. It also has got numerous advantages, one among which is the outstanding property of fast and active detection of microbial contamination. Other major advantages offered by nanomaterials are target delivery, precision, and efficient functioning. Nanosize antibacterial vaccines are a recent novel innovation added to the field of medicine. Nanomaterials have been designed for specific site delivery, slow release, complex delivery, and environmentally safe delivery (Gao et al. 2014; Rosenholm et al. 2012). Nanomaterials can be used as delivery agents to curb the shortcomings faced under traditional system of antibiotics in drug delivery (Gutierrez et al. 2017). In such systems the conventional antibiotics are either encapsulated, adsorbed, or chemically bound with the nanoparticle carriers for efficient delivery by improving therapeutic key and pharmacokinetics of the drugs used. Metal and their corresponding oxides are known for their antibiotic property since time immemorial. There are numerous organic and inorganic nanomaterials used as bactericidal agents. In inorganic group of nanomaterials, silver nitrate is the most intensely engaged metal group, and such kinds are less likely to develop resistance by the microbes.

There are multiple nanomaterials exploited for antibacterial assay as polymeric nanoparticle utilization aids effortlessly in providing an environmentally benign approach than the other methods. Polymers can be formulated in the forms of vesicles (Yang et al. 2017), micelles, star-shaped polymers, and metallic or inorganic hybrids of polymer in varied size and shapes. Such characteristic modification enhances efficiency in drug delivery than linear simple polymers (Hinde et al. 2017; Zhu et al. 2017; Callari et al. 2014; Ahmed and Narain 2013). Further these modified forms offer effective binding than other linear forms of polymers (Zhang et al. 2014). The most extensively studied and used polymeric nanoparticles is constituted by "liposomes." They are spherical-shaped vesicles with double-membrane layer consisting of both hydrophobic and hydrophilic molecules. It is studied by scientific researchers and integrated in several antimicrobial assays. Among polymeric nanomaterials, the lipid form of nanounits is extensively exploited as antibacterial delivery agents. Such lipid formulation of nanoparticles enables their designing as NLC (nanostructured lipid carriers), SLN (solid lipid nanoparticles), and liposomes. Liposomes are automatically designed to create fusion and hence, are called fusogenic agents. These fusogenic lipids have the ability to disrupt the cell membrane of the bacteria and infuse their active drugs inside the host bacteria, thus proving effective killing (Watarai et al. 2014; Nicolosi et al. 2010). Liposomic encapsulation of drugs prevents alteration of chemical composition, keeping drugs' property intact (Hallaj-Nezhadi and Hassan 2015). SLNs

provide efficient production and long-lasting stability upon incorporation with this solid lipid complex treatment, but they possess low chemical loading capacity, making it disadvantageous in mass applications. These limitations of low loading capacity of hydrophilic drugs can be solved by the use of NLCs which also provides higher stability. This type of polymeric nanoparticle provides stability, intact contents, and restricted release of drugs (slow release). The fundamental principle of active microbial killing by polymeric NPs is through strong cationic interface interaction with the microbial cell membranes ultimately disrupting the cell wall causing internal cell content release and death of cell. The mechanism of cell wall breakage by cationic pull by applied polymeric nanomaterials is through actions of long chains of hydrophobic agents that enables cell penetration and mediating negatively charged cell wall bursting (Fig. 12.5).

Few most commonly used polymeric nano-agents are ammonium polyethyleneimine nanopolymers (Beyth et al. 2008; Beyth et al. 2006), lipid molecules, peptide NPs (Gazit 2007), and chitosan (Qi et al. 2004). Some of the commonly exploited nanopolymers in antimicrobial assay are discussed below:

12.4.1 Micelles

These are self-assembled amphiphatic nanopolymers widely used in antimicrobial assays (Shahin et al. 2014). This structure is the most studied form of polymeric nanomaterials till date. Polymeric micelles of poly(2-alkyl-1,3-oxazolines) were used effectively against *Staphylococcus aureus*, and results were shown to inhibit microbial growth. The antimicrobial activity is believed to be contributed by the chemical structure of the polymers used (Waschinski et al. 2005).

12.4.2 Vesicles

They are also known as polymersomes and are spherical-shaped polymeric capsules composed of bilayer members comprising of amphipathic copolymers (Brinkhuis et al. 2011). This type of polymer vesicles is less studied for antimicrobial therapeutics than micelles group.

12.4.3 Star Polymeric Nanoparticles

This group possesses unique structure and is gaining quite a popularity recently. They are covalently joined unimolecular nanostructures with branching forming 3D globe-like structure (Sulistio et al. 2012). Through such interventions, high-

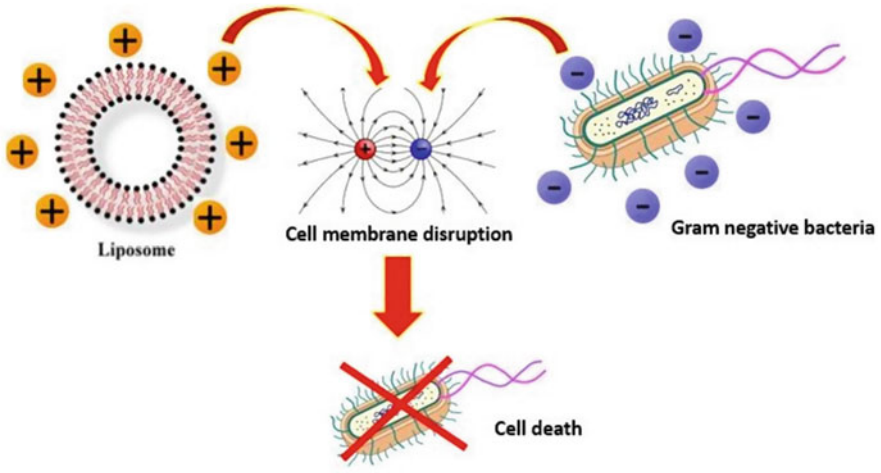

Fig. 12.5 Microbial cell damage by ionic interaction

molecular-weight star-shaped polymeric nanomaterials can be produced, conserving original solubility and viscosity (Wiltshire and Qiao 2007).

12.4.4 Metallic/Inorganic Polymer Hybrid Nanomaterials

It usually consists of metal- or silica-based core and polymeric shell covers (Ghosh Chaudhuri and Paria 2012). Oxidation rates are often checked under such polymeric complexes retaining metal integrity that is antimicrobial in nature. This type of hybrid system has recently received great attention. Hybrid polymeric complexes provide more microbial control than sole use as metallic or polymeric antimicrobial therapeutic agents. It is used most effectively against bacterial disease controls (Fig. 12.6).

12.5 Various Metallic Nanoparticles Used as the Antibacterial Agents

Several chronic infections are mostly caused by bacteria. Different antibiotics are employed for the treatment of bacterial infections due to their cost-effectiveness and positive results. Unfortunately, it has been observed that some bacterial species are able to tolerate or resist the antibiotics. Additionally, super-bacteria which are resistant toward almost all antibiotics have been found and revealed that they carry a gene called NDM-1 (Hsueh 2010). Antibiotics are used to inhibit or disrupt synthesis of cell wall, translational machinery and DNA replication machinery.

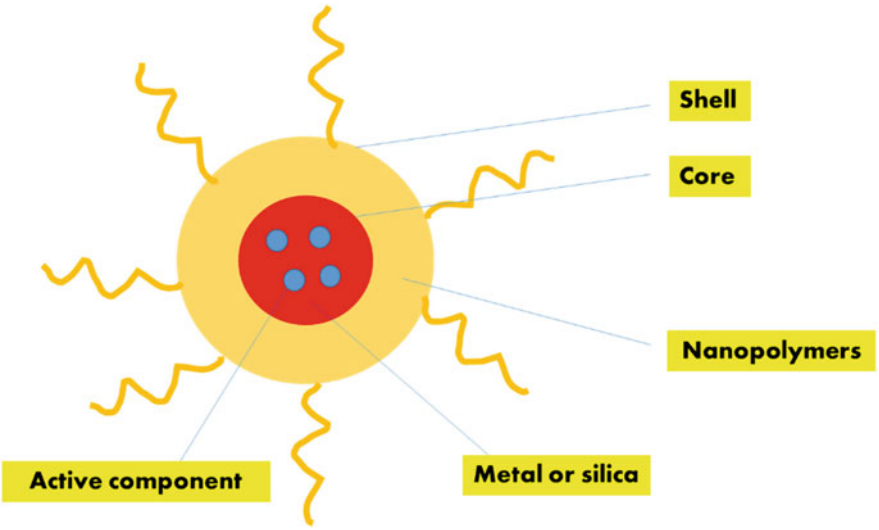

Fig. 12.6 Metallic/inorganic polymer hybrid nanomaterials

But some bacteria have resistance characteristics against these mechanisms. The reasons behind this are the enzyme modifications or degradation of antibiotics like β-lactamases and aminoglycosides (Poole 2002). Another reason is the modification in cellular components as seen in the case of vancomycin resistance, tetracycline resistance (Jayaraman 2009) and development of efflux pumps (Knetsch and Koole 2011). This issue can be dealt with the help of NPs as mechanisms of antibiotic resistance are irrelevant to mechanisms of NPs. Like the antibiotic, NPs also directly interact with the cell wall of the bacteria without penetrating into the cell. Hence, it attracts more attention of the researchers for producing novel and exciting nano-based materials with antibacterial activity. Nanomaterials are those materials which have at least a single dimension within the size range of 1–100 nm (Edmundson et al. 2013). They show antibacterial activity against a broad-spectrum of Gram-positive and Gram-negative bacteria (Wang et al. 2017). In this present period, metallic nanomaterials have become very popular and most emerging materials in multiple scientific areas. It has gained success due to its shape, size, high surface area, catalytic, electrical, and optical properties. Therefore, metallic NPs play a big role in advanced applications of modern medicines and are increasingly used for various purposes like antifungal, antiviral, antibacterial, anti-inflammatory, and antiangiogenic activities (Zafar et al. 2016). Metallic NPs or metal-based nanomaterials include quantum dots, nanogold, nanosilver and oxides with metal bases (Kreyling et al. 2010) such as titanium dioxide. Generally, metal-based nanomaterials receive more focus in biomedical and pharmaceutical industries (Mody et al. 2010). Among metallic NPs, silver NP is one of the widely used metals owing to its distribution of size, stability, morphology, and surface charge (Popa et al. 2007). Antimicrobial and antifungal activities are also among its important

features (Prabhu and Poulose 2012). Nano golds are also the most promising particles which are famous for their benefit in diagnostics, drug delivery, medical therapy, gene therapy, cancer treatment, sensing, etc. (Babu and Doble 2018; Giasuddin et al. 2012). A major advantage of AuNPs is its simple formation by chemical reduction process and less toxicity. Various kind of methods are also exploited to enhance the capability of AuNPs (Das et al. 2012). It provides different scopes for NPs with many functions (Sun and An 2011). Another type of prominent nanometal is copper (Cu) that offers similar properties as gold and silver. However, upon comparing with the other two, it has one disadvantage that it undergoes into the oxidation state during the synthesis process. Meanwhile, Cu NPs are considered as the ideal group for its novel properties and cost, thus proving itself as a potential material for biomedical researches of antimicrobial action (Galletti et al. 2013). The production of these NPs are achieved using various methods. Metallic NPs can be synthesized in three ways. They are through physical methods, chemical methods, and biological methods. In physical methods, metallic aggregates are differentiated mechanically using laser ablation, vapor deposition, wire discharge, and mechanical milling, while in chemical methods, metallic atoms give out nucleation and later growth occurs. In biological methods, metallic NPs are produced with the help of reduction of metallic salts with any plant serum. Among the three, the physical methods can be applied in different particle sizes and give out various yield dispersions. However, salt reduction in chemical methods are preferred for the ability to control the size of the particles (Grace and Pandian 2007).

From olden periods, silver pots were used for drinking water which were popular for its antibacterial properties (Ivask et al. 2014). Antimicrobial mechanism of metallic NPs has three modes of action. They are the generation of reactive oxidative species (ROS), liberation of ions, and interaction of NPs with the cell membrane. (Figure 12.7 shows the action of nanoparticles in the bacterial cell.) Metal and metallic salts were quite famous for antimicrobial properties, but metallic NPs have better potential to act against bacteria (Pelgrift and Friedman 2013). The first step in antibacterial activity is entry into the cells. The tiny nanoscale metal ions interact with the cell transmembrane protein. After incorporation into the bacterial cells, they modify the structure of the cell membrane and shut off all the transport channels (Dutta et al. 2012). However, the entire process may vary on the basis of size. Meanwhile, it has been revealed that smaller NPs are more efficient than larger ones. Additionally, smaller NPs are found to have higher surface area which allows better adhesion property through van der Waals forces. Subsequently, when NPs are incorporated, they produce ionization inside the cell and change the intracellular structures which leads to cell death. The second mechanism is the generation ROS by metal NPs. It forms a critical basis for their antibacterial function. ROS are oxidants like superoxide radicals (O^{-2}), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^{-1}), and singlet oxygen (O^{-2}) (Baek and An 2011). The reactivity rate of ROS is very high and sometimes cause destruction to peptidoglycan in the cell membranes, ribosomes, mRNA, DNA and proteins. In addition to this, ROS can also hinder the mechanisms of enzyme activity, protein synthesis, and the electron transport chain (Raffi et al. 2008). The last mechanism is the deactivation of protein

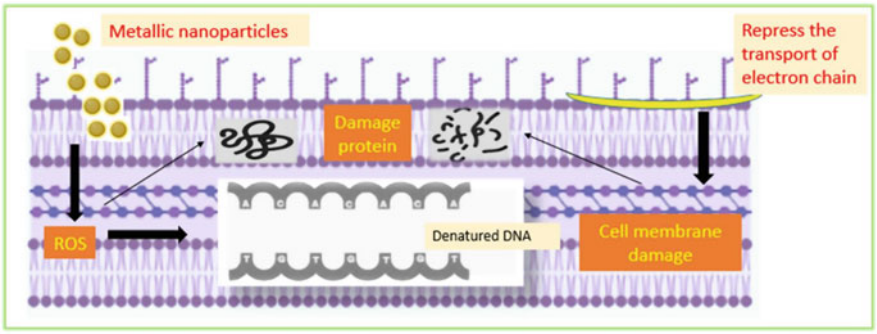

Fig. 12.7 Action of nanoparticle in the bacterial cell

and destruction of DNA. Metal atoms have high affinity toward the thiol group of enzymes; thus they attach and later deactivate the activity of enzymes. It has also been reported that metallic ions have the capability to attach themselves between the purine and pyrimidine bases which results in the damage of the hydrogen bond and destroy the DNA (Jung et al. 2008). In some reports, it is shown that silver NPs synthesized by laser ablation and chemical methods can act against Gram-positive bacteria such as *Bacillus subtilis* and *Staphylococcus aureus*. Likewise, it is also capable to act upon Gram-negative bacteria such as *Salmonella*, *Escherichia coli* and *Klebsiella pneumonia* (Zafar et al. 2016). In another report, gold nanoparticles (AuNPs) were used for their bactericidal against Gram-negative bacteria like *E. coli* and *K. pneumonia*. They were also tested against Gram-positive bacteria such as *S. aureus* and *B. subtilis* (Shamaila et al. 2016). Besides these, CuNPs were used to study the antibacterial action by the turbidity and disk diffusion method and was found that they can act against *E. coli* and *S. aureus* (Khalid et al. 2016).

12.6 Future Prospective and Conclusion

Bacterial infections when faced together with antibiotic resistance can turn into several times more challenging to treat and even prove to be fatal and the use of nanomaterials possessing antibacterial properties whose mechanisms are different from the mechanisms of antibiotics is better suitable to manage the problem. Therefore, the research aimed at developing novel NP-based PNCs with antibacterial activity is of great importance to both the scientific community and the society. Multiple PNCs have been already developed and investigated for their antibacterial properties which have demonstrated unique and eco-friendly results. In the future, further development and improvement in nanocomposites are expected to be seen with enhanced surface properties of active NPs by the use of nanostructures and nanopatterns. Different approaches may also be implemented in a combined manner

to achieve multifunctional activity or mechanisms of action for better efficacy and results.

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Conflict of Interest Authors declared no conflict of interest.

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Chapter 13
Metallic Nanoparticles and Their Composites as Alternative Antibacterial Therapeutics

Farwa Arshad, Md Palashuddin Sk, and Manab Deb Adhikari

Abstract Emerging infectious diseases and the continuous increase in the incidence of drug-resistant bacteria are burgeoning issues of great concern in modern healthcare. Further, many pathogenic bacteria form biofilms during chronic infections, are often recalcitrant to antibiotic treatments, and leave almost no therapeutic option. These threats prompted the development of new antibacterial agents and therapeutics that can treat bacterial infections. Recent advances in nanotechnology promise new developments in diverse areas of biology and medicine, due to its capability of transforming metals into their nanosize, which significantly changes the chemical, physical, and optical properties of metals. The use of nanoscale materials as antimicrobial agents holds considerable therapeutic potential for the amelioration of drug-resistant pathogenic bacteria. Recent research has shown that metal-based nanoparticles (silver, copper, zinc, etc.) have the potential to be efficient platforms for treating and preventing infections caused by bacterial pathogens. The antibacterial activity and mechanism of action depend on the interaction between the microorganism and the nanoparticles as well as on particle size, shape, specific surface area, and surface curvature of the nanoparticles. In this chapter, an effort is made to summarize the research progress concerning the antibacterial activity of metal nanomaterials together with their proposed mechanisms of action. Studies on the toxic effects of nanoparticles on humans, which are necessary for their use as therapeutic agents, were also discussed. The current in-depth review of the antibacterial metal nanoparticles or nanocomposites may contribute to the development of efficient antibacterial agents which can be used for controlling and treating different infectious diseases in the future.

Keywords Antibacterial · Metallic nanoparticles · Antibiotic resistance · Toxicity · Nanomedicine · Antibiotics · Reactive oxygen species · Pathogenic bacteria ·

F. Arshad · M. Palashuddin Sk
Department of Chemistry, Aligarh Muslim University, Aligarh, Uttar Pradesh, India

M. D. Adhikari (✉)
Department of Biotechnology, University of North Bengal, Darjeeling, West Bengal, India
e-mail: madhikari@nbu.ac.in

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Biofilm · Nanotechnology · Nanomaterials · Silver nanoparticles · Gold nanoparticles · Copper and copper oxide nanoparticles · Zinc oxide nanoparticles · Iron nanoparticles · Nanocomposite

Abbreviations

AgNPs	Silver nanoparticles
AuNPs	Gold nanoparticles
CuNPs	Copper nanoparticles
CuO NPs/Cu_2O NPs	Copper oxide nanoparticles
FeNPs	Iron nanoparticles
MDR	Multidrug resistant
MIC	Minimum inhibitory concentration
NPs	Nanoparticles
ROS	Reactive oxygen species
ZnO NPs	Zinc oxide nanoparticles

13.1 Introduction

The emerging infectious diseases, development of drug resistance in the pathogenic bacteria and fungi to antibiotics, and the poor development of new antibacterial agents are creating a major health issue worldwide. The fast rise of antimicrobial-resistant strains has complicated and lengthened the treatment of infections, resulting in greater healthcare costs, mortality risks, and a reduced life expectancy. The issue of treating infections caused by drug-resistant bacteria necessitates a paradigm shift in therapeutic strategy and emphasizes the necessity for the development of powerful antibacterial medicines that are fundamentally different from traditional antibiotics. The use of nanoscale materials as bactericidal agents is a particularly novel approach in this context. Recent advances in nanoscience and nanotechnology have opened up the route for miniature inorganic particles to find new applications in industries including medicine and therapeutics, synthetic textiles, and food packaging. The metallic nanoparticles are thoroughly being explored and extensively investigated as potential antimicrobials. The antimicrobial activity of the nanoparticles is known to be a function of the surface area in contact with the microorganisms. The small size and the high surface to volume ratio, i.e., large surface area of the nanoparticles, enhance their interaction with the microbes to carry out a broad range of probable antimicrobial activities. The antimicrobial activity of the nanoparticles is known to be a function of the surface area in contact with the microorganisms. The small size and the high surface to volume ratio, i.e., large surface area of the nanoparticles, enhance their interaction with the microbes to carry out a broad range of probable antimicrobial activities. Nanoparticles have unique features that make them ideal for

delivering medications to fight infection-causing bacteria. Enhancement in drug solubility and stability, biocompatibility with target agents, ease of synthesis, and controlled release are only a few of these advantages. Metal ions produced by the nanoparticles (NPs) may have a cumulative effect when antibiotics are coupled with metallic NPs. In this chapter, various metallic NPs were discussed for combating the bacteria and their synthetic methods, and the general mechanism behind their antibacterial activity is discussed.

13.2 Metallic Nanoparticles as Antibacterial Agents

Metals has a vital role to play in the biological system; chemistry involving metals cannot be replaced merely by the organic moieties in the biological activities, thus making the metals an important prerequisite micronutrient for the living organism (Lemire et al. 2013). Since, thousands of years back, metals have been known for their antimicrobial properties and were used for multifarious applications such as for the water disinfection, for preserving the water during the wars for preventing dysentery, using metal salt as an astringent, for preventing the eye infections in newborns, in preventing the infections of the surgical wounds, etc. (Pal et al. 2014; Borkow and Gabbay 2009; Alexander 2009). Until the discovery of the antibiotics in the 1920s, there was a boom in using metals for the medicinal aspects, but with the mark of the twenty-first century, there is again a renaissance in using the metal as an antimicrobial agent due to the proliferating risk of using multidrugs (WHO 2017; Coates et al. 2011; Lemire et al. 2013). With the blossoming era of nanotechnology, nanomaterials have been the center of attraction among the scientific community owing to their phenomenal properties. Owing to the rapid phase development in nanotechnology, scientists have explored metal nanoparticles which have exceptional optoelectronic and the magnetic properties (Arjunan et al. 2017). In the present times, metal-based compounds such as metallic nanoparticles (NPs) have a broader area of applications as antimicrobial agents (Lemire et al. 2013; Hemeg 2017; Wang et al. 2017). Studies on metal NPs have revealed the fact that metal NPs have a great potential to fight against the bacteria; they can reduce bacterial antibiotic resistance (Huo and Li 2018; Barui et al. 2018). The infectious disease triggered by bacteria resulted in serious health issues on the global level and is considered as a threat to human health. This issue has extended its arms as an economic obstacle as well as social impediment. Increment in the infections by the pathogenic strains, the resistance of the bacterial toward the antibiotics, the evolution of new bacterial mutants, and underdeveloped countries facing the crisis of the developed vaccines, all these problems are internationally affecting the human health. Thus there is a need of an hour to come up with the antibacterial agents that has the capacity to fight up with the pathogenic and nonpathogenic bacteria satisfying the utmost demand globally.

Being an antibacterial agent, metallic NPs have been considered as the paramount in fighting contagious disease (Wang et al. 2017). There are essential metals that are necessary for the normal functioning of the organism, and the nonessential metals

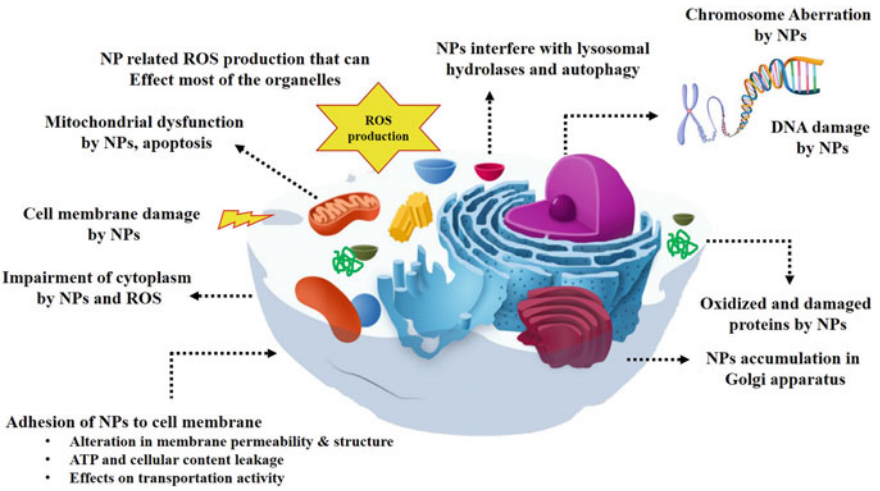

Fig. 13.1 Antibacterial activity of metal NPs. (Reproduced with permission from Attarilar et al. 2020. Copyright © 2020 Attarilar, Yang, Ebrahimi, Wang, Liu, Tang and Yang)

such as mercury, lead, cadmium, and tin, which if present in the concentration higher than the required, can have toxic effects (Nies 1999). Metals can destroy bacteria, but the destruction of the bacteria is highly dependent on the bacterial strains (Godoy-Gallardo et al. 2021; Schröfel et al. 2014). The bacteria have a cell wall that provides strength and rigidity in the shape that makes bacterial cells strong enough for the osmotic rupture and the damage done mechanically (Hajipour et al. 2012). Several factors come into play in the antibacterial activity of the metal NPs such as the morphology of metal NPs; for smaller metal nanoparticles, they show higher antibacterial activity (Lemire et al. 2013; Huo and Li 2018; Samu et al. 2017). Another reason for the effectiveness of NPs against the pathogens are large surface area and high degree of dispersion which enhances their catalytic activity along with their penetration through bacterial membranes, thereby leading to bactericidal effects. These factors contribute to ROS generation which damages DNA molecules (Kohanski et al. 2010). These metallic NPs poisoned the bacteria by impaired functioning of the cellular membrane, by interfering with the nutrient assimilation, and also by the genotoxicity (Gordon et al. 2010; Dibrov et al. 2002; Hong et al. 2012; Macomber et al. 2007). Sometimes these metallic NPs are combined with antibiotics to fight against the multidrug resistance (Cavassin et al. 2015; Singh et al. 2014). The various mechanisms of action of various metal nanoparticles are schematically shown in Fig. 13.1.

13.2.1 Silver Nanoparticles (AgNPs) and Its Nanocomposite

Since ancient times silver is known for its therapeutic applications. Owing to the unique physicochemical characteristics such as thermal stability, their environment-friendly characteristic feature, and their low volatility, AgNPs have been used as antibacterial agents, and hence AgNPs have shown antibacterial properties against various microorganisms (Hamad et al. 2020). On the account of multifarious applications of these AgNPs, they, in distinct shapes, different sizes, and in the different formulations, were marked by their antimicrobial effect which led these NPs to fall into the category of the new-generation antibiotics (Bruna et al. 2021). In the form of the gel and cream, AgNPs are used to heal chronic wounds by reducing bacterial infection. Apart from all this, AgNPs have one of the noble features that is the enhancement in the biochemical activity which led to these nanoscale particles having higher antibacterial activity then their bulk correspondence (Bruna et al. 2021; Kim et al. 2007). In comparison to antibiotic, the antibacterial mechanism of AgNPs on bacterial membrane is depicted in Fig. 13.2.

Generally, there are different mechanism that have been noticed for the antibacterial action which is exerted by the AgNPs. (1) The AgNPs interact with the microorganism by adhering them to the cell wall or the cell membrane of the bacterial cell. This interaction between the AgNPs and the cell wall of the bacteria is because of the electrostatic interaction between the negatively charged bacterial cell and the positively charged AgNPs. This interaction thus triggers the change in the structure of the bacterial cell membrane increasing membrane permeability which caused the leakage of cell content and eventually the death. (2) AgNPs also damage

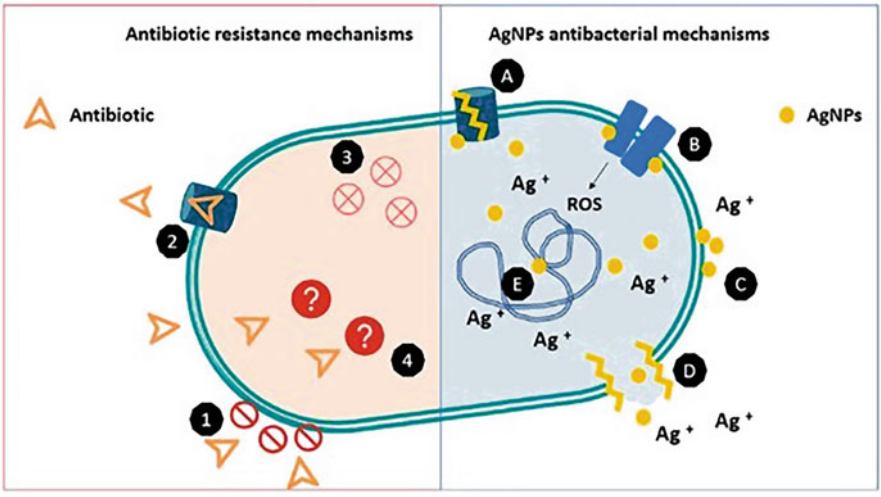

Fig. 13.2 AgNPs' antibacterial mechanism in the comparative schematics with respect to antibiotic action on bacterial membrane. (Reprinted (adapted) with permission from Bruna et al. 2021. Copyright 2021, Licensee MDPI, Basel, Switzerland)

the cell by penetrating into them and affecting their crucial functions by interacting with the sulfur groups or the phosphorus groups present in the DNA and proteins. Also, the AgNPs can enter into the respiratory chain which is present in the inner membrane by interacting with the thiol groups of the enzymes, producing the free radicals and reactive oxygen species (ROS), thus creating the imbalance between the ROS and the antioxidants and hence leading to the oxidative stress. This results in the destruction of the intracellular machinery, triggering the cell death. (3) AgNPs also release the Ag^+ ion which penetrates into the components of the cell such as the DNA, protein, and the peptidoglycan. This Ag^+ ion release is through an oxidizing agent that is responsible for the oxidation of the elemental silver (Hamad et al. 2020).

In 2014, Puiso and co-workers synthesized AgNPs from cranberry and investigated its antibacterial activity against the reference strains of *Staphylococcus aureus*, *Salmonella typhimurium*, *Listeria monocytogenes*, *Bacillus cereus*, *E. coli*, *B. subtilis*, and *Candida albicans*. AgNPs were found to be most effective against the reference culture of *S. aureus*, *B. subtilis*, and *B. cereus* and were least effective against the *C. albicans* and foodborne *B. cereus* (Puišo et al. 2014). In 2018, Yan et al. investigate the antibacterial effect of AgNPs on the pathogenic bacteria, i.e., *P. aeruginosa*, by employing the proteomic methods to analyze the silver-regulated and silver-binding protein. Bioinformatics analysis shows that the cell membrane was targeted by AgNPs and ROS was generated which caused the cellular oxidative stress. Furthermore, the silver ion release synergistically with the particle-specific effects caused the antibacterial action of the AgNPs (Yan et al. 2018).

In 2020, Pishkar and co-workers synthesized the AgNPs using the extract of *Tragopogon collinus*. The antibacterial property was investigated against the gram-positive *S. aureus* and the gram-negative bacteria *E. coli*. It was found that the AgNPs were more effective against the *S. aureus* (Seifipour et al. 2020). In 2021, Slavin et al. synthesizes the lignin-capped AgNPs, where the nontoxic lignin was employed both as the reducing and the capping agent. The resultant lignin-AgNPs were found to be able to combat the gram-positive and gram-negative multidrug resistance clinical isolates and the bacterial strains, namely, *S. aureus*, *Staphylococcus epidermidis*, *P. aeruginosa*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii* (Slavin et al. 2021). AgNPs were synthesized from different routes like using the green extracts, chemical reduction method, and by the inert gas condensation method and were biosynthesized (Yaqoob et al. 2020). These AgNPs were found to be effective against various bacterial strains such as the *E. coli*, *B. subtilis*, *K. pneumonia*, and *P. aeruginosa* (Bruna et al. 2021; Li et al. 1997).

Hence from the aforementioned discussion, it was observed that AgNPs have established themselves as a potent tool for combating different bacteria and came as an alternative to antibiotics. Also, research has been carried out on using AgNPs as antibiotic agent, but there is a major issue that needs to be addressed, that is, some bacteria become susceptible to AgNPs after continuous exposure (Panáček et al. 2018). However, at the same time, AgNPs synergistically with plant extract, antibiotics, and polymers were found to combat the multidrug resistance without the risk of any resistance to bacterial strains and also the development of new bacterial

strains (Khatoon et al. 2019; Kaur and Kumar 2019; Alizadeh et al. 2017; Vazquez-Muñoz et al. 2019).

Hence it was discovered that in recent studies, the synergistic effect of AgNPs and antibiotics can combat the gram-positive and gram-negative bacteria. The antibacterial activity of AgNPs with ampicillin was tested for the multidrug-resistant (MDR) *P. aeruginosa* and *K. pneumonia* which shows the MIC value of 20 μg/mL and 28.12 μg/mL, respectively. Also, AgNPs in conjunction with chloroamphenicol, kanamycin, biapenem, aztreonam, and ampicillin were used to treat *E. coli*, *S. typhimurium*, *S. aureus*, and *B. subtilis* (Khatoon et al. 2019). AgNP bio-composites also have gained attention for their antibacterial activity. The chitosan-and-AgNP-based composite was found to be effective against *S. aureus* due to the synergistic interaction between the two components (Potara et al. 2011; Son et al. 2009). Also, coating of the AgNPs with the biopolymer provides steric stabilization. AgNP-based nanocomposite fabrication for antibacterial activity depends upon the material's nature and the synthetic condition impacting the end products' properties (Raza et al. 2021).

Hence, AgNPs and its nanocomposites have remarkable antibacterial activity (Wahab et al. 2021). It was also observed that the properties such as size and coating of the surface affect the antibacterial activity of AgNPs (Hamad et al. 2020; Bruna et al. 2021; Kim et al. 2007; Wahab et al. 2021; Silva et al. 2017). With all the antibacterial applications of the AgNPs and its nanocomposites, their toxic impact on the human should also be kept in consideration (Wahab et al. 2021).

13.2.2 Gold Nanoparticles (AuNPs) and Its Nanocomposite

AuNPs are emerging as the versatile material for the next-generation bioscience as they have the controlled geometrical, surface, and the optical properties (Dykman and Khlebtsov 2012). They are biocompatible, have no toxicity, and can be easily functionalized, thus providing a platform for multifarious application ranging from targeted drug delivery, biomedical diagnostics, photodynamic therapies, and biomedical analytics (Yeh et al. 2012; Gu et al. 2020). AuNPs have numerous characteristic features that make them a potent tool for the range of the biomedical applications (Dykman and Khlebtsov 2012; Gu et al. 2020). Also, AuNPs were used as an antibacterial agent (Tao 2018). The route for the synthesis of the nanoscale gold depends on chemical reduction method, the seed growth method, or on the biological means by using plant extracts (Geetha et al. 2013; Singh et al. 2018; Maruyama et al. 2015; Camas et al. 2018; Ahmad et al. 2013). Generally, the AuNPs are functionalized by changing the morphology, optimizing the size of the particle and the dispersibility.

Boomi and Prabu (2013) synthesized polyaniline/Au nanocomposite and polyaniline/Au-Pd nanocomposite by chemical oxidative polymerization method and tested their antibacterial activity against four bacterial strains, namely, *E. coli*, *Staphylococcus* sp., *Streptococcus* sp., and *Klebsiella* sp. Both the nanocomposites

of polyaniline/Au and polyaniline/Au-Pd show good zone of inhibition against *E. coli*. The antibacterial effect of polyaniline/Au-Pd nanocomposite is more enhanced as compared to the polyaniline/Au nanocomposite due to the different size of the nanoparticle and their distribution (Boomi and Prabu 2013). As the antibacterial activity is strongly dependent on size and thus the smaller NPs show better antibacterial activity than the bigger ones (Gu et al. 2020). Sekar and co-workers synthesized AuNPs using conventional bio-reduction method, and antibacterial activity here is tested against *S. aureus*, *S. epidermidis*, *E. coli*, and *P. aeruginosa*. The AuNPs show strong antibacterial activity against the gram-negative bacteria *E. coli* and *P. aeruginosa* at different concentration (Vanaraj et al. 2017). Lee et al. synthesized AuNPs using the plant extract of *Inonotus obliquus*, and the antibacterial activity was tested against the gram-positive bacteria, namely, *B. subtilis* and *S. aureus*, and the gram-negative bacterium *E. coli* (Lee et al. 2015).

Perry and co-workers synthesized AuNPs using cefaclor which is a second-generation antibiotic and was tested for the antibacterial activity against *S. aureus* which is a gram-positive bacteria and the *E. coli* gram-negative bacteria. Here, the mechanism behind the antibacterial activity is described as AuNPs that makes holes in the cell wall of the bacteria causing cell leakage leading to death of cell (Rai et al. 2010).

Bunz and co-workers synthesized hydrophobic cationic monolayer-protected AuNPs of different sizes, i.e., 2 nm and 6 nm. The antibacterial nature of AuNPs was checked by making them to interact with the cell membranes of the *Bacillus subtilis* which is gram-positive bacteria and the *Escherichia coli being gram-negative bacteria*. Interestingly, depending upon the size of the NPs, they form spatiotemporal aggregation patterns. The 6 nm AuNPs aggregate onto the particular loci on the bacterial surface, whereas the 2 nm AuNPs lyse the gram-positive bacteria but not the gram-negative bacteria. The distinct pattern here arises due to hydrophobic region that is induced by the NPs on the cell membrane to combine or due to the aggregation of the NPs on the anionic and the hydrophobic hotspots present on the bacterial cell (Hayden et al. 2012). Li et al. (2014) synthesized AuNPs by Brust-Schiffrin two-phase methodology, and the functionalized AuNPs were used to attack the multidrug resistant pathogenic bacteria. By tuning the surface of the AuNPs with different functional groups, they were observed to be competent for the gram-positive and the gram-negative pathogens and also the multidrug-resistant pathogens.

Benitez and co-workers used AuNPs that have shown the antibacterial properties of the AuNPs against the *Streptococcus pneumoniae*. Various strains of these pathogens have serious effect on the human health; hence, combating this pneumococcal infection are necessary. They found that AuNPs can kill the bacteria *S. pneumoniae* by binding with carbohydrate, lipid, and proteins. These biomolecules form a pore that is irreparable by the bacterial cells which result in the lysing of the bacterium (Ortiz-Benítez et al. 2019). Lee and Lee (2019) demonstrated the AuNP antibacterial activity against the *Salmonella* species and conventional antibiotics. The minimum inhibitory concentration (MIC) of AuNPs in the antibacterial

susceptibility test against the *Salmonella* species was found to be 2.5–5 μg/mL. AuNPs cause the collapse of the intracellular divalent cation homeostatic, and then there is ROS accumulation by the antibiotics which caused the apoptosis-like death in the cell of *S. typhimurium*. Gold NP conjugates show better antibacterial property than the individual gold NPs (Payne et al. 2016).

Hence, from the aforementioned points, it is observed that the antibacterial properties of AuNPs are dependent on the size of the particle, modification of the surface, and the their dispersibility. AuNPs cause the death of the bacterial cell or the cell lysis either by damaging the bacterial cell membrane, by electrostatically adsorbing on the surface of the bacteria, and by the ROS destruction of the proteins and the DNA (Dykman and Khlebtsov 2012; Yeh et al. 2012; Gu et al. 2020; Tao 2018). Presently, most of the research studies with AuNPs are usually focused on the conventional bacteria with a very few on the bacteria that are multidrug resistance. Hence, there is still a large room for the research in this direction.

13.2.3 Copper and Copper Oxide Nanoparticles (CuNPs/CuO NPs/Cu_2O NPs) and Its Nanocomposite

Copper is one of the essential elements which is necessary for human body and for various other mammals. In comparison to gold and silver, copper is much inexpensive and is more simply available (Roy et al. 2016). Simultaneously, its NPs are biocompatible, and their synthetic methods are environmentally friendly. The dissolving power of CuNPs is quicker in comparison to noble metals through the release of ions in the surroundings (Sánchez-López et al. 2020). Several industries have utilized copper-based NPs in many areas, viz., additives in skin product, metal coatings, inks, and plastic for food packaging (Ramteke et al. 2020). CuNP-based alloys is used for antimicrobial coatings in dentistry and virus disinfection (Poggio et al. 2020). The important advantage of CuNPs over their other metallic counterpart is their cheaper and easier production (Nikolova and Chavali 2020; Gawande et al. 2016). With respect to their antimicrobial activity, oxidative stress is generated by the CuNPs, causing the dismemberment of bacterial membranes, and can obstruct its activity (Broglie et al. 2015).

CuNPs are synthesized by five techniques: (1) chemical treatment, (2) thermal treatment, (3) electrochemical synthesis, (4) photochemical methods, and (5) sonochemical techniques. The "chemical treatment" being used mostly among different methods and some more modern techniques have used this method in Cu-based NP synthesis (Gawande et al. 2016). Environmental concern is on the rise; thus, green synthesis without the production of toxic waste products has been the focus nowadays. In green synthesis biological routes are used for synthesizing NPs such as from bacteria, fungi, plants, and enzymes or their byproducts, such as proteins (Singh et al. 2016). $CuSO_4$, $CuCl_2{\cdot}2H_2O$, $Cu(NO_3)_2$, or $Cu(CH_3COO)_2$ are the most commonly used precursors for the synthesis of the Cu/CuO NPs. There are

two steps involved in the synthesis of CuO NPs (Sánchez-López et al. 2020). In the first step, there is reduction of Cu^{+2} into Cu^{0} which is highly unstable; hence, it undergoes oxidation in the presence of the oxygen forming CuO which is elucidated below (Sánchez-López et al. 2020).

$$Cu^{2+} + 2e^{-} \text{ reduction } CuO + Air(O_2) \text{ oxidation } CuO$$

CuNPs have the great potential to inhibit the growth of bacteria which is ascribed to the better transfer of the electron between the CuNPs and the bacteria (Usman et al. 2013; Mahmoodi et al. 2018), as the bacteria and CuNPs both act as the electron acceptor thus corresponds to the transfer of the electron and the bacterial membrane rupture. This antibacterial aspects of the copper is observed after the contact of the released Cu^{2+} ions and the membrane of the bacteria (Mahmoodi et al. 2018). The instability of the CuNPs made them susceptible to the oxidation, but this problem was surpassed by converting CuNPs to CuO NPs (Ermini and Voliani 2021). Although CuO NPs have better stability than CuNPs, they are less active, and also their antibacterial mechanism is not explained yet. It is assumed that their adherence to the cell wall of the bacteria is initiate by the electrostatic attraction. Further the ROS is generated by the Cu^{2+} dissociation. These ions can enter the cell and cause the damage of the membrane by destroying the inner contents and leakage of the bacterial cell (Singh et al. 2016; Usman et al. 2013; Mahmoodi et al. 2018; Ermini and Voliani 2021). Rasool et al. synthesized the CuNPs using marine endophytic actinomycetes which were isolated from seaweeds. The antibacterial activity of the synthesized NPs were analyzed through the zone of inhibition against *Klebsiella pneumoniae*, *Proteus mirabilis*, *E. coli*, *Salmonella typhimurium*, and *MRSA* with respect to $CuSO_4$ as the negative control and gentamicin being the positive control (Rasool and Hemalatha 2017). Amorim et al. synthesized CuNPs by chemical reduction method and was stabilized by utilizing the cashew gum as the natural polymer stabilizer. Further their antibacterial activity was tested against the bacterial strain of *S. aureus* with a minimum inhibitory concentration (MIC) of 0.64 nm. Minimal bactericidal concentration (MBC) was equal to MIC that inhibits the growth of bacteria; as well as it can kill the bacteria effectively (Amorim et al. 2019). Bocarando-Chacón and co-workers synthesized CuNPs by using the green synthesis from the extract of *Opuntia ficus-indica* and by using *Geranium* extract. The antibacterial activity was studied against *Escherichia coli*. Bezza and co-workers fabricated the Cu_2O NPs by reverse micellar technique by using the benign lipopeptidal surfactant which is acting as stabilizer. The stabilized Cu_2O NPs show significant antibacterial activity for *B. subtilis* CN2 and *P. aeruginosa* CB1 with a MIC value of 62.5 μg/mL. The surfactant-stabilized Cu_2O NPs exhibited effective ion-releasing characteristics than the bare Cu_2O NPs. The enhanced antibacterial activity of the surfactant-stabilized Cu_2O NPs is due to the higher surface area of the NPs which is provided for interacting with bacterial cells, also the increased solubility of Cu ions and the more interacting atoms with the bacterial membrane (Bocarando-Chacón et al. 2020). Li and co-workers synthesized the

nanocomposite of CuNPs loaded with molybdenum disulfide (MoS_2). The sheets of MoS_2 inhibited the aggregation of CuNPs. Its antibacterial activity was studied in vitro and in vivo against the *Xanthomonas oryzae* pv. *oryzae* (Xoo) through agar disk diffusion and MIC. It was observed that the nanocomposite exhibits 19.2 times higher antibacterial property than commercial copper bactericide (Li et al. 2020). Also, Nithya and co-workers synthesized the nanocomposite of copper with chitosan (CS-Cu) without any external reducing agents. It also shows the antibacterial activity against the gram-positive (*S. aureus*, *S. pneumoniae*) and the gram-negative (*P. aeruginosa*, *P. vulgaris*) bacteria. The antibacterial mechanism of the nanocomposite was ascribed to the surface area of the catalyst. Thus CS-Cu nanocomposite can easily attach themselves to bacterial cell and can lead to the leakage of the intercellular content and the disruption in the normal functioning of the cell leading to their death (Arjunan et al. 2017).

There are a number of evidences that prove the antibacterial property of CuNPs, but there are still some challenges that need to be addressed. Firstly, Cu is more susceptible to oxidation compared to other metal counterparts like Ag and Au, rendering its nano-synthesis to be difficult. Furthermore, thermodynamically also copper oxides are stable than copper in air which decreases the effectiveness as an antibacterial agent. In addition, CuNPs show agglomeration (Usman et al. 2013). In spite of being an essential element for mammalian and life of humans, involvement in pathways for regeneration of tissues, it gets accumulated by releasing the ions which resulted in the toxic effects (Rubilar et al. 2013; Zhou et al. 2018; Hong et al. 2017). In the future, more investigations should be done on the design of CuNPs, and their interaction with different microorganism is also highly needed.

13.2.4 Iron Nanoparticles (FeNPs) and Its Nanocomposites

As humans, we are susceptible and prone to various diseases and sickness by a multitude of agents. One of them is bacteria which can exist in a wide variety of forms, each generating their own set of diseases. The greatest remedy for bacterial agents used currently are antibiotics which have side effects and also make bacteria immune to them in the long run. Thus various alternatives are given considerations among which are the use of metal/metal oxide nanoparticles which is on the rise due to their significance (Thukkaram et al. 2014; Aisida et al. 2020; Gudkov et al. 2021). One of the nanoparticles under consideration is iron/iron oxide nanoparticles (Gabrielyan et al. 2019). There are various approaches to synthesize this iron/iron oxide NPs, viz., chemical route and green synthesis route, and a multitude of research has been done on each route (Gordon et al. 2011). The green synthesis route is eco-friendly and cost-effective compared to the chemical route. Samson successfully synthesized and demonstrated bactericidal effect from iron oxide NPs using *Moringa oleifera* leaf (Aisida et al. 2020). Although a complete list is far beyond the scope, a list in tabular form is appended below.

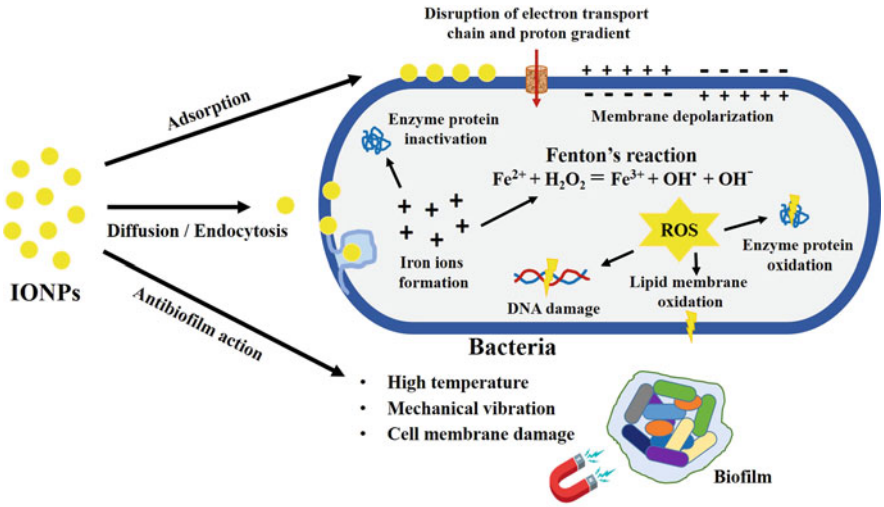

Fig. 13.3 Antibacterial mechanism of IONPs. (Reprinted from Gudkov et al. (2021))

There are multiple studies on these NPs showing their remarkable properties against a range of bacterial pathogens, viz., *E. coli*, *Enterococcus hirae*, etc. (Gudkov et al. 2021). For instance, Fe_3O_4 NPs have been shown to decrease the growth-specific rate of *E. coli* and *E. hirae*. However, the concentration for which this bactericidal effect was observed is not the same for both. In another work demonstrated that the antibacterial activity even on biofilm formed on bio-implants which caused major complications and infections among the recipients (Thukkaram et al. 2014). Hence, by the influence of the iron oxide nanoparticles (IONPs), a notable reduction in the growth of biofilm was observed on the biomaterial surface. Das and co-workers have synthesized the IONPs by the thermal decomposition of diiron(III) complex and have shown the antibacterial property of IONPs on *Staphylococcus aureus*. This bactericidal property of IONPs is due to their electromagnetic interaction with *S. aureus* which led to the development of the ROS and put an oxidative stress by the oxidoreduction process (Das et al. 2020). Various antibacterial modes of action of IONPs are depicted in Fig. 13.3. Research has shown that antibodies can be conjugated on the metallic NP surface which are specific to bacteria for the selective targeting and bacterial separation. Metallic NPs were modified with H- or O-antibodies for *S. typhimurium* separation (Sakudo et al. 2015; Kim et al. 2016; Kuang et al. 2013). NPs are not only directly utilized; in many cases, they are used along with other drugs to enhance their effectiveness. Aparicio-Caamaño et al. used this strategy where they coupled the FeNPs with erythromycin, and the antibacterial property was analyzed on *S. pneumoniae*, and an enhancement in the antibacterial property was further observed (Aparicio-Caamaño et al. 2016). So far, only the advantages and the properties of FeNPs are discussed because of which they are boon in the biomedicine field. However, the study would be incomplete and rendered ineffective if we don't

check the side effects it may cause to the human body. If the side effects outweigh the advantages, then that technology will never see the light. In this regard cytotoxicity experiments are a must in each case. It is shown that IONPs are biocompatible and biodegradable. Particularly the intravenous injection of Fe_2O_3 NPs did not affect the rats' weight gain or apoptosis activation in HUVEC cells. After intravenous injection, NPs were found in rat lungs, liver, and kidneys but not in the brain or heart. A significant proportion of NPs was eliminated with urine after 72 h (Hanini et al. 2011). Generally, the IONPs exhibit low cytotoxicity on the cell culture. For example, IONPs that are coated with polyethyleneimine, dimercaptosuccinate, or citrate display no unfavorable effect on primary rat cerebellar cortex astrocytes and cultured murine astrocytes (Yiu et al. 2012; Zhang et al. 2016).

IONPs in conjugation with PEG-phospholipids have no influence on the viability of the B16-F10 cell line with doses up to 0.75 mg Fe/mL (Yiu et al. 2012). Fe_3O_4 NPs show a bacteriostatic effect without exerting the hemolytic action (Irshad et al. 2017). IONPs cause the enhancement in the Casp3-dependent apoptosis severally in the case of HUVEC cells, resulting in the ROS generation, damage in the membrane, c cytoskeleton changes, and so on (Valdiglesias et al. 2016). The cytotoxic activity of IONPs on human lung cancer cells was shown where the IONPs induce the mode of apoptosis for destroying the cancerous lung cell (Das et al. 2020). Generally, the cytotoxic characteristics in IONPs are displayed at higher concentration in comparison to the antimicrobial characteristics.

13.2.5 Zinc Oxide Nanoparticles (ZnO NPs) and Its Nanocomposites

According to the US Food and Drug Administration (21CFR182.8991), ZnO is considered "safe." At nanoscale, ZnO exhibits significant antibacterial activity for a wide range of bacterial species (Raghupathi et al. 2011; Zheng et al. 2020; Sirelkhatim et al. 2015; Tayel et al. 2011; Liu et al. 2009). Owing to the thermal stability and high antimicrobial activity, they are considered as potential antibacterial agents (Sirelkhatim et al. 2015). Its antibacterial activity has been studied for pathogenic and nonpathogenic bacteria. ZnO NPs are nontoxic, biocompatible, and were effectively used in cosmetics and for the drug delivery purposes (Xie et al. 2011). Considering the nontoxicity of the ZnO NPs, it is necessitated as antibacterial agents to the human cells, and they are noxious to the microorganism (Colon et al. 2006). The antibacterial activity of ZnO NPs depends upon its morphology, i.e., shape and size (Sirelkhatim et al. 2015). In order to enhance the performance of ZnO as an antibacterial agent, they are generally tuned with inorganic or the organic particle, their particles size were tuned, and also the concentration of ZnO NPs were varied (Sirelkhatim et al. 2015; Qi et al. 2017).

Generally, there are various aspects of the antibacterial mechanism of ZnO NPs, but the exact mechanism behind the antibacterial activity is still controversial.

Different mechanisms were put forward such as (1) generation of ROS, (2) release of the Zn^{2+} ion, (3) dysfunction of the membrane, and (4) the internalization of the NPs.

13.2.5.1 Generation of ROS

The oxidizing nature and the high reactivity of the ZnO NPs produce increased levels of ROS (Zhang et al. 2008; Padmavathy and Vijayaraghavan 2008). There was a contradiction in the number of studies regarding the ROS generation by UV illumination or in the dark (Sirelkhatim et al. 2015). Further, research was carried out to bring forth the ROS production under UV light or in the dark. Padmavathy et al. check upon the antibacterial activity of ZnO NPs with different sizes, and it was found that the particle with smaller size was found to be efficient than the bigger particles (Sirelkhatim et al. 2015). Also, ROS was generated both under UV illumination and without it, i.e., in visible light. The researchers further explained the ROS production on the surface of ZnO and the correlation between the antibacterial activity and the photon reactions. They proposed that the interaction between the electron and hole with the water produce •OH and H^+. Additionally, the oxygen molecule which is suspended in the mixture containing the bacteria along with ZnO produces the superoxide anion which further reacts with the H^+ producing the $HO_2^{\bullet}$. This $HO_2^{\bullet}$ generates $^{\bullet}HO_2$ after interfering with the electron which further produces the H_2O_2 molecule after reacting with the H^+. The H_2O_2 molecule destroys the bacteria by entering into the cell membrane (Sirelkhatim et al. 2015; Qi et al. 2017). The chemical equations expressing the ROS generation are as follows:

$$ZnO + h\nu \rightarrow e^- + h^+ \tag{13.1}$$

$$h^+ + H_2O \rightarrow \bullet OH + H^+ \tag{13.2}$$

$$e^- + O_2 \rightarrow O_2^{\bullet -} \tag{13.3}$$

$$O_2^{\bullet -} + H^+ \rightarrow HO_2^{\bullet} \tag{13.4}$$

$$HO_2^{\bullet} + H^+ + e^- \rightarrow H_2O_2 \tag{13.5}$$

It was observed that the hydroxyl radicals and the superoxides were inactive in killing the bacteria owing to their negative charges (Xie et al. 2011). Also, the ZnO NPs after interacting with the cell membrane is adsorbed on the surface of the bacteria which is either killed or left over and further block the extra antibacterial activity (Sawai et al. 1998). Once these NPs are in the growth media, they release the peroxides which cover the entire surface of the bacteria which is dead. Hence, this continuous release of the peroxides contributes in the higher bactericidal efficacy (Sirelkhatim et al. 2015; Qi et al. 2017).

13.2.5.2 Release of the Zn^{2+} Ion

This released Zn^{2+} ion has a major role to play in the disruption of the enzyme system and also in the metabolism of the acid (Heinlaan et al. 2008; Li et al. 2011). It was also believed from various research studies that the toxicity and dissolution of the ZnO NPs is due to the leakage of the Zn^{2+} ion. Hence, the toxicity of the ZnO NPs can be modified by the manipulation of the dissolution rate (Wong et al. 2010; Wu et al. 2010). There is interaction between the membrane of the bacterial cell and the Zn^{2+} ion which leads to the easy entry of the released Zn^{2+} ions into the bacterial cell, where they interact with the amino, sulfhydryl, and hydroxyl group (Bulcha et al. 2021). This interaction with the functional groups terminate bacteria due to the unbalanced metabolism caused by the structural change and protease performance.

13.2.5.3 Dysfunction of the Membrane

ZnO NPs prevent the growth of bacteria; thus, they cause disorder in the membrane resulting the membrane dysfunction (Qi et al. 2017). There is an electrostatic attraction between the ZnO NPs bearing the positive charge and the bacteria cell with the negative charge causing the ZnO NPs to adsorb on the membrane of the bacteria (Applerot et al. 2009). Thus there will be the disturbance in the charge balance on the surface of the cell which resulted in the disruption of the cell (Wang et al. 2014). The interaction of the cell wall and the ZnO NPs resulted in toxicity (Papavlassopoulos et al. 2014). Research studies showed that the interactions are not only electrostatic but also the weaker interactions, namely, the hydrophobic interactions, van der Waals force, or the ligand-receptor interaction (Papavlassopoulos et al. 2014; Stoimenov et al. 2002). Also, the membrane viscosity is affected by the adsorption of the ZnO NPs on the cell membrane (Chen et al. 2012).

13.2.5.4 The Internalization of the NPs

The internalization of ZnO NPs take place due to the dysfunction of bacterial membrane. This result in the inhibition of matter or the energy exchange metabolism with bacterial environment (Chen et al. 2012). This caused the membrane to lose its integrity and thus cause permeability barrier malfunction (Brayner et al. 2006). Also, it was observed that ZnO NPs with larger surface area and higher surface energy resulted in its adsorption and in addition terminated the bacteria which are present (Sabir et al. 2014). Keeping in view the antibacterial mechanism of ZnO NPs, also various strategies were proposed to enhance the antibacterial activity of these ZnO NPs. The coating of the ZnO NPs with the inorganic photocatalytic promoters such as the Au and Ag or by loading with the organic antimicrobial agents can also enhance the antibacterial activity of the ZnO NPs (Sadeghi 2014; Wang et al. 2009). These organic antimicrobial agents such as the chitosan have an immobilized nature,

or they embedded on the surface of the ZnO NPs (Sudheesh Kumar et al. 2012). The chitosan molecule and the cell membrane interact with each other and thus result in the leakage of the intracellular and the proteinaceous contents. Doping the impurity atoms such as Fe and Mn into the host lattice of the ZnO can result in the tunability in its physicochemical properties which further enhance its antibacterial activity (Nair et al. 2011).

It was also noticed that the antibacterial activity of the ZnO NPs is size dependent; the smaller the NPs size, the greater the antibacterial activity (Nair et al. 2009). The particle morphology also affects the antibacterial activity of ZnO NPs. By the proper synthetic technique, the morphological characteristics of ZnO NPs can be controlled (Darwish et al. 2017; Talebian et al. 2013). There is also the correlation between ZnO NPs and their concentration. It was observed that the higher the concentrations, the antibacterial activity will also be higher (Yan et al. 2011; Li et al. 2013). Thus the fascinating characteristic features of the ZnO make them potent for use as an antibacterial agent. Further research should focus on using ZnO NPs as a smart weapon to the MDR and also using ZnO NPs as a substitute to the traditional antibiotics.

13.3 Toxicity of Nanoparticles

Despite the fact that metal nanoparticles and nanocomposites have a wide range of biological applications, little is known about the long-term effects of nanoparticle exposure on human health and the environment. Because of their unique physicochemical properties, nanoparticles are appealing materials in the pharmaceutical, drug delivery, biomedical imaging, biosensing, and food industries. When employing nanoparticles in therapeutic applications, biocompatibility, toxicity, and the capacity to enter cells are three important factors to consider. Nanoparticles can harm cells by releasing harmful ions and nonspecifically binding to the biomacromolecules. Size and uniformity, composition, morphology, crystalline structure, surface functionalization and charge, and magnetism are all factors that influence the toxicity of metallic NPs (Marambio-Jones and Hoek 2010; Rudramurthy et al. 2016). The degree of cytotoxicity is determined by the route of administration and the accumulation site (Naahidi et al. 2013). NPs have been shown to exhibit neurotoxic properties in several studies. The mechanism and channels via which NPs cause neurotoxicity; however, they are still largely unknown.

Nanoparticle toxicity appears to be caused by the generation of reactive oxygen species (ROS) and oxidative stress. Stress, inflammation, DNA damage, and apoptosis are only a few of the physiologic and cellular phenomena triggered by ROS and oxidative stress. NPs have been shown to produce ROS through various mechanisms such as direct formation of ROS from the surface of the NPs or from leached ions as a result of exposure to an acidic environment, such as the lysosomes. Further, NP interaction with cellular organelles like as mitochondria, which might alter mitochondrial function (Soenen et al. 2011). Additionally, ROS harmful effects

are not limited to specific cells or organs but also disrupt a variety of physiological systems and processes, including the central nervous system, respiratory system, and cardiac conduction (Beyth et al. 2015).

Silver has been discovered to have detrimental environmental repercussions due to the existence of free silver ions in the aqueous phase. Free silver ions, which can cause persistent bluish gray discoloration of the skin or eyes, as well as liver and kidney damage from soluble silver compounds, can affect all living species (Panyala et al. 2008).

The liver is one of the organs that copper oxide nanoparticles target once they enter the body through any of the many routes (Nishimori et al. 2009). According to reports, ZnO NPs can reach numerous organs after systemic distribution and cause harm to the lungs, kidneys, liver, stomach, pancreas, tests, thymus, heart, spleen, brain, and blood (Cho et al. 2011; Li et al. 2012; Vandebriel and De Jong 2012). Despite the fact that nanoparticles have been found to have negative effects on living organisms, there is still a lack of information on their toxicity.

13.4 Future Prospects

Nanoparticles have demonstrated enormous potential as antimicrobials and therapies due to their unique physicochemical characteristics. The diverse mechanism of action of nanoparticles against microorganisms appear to have the advantage of preventing or delaying the development of resistance, making them preferable to conventional antibiotics. The multivalent target bactericidal activity of NPs would be excellent for treating and killing bacterial pathogens because multidrug-resistant bacteria are unlikely to establish numerous defenses at the same time. Various clinical trials of different nanomaterials have been now directed, including hand gels, medications focused on HIV diseases. Given the clinical promise of antimicrobial nanomedicine, conducting clinical studies is a critical step in possibly incorporating nanomedicine into everyday medical practice. However, nanomedicine research is still in its early stages, and little is known about the behavior of nanoparticles inside the human body or the long-term repercussions of their release into the environment. This is one of the primary obstacles that must be overcome before nanoparticles may be used as routine therapeutic agents.

13.5 Conclusion

Metal-based NPs have proved themselves as the potent antibacterial agents and are being used in the field of biological sciences and engineering. In this chapter, we have discussed about various NPs such as AgNPs, AuNPs, CuNPs, FeO NPs, and ZnO NPs and their composites. Their antibacterial properties and mechanism are also discussed. These NPs and nanocomposites have shown their effectiveness

against gram-positive and gram-negative bacteria. Their use as an alternate to antibiotics has been discussed in detail. Considering the intensive use of NPs for killing bacteria, this field seems to be on the threshold of intensive research studies. Inorganic NPs have the potential to be a long-term antibacterial class, capable of eradicating illnesses by preventing the formation of both susceptible and resistant germs while decreasing the selection of resistance associated with the use of broad-spectrum antibiotics. Therefore, it's high time for researchers to use the antibacterial property of metallic NPs to the clinical fields.

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Conflict of Interest The authors declare that there is no conflict of interest.

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Chapter 14
Carbon Nanoparticles as the Next-Generation Antimicrobial Agents

Sujoy Deb and Divya Sriram

Abstract In the wake of increasing antimicrobial resistance due to the indiscriminate use of antibiotics, the need of the hour has been to look for novel antimicrobial agents that have least potential for eliciting resistance from microbes. Current antibiotics that target bacterial cell wall or biochemical processes in the cell have thus largely failed as microbes evolve faster to counteract these mechanisms. Carbon nanomaterials (CNMTs) have been studied as a novel class of antibiotics in the last decade, and functionalized carbon nanoparticles and nanocomposites have shown to possess antimicrobial activity. Carbon nanocomposites like nanotubes, fullerenes, and graphene and their derivatives have also shown great potential to act as antimicrobial compounds. One of the main contributors to antimicrobial activity is the large surface to volume ratio of CNMTs that allows easy and efficient binding to microbes to elicit the antimicrobial activity by various mechanisms including physical cell membrane damage, oxidative stress, affecting bacterial enzymes, and respiration. This chapter focusses on the antimicrobial properties of various types of CNMTs, their mechanism of action, their advantages for combating antimicrobial resistance, and challenges in use as next-generation antimicrobial agents.

Keywords Antimicrobial resistance · Antimicrobial agents · Bacterial cell wall · Antibiotics · Carbon nanocomposites · Carbon nanoparticles · Carbon nanotubes · Membrane disruption · Nanoliposomes · Oxidative stress · Antioxidant · Reactive oxygen species · Photosensitizers · Multidrug resistance · Fullerenes · Photodynamic therapy · Gram-positive bacteria · Gram-negative bacteria · Liposomes · Graphene

Abbreviations

CNTs	Carbon nanotubes
DWCNTs	Double-walled carbon nanotubes
GO	Graphene oxide

S. Deb · D. Sriram (✉)
CSIR-Centre for Cellular and Molecular Biology, Hyderabad, Telangana, India

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Gt	Graphite
GtO	Graphite oxide
MDR	Multidrug resistance
MWNTs	Multiwalled nanotubes
PDT	Photodynamic therapy
rGO	Reduced graphene oxide
ROS	Reactive oxygen species
SWNTs	Single-walled carbon nanotubes

14.1 Introduction

Antimicrobial resistance is a global crisis, affecting lives of millions of people suffering from various infections. Developing countries are the worst affected. Indiscriminate use of antibiotics and the lack of new effective drugs have made multidrug-resistant infections common, impacting the clinical outcomes of many especially in hospitals and intensive care units, surgery patients, and immunocompromised patients including cancer patients and transplant patients (Ventola 2015). Typically treating drug-resistant bacteria is not only cost-intensive but extremely long, impacting not only the affected individual but also hospitals and governments. This is especially causing endemics in the case of communicable infectious diseases, especially in developing countries like Africa, India, South America, and Southeast Asia, making it a worldwide public threat (Mogasale et al. 2021).

With the increasing global population, and pollution in the environment and lack of sanitation, there is a wide spread of infectious diseases, and therefore, antibiotics are being indiscriminately used. However, over the years, bacteria both individual and those in biofilms have developed resistance through adaptations, selection of mutants. There are several mechanisms by which bacteria protect themselves—some secrete enzymes that destroy antibiotics, and others produce efflux pumps that pump out antibiotics from cellular interior. Others excrete extracellular polymeric substances that preserve them from host responses. Due to existence of bacteria in biofilms in respiratory infections, chronic wounds, infective endocarditis, and osteomyelitis, bacteria are protected due to advantages of spatial and chemical heterogeneities allowing for persistence of infections for reasons other than resistance mutations.

Currently treatment of MDR requires multiple or high dosages of antibiotic agents that result in long and expensive treatments, with the possibility of adverse effects and uncertain outcomes. Nanomaterials are not naturally present in the environment and hence are new materials for bacteria, and any mechanisms against them have not yet been developed. This presents an opportunity for carbon nanomaterials to be used as nanocarriers to address multidrug-resistant microbes—both planktonic and in biofilms in therapeutics (Mulani et al. 2019).

Carbon allotropy is one of the most interesting phenomena in material science giving rise to a variety of nanostructures that differ in their properties while all being composed of the same carbon atoms. In the last 20 years, apart from the two well-known natural allotypes, graphite and diamond, there have been other allotypes synthesized in labs, exploiting the versatile sp2 and sp3 bonding ability of carbon nanotubes (CNTs), graphite, graphene/graphene oxide (G/GO), and fullerenes (Rajeswari et al. 2021; Wang et al. 2014a). This chapter focuses on each of the different types of carbon nanotubes, their antimicrobial properties, and their mechanism of action allowing them to be effective as next generation of antibiotics.

14.2 Carbon Nanotubes and Their Antimicrobial Properties

Carbon nanotubes are hollow carbon-walled structures that are broadly classified into single-walled carbon nanotubes (SWNTs), double-walled carbon nanotubes (DWCNTs), and multiwalled nanotubes (MWNTs) (Iijima 1991). The differences are in the wall diameter and thickness and properties that are conferred due to addition of cylinders. Their methods of synthesis have been detailed in Azizi-Lalabadi et al. (2020). They are known for their high electrical conductivity (~3000 W/m/K) high adsorption (17.44 mg g^{-1}), and surface/volume ratios. These structures not only possess high electrical and thermal conductivity but also mechanical resistance and photoluminescence, making them useful for many biosensing applications at the interface of electronics and have thus been used in a variety of biosensing applications in agriculture and medicine (Rdest and Janas 2021; Chik et al. 2019; Wang et al. 2017; Cataldo and Da Ros 2008).

The antimicrobial properties of carbon nanotubes have come to light while investigating the possible interactions of carbon nanotubes with human cells for assessing toxicity. These studies were important as carbon nanotubes have wide applications in electronics and could be used as biosensors, hence could contaminate our environment. Their constant presence and interactions would help us evaluate their applications which are in vivo sensing capabilities. Carbon nanotubes are hydrophobic in nature. Therefore, in order to use carbon nanotubes for biological applications, they need to be functionalized. Functionalization is carried out using solubilizing agents like surfactants, harsh chemical treatments that could be toxic to human cells and elicit immune responses if used in medical applications (Vardharajula et al. 2012; Sun et al. 2002). Additionally, physicochemical properties of SWCNTs, such as properties such as structure, diameter, and cleanliness (e.g., % metal), also contribute to toxicity (Mohanta et al. 2019; Allegri et al. 2016; Jafar et al. 2016). These studies propelled groups to look into carbon nanotubes' effect on microbes (Kang et al. 2007).

14.2.1 Carbon Nanotubes Damage Bacterial Cell Membranes by Direct Contact

In 2007, for the first time, the antimicrobial properties of commercial SWCNTs (diameter: 0.75 nm to 1.2 nm; length: ~13 μm) were described on *E. coli* using fluorescence-based cytotoxicity assays utilizing dyes like propidium iodide (PI) and 4′6diamidino2phenylindole (DAPI) whose emission spectra did not overlap with SWCNTs. *E. coli* treated with SWCNTs were found to exhibit severe cell membrane damage (Kang et al. 2007). They had concluded that direct contact of SWCNTs with cells was essential and that the membrane damage increased with time. Damage observed in the first 30 min was associated with loss of viability at 73.1% that increased to 87.6% viability loss in 120 min. Direct contact is dependent on many factors: size and concentration of nanotubes and time of treatment (Kang et al. 2007). This was confirmed from many subsequent studies showing direct contact by microscopical observations under different treatment conditions and from different types of nanotubes and bacteria studied. The main factors contributing to direct contact required to elicit antimicrobial activity against bacteria are discussed below.

14.2.2 Antimicrobial Activity Is Dependent on CNT Concentration, Buffer, and Treatment Time

Antimicrobial activity of SWCNTs and MWCNTs was studied against various groups of bacteria—gram-positive and gram-negative cocci/rods (Arias and Yang 2009). There was no significant difference in activities of nanotubes against various groups of bacteria studied. All of them showed approximately a 7-log reduction in cell count in viability testing.

This study also shed a light on the following properties of carbon nanotubes and their effect on antimicrobial activity: surface charge due to functionalization (presence of –OH and –COOH groups), concentration of nanotubes, and the buffer composition. They concluded that surface charge due to –OH and –COOH groups does not seem to be a factor for determining toxicity as SWNTs and MWNTs with the same surface charges behaved differently. While charged SWCNTs exhibited strong antimicrobial activity, MWCNTs did not exhibit significant antimicrobial property. They found that increasing concentration of nanotubes increased antimicrobial activities on both types of charged SWCNTs (with surface groups of −OH and –COOH), starting at 50–250 μg/ml (at the highest concentration, these could inactivate 10^7 cfu/ml *Salmonella* cells in 15 min). Interestingly, buffer composition was also found to be a critical factor in eliciting antimicrobial properties. SWCNTs in deionized water or 0.9% NaCl solution exhibited extremely strong antimicrobial activity but lost activity in PBS buffer and brain heart infusion broth.

Another study investigating antimicrobial property of SWCNTs against *Salmonella enteric*, *E. coli*, and *Enterococcus faecium* at concentrations 0.3–1 mg/ml showed that antibacterial activity was positively dependent on nanotube

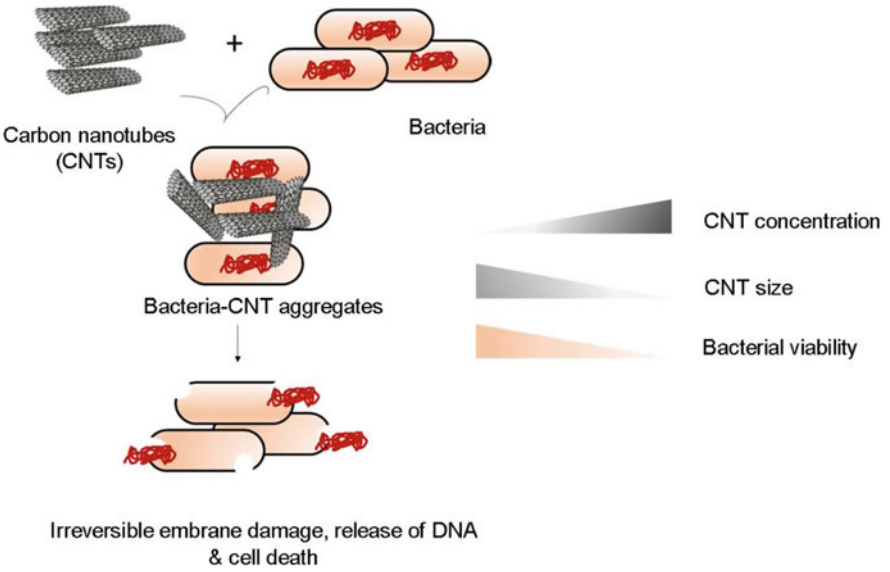

Fig. 14.1 Antimicrobial mechanisms of CNTs. When introduced to suspensions containing bacteria, CNTs aggregate with them and cause membrane damage, resulting in the release of DNA (red) and microbial cell death (left). Bacterial viability decreases with increasing CNT concentration and decreasing size (right)

concentration (Dong et al. 2012). SWCNTs are generally treated with surfactants for dispersing bundles into individual tubes for applications. Many of these surfactants themselves show antimicrobial activities, and hence the use of the right one is essential for applicability in biomedical applications. A study of various surfactants—sodium dodecyl benzenesulfonate (SDB), sodium cholate, and sodium dodecyl sulfate (SDS) in combination with SWCNTs—showed that sodium cholate exhibited least inhibitory effect on bacterial growth at concentrations of 1% while allowing for SWCNT dispersion effectively; SDS and SDB showed total effectiveness against antimicrobial growth at 1% (Dong et al. 2009). Moreover, sodium cholate shows lower toxicity to 1321Ni human astrocytoma cells and hence is optimal for use in biomedical applications. This allows for use of sodium cholate SWCNTs in drug delivery applications especially those that require long treatment times like those against multidrug-resistant microbes. In contrast to previous study, the time of treatment from 5 min to 2 h did not seem to affect antimicrobial activity (Dong et al. 2012). The antimicrobial mechanisms of CNTs are depicted in Fig. 14.1.

14.2.3 The Size of Nanotubes Determines Antimicrobial Activity

Nanotube size depends upon its diameter, length, and volume/surface area. A detailed study on the types of NTs and their effect showed that SWCNTs displayed

better antibacterial activity compared to MWCNTs owing to their smaller nanotube diameter and large surface area allowing for better interaction with bacterial membranes (Maleki Dizaj et al. 2015; Yang et al. 2010).

SWCNTs of similar weights but varying lengths were studied, and 5 μm long were found to exhibit higher antibacterial activity compared to smaller-length tubes of <1 μm and <5 μm. The longer nanotubes aggregated better with bacterial cells, while shorter ones were more prone to self-aggregation excluding the bacterial cells. Interestingly, longer nanotubes also showed better antibacterial activity that increased with nanotube concentration and treatment time (Vecitis et al. 2010; Kang et al. 2008).

14.2.4 Carbon Nanotube Composites and Their Antimicrobial Properties

Carbon nanotube composites with biopolymers and nanoparticles like CuO, Ag, TiO2, ZnO, etc. are known for their high antimicrobial activity (Kim et al. 2008; Cha et al. 2005). However, the utilization of these NPs requires much care considering their toxicity. To resolve this problem, carriers or supporters such as polymers, magnetic NPs, or CNMs like CNTs, GO, and fullerenes can be used, which can potentially boost the antimicrobial activity of the NPs too (Ballatore et al. 2015; Duri et al. 2017). CNMs have good functionalization potentials considering their chemical groups and excellent dispersion capability. The synergistic effect in antimicrobial properties is very important. CNT chitosan, CNT Ag, GO–Ag, and C60 ZnO and C60 CuO show increased antibacterial properties against microbes, including *E. coli* (Hussain et al. 2019; Sakib et al. 2019; Dinh et al. 2015; Das et al. 2011). This synergist effect is related to their mechanisms in disrupting the cell wall or membrane which permits easier availability for other small antimicrobial materials to penetrate into cells. AgNP deposition on MWCNTs with polyamidoamine has been shown to induce bacteriostatic effects against *S. aureus*, *E. coli*, and *P. aeruginosa* (Yun 2013; Yuan et al. 2008).

14.2.5 Carbon Nanotubes for Delivery of Antibiotics

Due to the high adsorption properties of carbon nanotubes, imparted on it by its high surface area, electronic properties, and stability, CNTs have been used as drug delivery systems in therapeutic use.

CNTs have been used for delivery of vectors and biomolecules (like DNA, RNA, and proteins) to cells, tissues, and organs (He et al. 2013). And they have been used as biosensors for diagnostic applications as well (Zhang et al. 2011).

Due to the hollow nature of carbon nanotubes, drugs can be loaded into two ways (Martincic and Tobias 2015). It can either be packed into the hollow CNT structure or covalently conjugated to the CNT surface (Heister et al. 2012). CNT has been shown to act as an antimicrobial both as a drug deliverer without internalization into cells and being internalized into the cells along with the drugs. This was found to occur via either by penetration followed by diffusion mechanism or the endocytosis pathway. The internalization of drug into CNT hollow tube has been shown to be more effective than surface attachment. CNT works by releasing conjugated drug inside the cells, once internalized. If the drug were to be attached to the surface, they are in danger of degradation by the physiological fluids prior to be internalized by the cells (Debnath and Srivastava 2021; Roldo 2016; Bianco et al. 2005). CNTs have therefore promising agents for antibiotic drug delivery.

14.3 Fullerenes and Their Antimicrobial Properties

Fullerenes are a group of cage-like carbon nanostructures of various shapes from hollow spheres to ellipsoids. C60 or buckminsterfullerene is a well-known and the most abundant form of fullerene, resembling a soccer ball. Due to their unique structure and electronic properties, fullerenes like C60 have been shown to possess high antioxidant activity among other functions—organic photovoltaics and biopharmaceuticals and treatment of water.

They show bacteriostatic properties against gram-positive (*B. subtilis*) and gram-negative (*E. coli*) bacteria in saline or buffer systems; the antimicrobial properties of fullerene decrease with certain types of buffers that result in aggregation of fullerenes in solution (Fortner et al. 2005).

14.3.1 Functionalized Fullerenes Affect Viral Proteases and Possess Bacteriostatic Properties

Functionalization is essential for fullerenes to be used in biomedical applications due to their insolubility (Li et al. 2012a). Thus, functionalization with different agents imparts different properties leading to different mechanisms of action against microbes.

Functionalization of C60 has shown to impart antimicrobial activity due to better interaction with microbes. Studies of C60 with viral proteases showed that similar to carbon nanotubes, direct contact was shown to be essential for antimicrobial activity of fullerenes (Zhu et al. 2003; Shoji et al. 2013).

Heightened antimicrobial and antiviral activity was seen due to incorporation of hydroxyl, carboxylic acid, and glycolic oxide groups in C60, due to enhanced interaction of C60 with virus protease active site cavity due to the formation of

various stable hydrogen bonds with supporting interactions between C60 and aromatic Phe53/Arg8 in protease active site (Barzegar et al. 2017; Thota et al. 2012). Amino derivatives of fullerenes have demonstrated potent antimicrobial activity in the water treatment. Fulleropyrolidine-containing amino groups have been shown to deactivate HIV-1 and HIV-2 proteases (Szunerits et al. 2015).

Hexakis carboxylic acid fullerene derivatives have shown to protect mice from *Streptococcus* infection (Tsao et al. 2001). Fulleropeptides, prepared by solid-phase peptide synthesis, have been shown to possess bacteriostatic activities and have been effective against *Streptococcus* and *E. coli*. Interestingly, fulleropeptides have shown better antimicrobial activity to gram-positive bacteria than parent peptide. But these showed decreased potency against gram-negative bacteria and yeasts (D'Souza and Kadish 2012). In silico studies have shown that fullerenes can be effective against SARS-CoV-2 virus, causative agent of COVID-19 pandemic (Hurmach et al. 2021; Serrano-Aroca et al. 2021).

14.3.2 Fullerenes Affect Microbial Energy Metabolism

Antimicrobial properties of fullerenes are due to multiple properties relating to structure, solubility, and electronic chemistry. Like carbon nanotubes, membrane contact is important for the mechanism of action of fullerenes, but they do not necessarily cause membrane damage. Below are detailed accounts of mechanism of action of fullerenes in context of antimicrobial activity.

14.3.2.1 Lipophilic Nature of Fullerenes Allow for Membrane Permeability

Due to their lipophilic nature, amino and other biofunctionalized fullerenes show membranotropic properties, i.e., easy absorption by cellular membranes (Kotelnikova et al. 1996). Studies show that even though direct contact is important, unlike carbon nanotubes, physical disruption of cell wall/membrane was not responsible for antimicrobial activity of fullerenes. Rather, fullerenes were shown to pass through cell membranes and interact with components of the cellular metabolism to affect their growth and survival (Zhang et al. 2021).

Fullerenes being capable of generating reactive oxygen species are thought to harm cell membrane metabolism. Studies in gram-positive bacteria *Bacillus subtilis* and gram-negative bacteria on the effect of C60 on cell membrane lipid composition showed that the presence of fullerenes changed the proportion of unsaturated fatty acids and saturated fatty acids and impacted membrane fluidity. *P. putida* responded by decreasing its unsaturated fatty acids and increasing cyclopropane fatty acids, while *B. subtilis* levels of iso- and anteiso-branched fatty acids or monosaturated fatty acids are dependent of the concentration of C60 (Fang et al. 2007).

14.3.2.2 Cationic Fullerenes Use ROS-Mediated Antimicrobial Mechanism

Functionalization of fullerene compounds has been carried out to generate positively charged, neutral, and negatively charged derivatives. Positively charged or cationic derivatives show increased antimicrobial activity on *Shewanella oneidensis* and *E. coli*, while the anionic derivatives are not that effective (Nakamura and Mashino 2009). Cationic fullerenes are thought to inhibit the bacterial respiratory chain by ROS production or direct reduction (Cataldo and Da Ros 2008).

Another study comparing a protonated amine and deprotonated carboxylic conjugated to fullerene cage via organic linkers showed that positively charged fullerenes bound effectively to *E. coli* and showed antibacterial activity, whereas those negatively charged did not bind nor show antimicrobial activity (Deryabin et al. 2014).

14.3.3 Fullerenes Used for Photodynamic Therapy Against Infections

Oxidative damage by ROS has been known to be toxic to human cells and microbial cells. Molecules that make ROS in response to light, called photosensitizers, have been used for generation of ROS for targeting cancers and microbes in vivo (Abrahamse and Hamblin 2016). This is called photodynamic therapy (PDT). Using localized light delivery, PDT can be performed to target specifically diseased tissue, cancer cells, or microbial cells while keeping the normal cells unharmed. Since functionalized fullerenes possess the ability to form excited singlet state, followed by transition to the long-lived excited triplet state, and can react with oxygen to make ROS, they are excellent candidates for photodynamic therapy (Li et al. 2012a). Figure 14.2 represented the PDT of microbe-infected tissue using fullerenes.

Functionalization of fullerenes with the addition of hydrophilic groups enhances production of superoxides which selectively increases cytotoxicity toward microbial cells compared to mammalian cells in PDT. Photoradiation of fulleropyrrolidinium salts resulted in toxicity of 99.99% of bacterial and fungal cells (Tegos et al. 2005). Sulfobutyl fullerenes were found to be effective against environmental bacteria (Lu et al. 2010). Cationic fullerenes are found to be effective against a broad range of microbes in PDT (Sharma et al. 2011). Cationic fullerenes with quaternary amino groups are found to be most effective against *S. aureus* and *E. coli* with most resistance from *C. albicans* (Cataldo and Da Ros 2008).

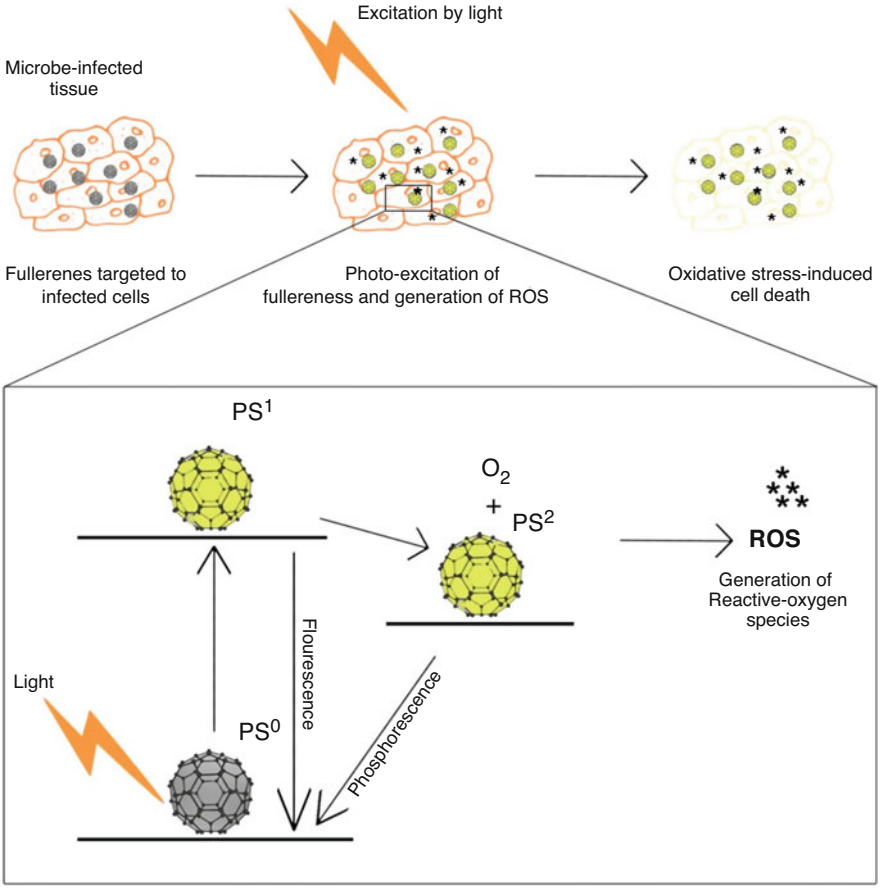

Fig. 14.2 Photodynamic therapy (PDT) of microbe-infected tissue using fullerenes. Microbe-infected tissues can be targeted using fullerenes that can specifically enter infected cells. In the presence of light, fullerenes, effective photosensitizers by nature, are excited to higher electronic states that react with oxygen to form reactive oxygen species that induce oxidative stress-induced bacterial cell death (**top**). The excitation states are shown in the boxed figure (**bottom**). Gray fullerenes represent unexcited or ground state fullerenes, and yellow-green fullerenes represent excited states. PS^1 and PS^2 are the two excited states that give rise to fluorescence and phosphorescence as they give off energy to return to the ground state (PS^0). * indicates ROS

14.3.4 Fullerene Nanocomposites as Antimicrobial Agents

Alekseeva et al. found that the fullerene/polystyrene film had bacteriostatic properties versus *S. aureus*, *E. coli*, and *C. albicans* (Alekseeva et al. 2013). Duri et al. (2017) investigated the effect of combining fullerenes (C60 or hydroxyC60) with polysaccharides such as cellulose, chitosan, and γ-cyclodextrin. The results indicated that the γ-cyclodextrin/chitosan/fullerene film had considerable antimicrobial activity against vancomycin-resistant enterococci. Thus, γ-cyclodextrin and chitosan

with fullerenes in composite films can be used in food packaging. Ballatore et al. (2015) assessed the inactivation of microorganisms in electrogenerated porphyrin-fullerene C60 polymeric films. These films exhibited photocytotoxic features versus *S. aureus* and *E. coli*; in the irradiated films, the microbial population decreased by as much as 4 logs after 30 and 60 min for *S. aureus* and *E. coli*, respectively. Therefore, porphyrin-fullerene films feature a fascinating, flexible, photodynamic, active surface that can annihilate microorganisms.

14.4 Antimicrobial Properties Graphene, Graphene Oxide (GO), and Their Derivatives

Graphene is the simplest form of carbon, made of carbon atoms arranged tightly in a monolayer, essentially thin sheets of graphite. It has unique electrochemical properties like high thermal conductivity, inertness to chemicals, very hydrophobic nature, and optical transmittance. The other graphite family members include graphene oxide, reduced graphene oxide, and graphite oxide.

A comparative study of antibacterial activity among graphite (Gt), graphite oxide (GtO), graphene oxide (GO), and reduced graphene oxide (rGO) toward *E. coli* showed that GO showed maximum antibacterial activity. After GO, rGO, Gt, and GtO showed decreasing antibacterial activity (Liu et al. 2011). Graphene oxide nanostructures are formed by functionalization of graphene with hydroxyl, epoxyl, or carboxyl groups (Sanchez et al. 2012). These hydrophilic functional groups along with hydrophobic graphenic regions allow for hydrophobic interactions in polar and hydrophilic solvents. Its large surface area also enables its use as an adsorbent or catalyst. The amphiphilic nature of GO makes them suitable for in vivo drug delivery of water-insoluble drugs and other applications in bioimaging and biosensing applications. Both graphene and graphene oxide are known for their antibacterial properties owing to two main properties: membrane damage and oxidative damage to bacterial cells. But recent studies have shown controversial results, indicating that other mechanisms may be responsible. In the coming sections, studies indicating the different mechanisms of action will be touched upon.

14.4.1 Graphene and Derivatives Cause Physical Damage to Microbial Membrane

Similar to carbon nanotubes, graphene and its derivatives show antimicrobial activity through direct contact and damage of cell membranes due to the sheets being sharp to penetrate membranes. This is why they are called nanoknives. Studies on both gram-positive and gram-negative bacteria showed that sheets caused membrane damage and consequent RNA efflux (Akhavan and Ghaderi 2010). Graphene

nanowalls, vertically standing graphene nanostructures, have also been found effective against *S. aureus* (Gurunathan et al. 2012). Damage of cell membranes by GO and rGO has been reported against *E. coli* (Hu et al. 2010). Another report suggests that apart from physical piercing of cell membrane, hydrophobic regions of GOs have been found to strongly interact with lipids on the cell membrane resulting in their extraction and further destruction of cell membrane. In *E. coli*, large amounts of phospholipids were found to be extracted by graphene nanosheets (Tu et al. 2013).

Interestingly a study in understanding GO uptake by *E. coli* through atomic force microscopy studies found that hydrophilic GO sheets face high-energy barrier before entry into the cell due to repulsive interactions with the outer cell membrane. This was thought to result in lower incidence of adhesion events possibly bringing into question the physical piercing of cell membrane as the mechanism of action of GO sheets (Romero-Vargas Castrillón et al. 2015). Nano-wrapping of bacteria by carbon nanowalls has been another proposed antimicrobial mechanism (Akhavan et al. 2011).

14.4.2 GOs Cause Oxidative Damage to Microbial Cells

GO being an oxidated state of graphene has also been shown to induce cellular damage by oxidation of bacterial cell membrane components. But the type of oxidation event has not yet been clear due to contrasting reports. On the one hand, oxidative stress has been thought to be caused by generation of ROS and on the other hand, through nonsuperoxide-mediated oxidation of cellular components directly by GO (Fig. 14.3).

Study on *P. aeruginosa* evaluating the antibacterial activities of GO and rGO showed that the presence of these nanostructures induces significant production of ROS, leading to cell death, in a time- and dose-dependent manner of GOs (Gurunathan et al. 2012).

Another report showed that ROS formation did not take place in the presence of GO and rGO, but oxidation of glutathione added for checking oxidation events in vitro indicated that GOs may be oxidating cellular substrates in microbial cells to induce oxidative stress (Gurunathan et al. 2012; Vecitis et al. 2010; Kang et al. 2008).

14.4.3 Size and Solubility of GO Determines Antimicrobial Activity

Solubility plays an important role in determining the antimicrobial activity in GO sheets. Depending on solubility antimicrobial activity of GO changes. In the case of GO suspensions, antimicrobial activity is through cell entrapment mechanism (Das

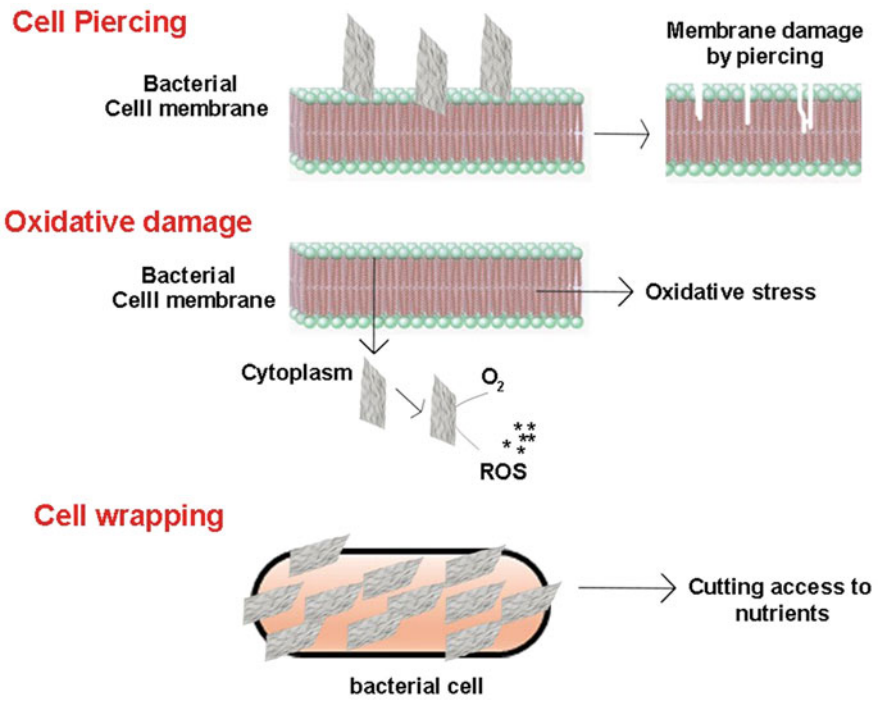

Fig. 14.3 Antimicrobial properties of GO sheets. Sheets of graphene oxide and derivatives use three mechanisms against microbes: (1) cell piercing due to the sharp edges; (2) oxidative damage to cells by production of ROS or direct reduction of cellular respiration contents; and (3) cell wrapping preventing access of microbes to nutrients

et al. 2011). This antimicrobial effect of GO increases with increasing sheet area and size. In other cases, the antimicrobial activity of GO is attributed to oxidative mechanisms, where decreasing sheet area or smaller size was found to increase antimicrobial activity by fourfold. The higher antimicrobial effect of smaller GO sheets is attributed to higher defect density of smaller sheets. The oxidative damage is also more permanent in nature, while cell entrapment is reversible (Perreault et al. 2015).

14.4.4 GO Nanocomposites in Antimicrobial Therapy

Nanocomposites of GO with metal ions and other molecules have been shown to be effective antimicrobials. GO nanostructures, graphene oxide chlorophyllin and graphene oxide chlorophyllin Zn, were found to act against *E. coli*. Membrane damage was their proposed mechanism of action. Surface chemistry by way of hydrogen bonding of tetrapyrroles with cellular surface and metal toxicity due to

Zn2+ leaching was thought to be additional antimicrobial mechanisms (Azimi et al. 2014).

14.4.4.1 Metal Nanocomposites Most Effective Antimicrobial Agents

GO-Ag nanocomposites have shown effective antibacterial activity against bacteria, gram- positive and gram-negative (Perreault et al. 2015; Yun 2013). GO-Ag composites significantly reduced *E. coli* and *S. aureus* populations by up to 99.99% and 99.96%, respectively. Membrane penetration, oxidative stress, ROS production, and disruption of bacterial DNA replication were found to be responsible. These were thought to be dependent on temperature, time, pH, and concentration.

A bacteriostatic activity was observed against *E. coli* by GO-ZnO nanocomposites (Wang et al. 2014b). Although gold NPs themselves have antimicrobial properties against *E. coli*, addition of GO or rGO resulted in enhanced action against *S. aureus* and *B. subtilis* as well. The antibacterial activity of GO-Ag and GO-ZnO against *E. coli*, *Enterococcus faecium*, *S. aureus*, and *Klebsiella pneumonia* was assessed. GO-Ag was found to be more effective than GO-ZnO in terms of antibacterial activity (Whitehead et al. 2017).

Nanocomposite of rGO, CuO, and poly-L-lysine (PLL) showed high antimicrobial activity against *E. coli* and *S .aureus* with a lethality rate of 99.9% (Ouyang et al. 2013). GO-Au or rGO-Au composites show enhanced activity against microorganisms such as *E. coli*, *S. aureus*, and *B. subtilis* (Hussain et al. 2014). Irradiated (758 nm) nanocomposites have also been found to be effective antimicrobial properties. GO-Au, GO-Fe, and rGO-TiO2Au were found to work against gram-positive, gram-negative, and fungal microorganisms. GO-TiO2 composites have been found to be cytotoxic to mammalian cells as well. They announced that this composite could damage the mitochondria, increase the number of lysosomes, and consequently disrupt and destroy the cell (Díez-Pascual 2020). rGO nanocomposites containing polyvinyl alcohol and Cu2O or TiO2 have shown bacteriostatic activity against *Streptococcus oralis*, *S. aureus*, *E.coli*, and *P. aeruginosa* (Dhanasekar et al. 2018). TiO2 particles and their nanocomposites have excellent potential to be used in food packaging applications. rGO-FeNP composites have shown bacteriostatic activity against *S. aureus*. These produce hydroxyl radicals inactivate vital cells and kill bacteria both in vivo and in vitro.

14.4.4.2 Other GO Nanocomposites and Their Antimicrobial Properties

Studies on polymer-GO composites like polyurethane-rGO-polyethyleneimine and GO- polyurethane composites showed that polyurethane-rGO-PEI composite had higher antibacterial property and hence has practical application in biomolecule encapsulation or immobilization (Tang et al. 2016). Agarose has also been used as an antibacterial hydrogel for its bacteriostatic properties. GO along with cationic surfactants such as benzalkonium bromide can be used to produce novel GO

hydrogels for their antibacterial properties (Wang et al. 2015; Guo et al. 2020). The combination of polyvinyl-N-carbazole (PVC) and GO shows enhanced bacteriostatic activity against *E. coli*, *Cupriavidus metallidurans*, *B. subtilis*, and *Rhodococcus opacus* and few gram- positive bacteria (Carpio et al. 2012). For this reason, GO-PVC composites could be used to prevent biofilm formation.

GOs with surfactants like Tween are shown to be effective in inhibiting the bacterial growth (Szunerits and Boukherroub 2016). A composite with GO, LF, and chitosan enhanced antimicrobial activity of GO against bacteria (Nanda et al. 2015). GO-lysozyme composite showed excellent activity against *E. coli*. Nisin-G composites produced an active matrix that was used to identify, separate, and disinfect water contaminated with methicillin-resistant *S. aureus*. Due to the numerous properties of GO, they are under consideration for medical applications. One of the major challenges is its low biocompatibility. Functionalized GO are hence the solution for applicability.

14.5 Carbon Dots as Emerging Class of Photosensitizers for Antimicrobial Therapy

Carbon dots, also known as quantum carbon dots, are extremely tiny, quasi-spherical nanoparticles with diameter below 10 nm. They are a new class of carbon nanomaterials with enhanced photosensitizing properties. They have the ability of specific detection and inactivation of different bacterial species. Like other carbon nanoparticles, they possess chemical stability and outstanding photoelectric properties. Interestingly, they show high water solubility, low toxicity, and hence are known to be extremely biocompatible. They are easy to prepare and are affordable and hence ideal candidates for antimicrobial therapy. Discovered in 2004, CDs have been found to have applications ranging from use in semiconductors to biomedicine and agricultural applications (Li et al. 2012b).

Their photosensitizing properties have been exploited for testing their use in photodynamic therapy, like GOs. Bright carbon dots (Cipro@Cdots) synthesized using microwave assistance and gum arabic have been shown to be used to deliver broad-spectrum antibiotic nad ciprofloxacin hydrochloride into mammalian cells where release of antibiotic was regulated under physiological conditions. Release of ciprofloxacin was found to be extremely regulated under physiological conditions. Cipro@Cdots conjugate also exhibited antimicrobial activity against gram-positive and gram-negative microbes. CDs hence could be used as nanocarriers with abilities for controlled drug release, contributing to high antimicrobial activity. Table 14.1 summarizes the different types of nanoparticles described in this chapter and their mechanisms of action.

Table 14.1 Summary of types of carbon nanoparticles and their antimicrobial mechanisms

Type of carbon nanoparticle (CNP)	Mechanism of action
Carbon nanotubes SWCNTs, MWCNTs, DWCNTs, and metal carbon nanotube composites	Physical damage of the cell membrane Oxidative stress by ROS production
Fullerenes Fullerene family of materials, C60 (common), and metal fullerene composites	Oxidative stress by ROS production Damaging cellular respiration directly
Carbon nanosheets Graphene, GO, rGO, GO polymer composites, and GO metal composites	Membrane piercing (physical damage) Oxidative stress by ROS production

14.6 Future Perspectives for the Use of Carbon Nanomaterials as Antibiotics: Advantages and Challenges

Nanoparticles by themselves or in conjugation with other molecules are the next-generation antibiotics due to their varied mechanisms of action against bacterial cells. Current antibiotics fail due to the fast rate of evolution of microbes against the mechanisms of action of antibiotics. This is especially true for gram-negative ESKAPE pathogens that are more resistant than others (Santajit and Indrawattana 2016). This resistance is therefore due to the biological warfare and continuous arms race between biological molecules. Hence the only way to put an end to this never-ending fight is the use of physical forces that bacteria cannot evolve against. This is largely absent in the current in vivo antimicrobial therapies. Carbon nanomaterials hence have an advantage due to their properties of piercing cell membranes and carrying out physical damage to microbial cells. Carbon nanotubes and graphene and its derivatives are very well known for this property and hence can be used as carriers with antibiotics or standalone for antimicrobial in vivo therapy.

One of the main issues in the fight against antimicrobial resistance is the lack of biotherapeutic molecules for use as antibiotics in the case of multidrug-resistant infections. Thus, combinatorial therapy with existing antibiotics can be carried out to generate new drug combinations. Combination of nanoparticles along with existing antibiotics show a better therapeutic strategy to combat multidrug-resistant infections, targeting different mechanisms of action against bacteria. This would also mean more combinations can be created in therapy to prevent the rise of resistant strains.

Non-toxic inert carbon nanoparticles are used as carriers of antibiotics to allow for more permeability into bacterial cells due to the lipophilic nature of carbon nanoparticles like fullerenes. This makes better drug permeability and availability and also allows for targeted drug delivery mechanisms.

Graphene and metal nanocomposites of carbon nanoparticles have been shown to be very effective against multidrug-resistant bacteria due to their thermo-plasmonic properties. Therefore, antibiotic functionalized carbon nanoparticles could be used for photothermal lysis to increase the efficacy of antimicrobial therapy. Some composites of carbon nanodots have been shown to regulate the release of antibiotics as well depending on physiological conditions, allowing for controlled release of antibiotics in vivo.

One of the major factors in antibiotic resistance is efflux of antibiotics by the increased number of efflux pumps on membranes of resistant bacteria, affecting drug availability in the cellular interior. Carbon nanotubes and fullerenes have been shown to inhibit the activity of efflux pumps and components in bacterial membrane and metabolism, neutralizing the resistance mechanisms. This is thus a huge advantage of using nanoparticles in conjugation with antibiotics that will allow for more drug availability.

Although antibacterial effects of carbon nanoparticles have been studied for two decades, only few have come into mainstream treatment. The three main reasons for it are the lack of comprehensive understanding of cytotoxicity of nanoparticles on biological systems, selection of narrow size range of nanoparticles, and emerging news of resistance against nanoparticles that have mechanism of action other than physical damage. Cytotoxicity of carbon nanoparticles essentially is imparted by chemical treatments carried out during their preparation to arrive at the right size range as well as to functionalize them for use in biological applications. Although toxicity of various carbon nanoparticles and their functionalized derivatives have been studied in mammalian and microbial cells, and even mice, there needs to be studies carried out in higher mammalian models to understand accumulation of carbon nanoparticles (CNP) in tissues, organs, their half-life and clearance, and potential long-term effects. The antimicrobial mechanisms of CNPs effective against the evolving microbes are summarized in Fig. 14.4.

Synthesis of carbon nanoparticles is fairly a complex process and extremely sensitive to various factors, including choice of precursors and method of synthesis to temperature and chemical environment. This makes their synthesis unpredictable as getting a narrow range of size of CNPs in nanoscale is a challenge, consistently, even with the same production method. Even if synthesis is achieved, slight variations may cause changes in their antimicrobial properties which need to be tested every time, unlike drugs that will behave similarly if a particular SOP is followed for its synthesis. Therefore, carbon nanoparticles used in therapy would require exhaustive structural and functional characterization studies.

Non-toxic carbon nanoparticles have been in use for nano-delivery and sustained release of antibiotics, to counter-attack toxicity from antibiotics themselves. Polymer-based and nonpolymeric nanoparticles as liposomes are used for nano-delivery for the treatment of infectious diseases (Zazo et al. 2016). There are now commercially available carbon nanoparticles used for treatment. Respiratory infections are generally difficult to treat and have longer treatment time that leads to systemic side effects. Broad-spectrum antibiotics like ciprofloxacin used for lung infections are now commercially available as a liposome formulation such as

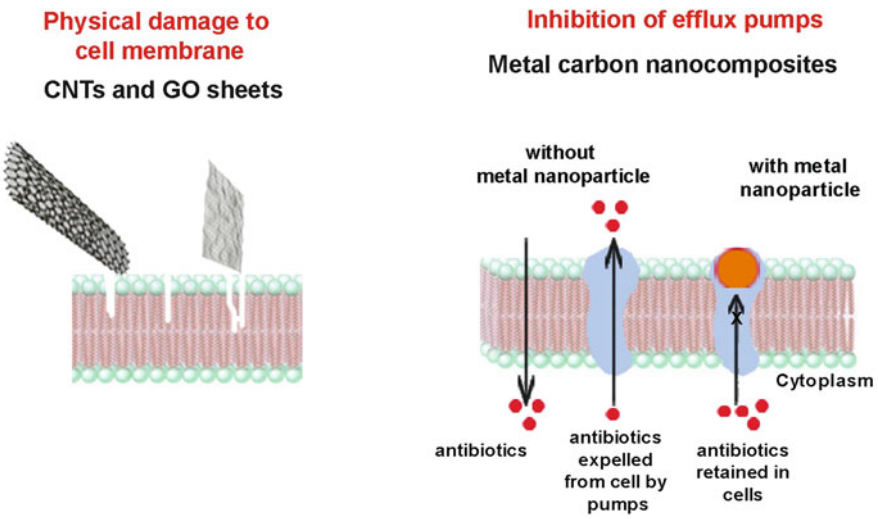

Fig. 14.4 CNP mechanisms effective in combating the evolving microbial defense. Microbes develop antimicrobial resistance to antibiotics by either evolving enzymes that breakdown membrane-targeting antibiotics or evolving transmembrane efflux pumps that pump out antibiotics from cell interior reducing drug availability. Carbon nanoparticles in combination with traditional antibiotics are advantageous as CNPs by themselves can carry out physical damage and prevent efflux of antibiotics by blocking bacterial membrane pumps

Lipoquin™, designed as an inhaled formula that releases drug in 24 h, eliminating the systemic effects of the high-dose antibiotic (Cipolla et al. 2016). Antifungal liposomal carrier AmBisome® (amphotericin B) reduces toxicity of amphotericin B and hence is used for highly immunocompromised patients with HIV infection and disseminated histoplasmosis (Meletiadis et al. 2008). Nano-drugs containing silver nanoparticles are also used in biosensors and medical devices like venous catheters to prevent biofilm formation (Wu et al. 2015; Wang et al. 2017). Other nanoparticles used in diagnosis or as medical devices include Endorem™ SPIONs, Verigene®, Silverline®, and Acticoat™ (Beal et al. 2013).

Recent reports indicating resistance against nanomaterials show an emerging threat against the use of nanomaterials as antibiotics. These concerns have arisen due to the widespread use of silver nanoparticles in consumer products as well as healthcare settings. Studies have shown the possibility of *Acinetobacter baumannii*, an opportunistic pathogen, causing nosocomial infections, to become resistant to silver nanoparticles. This raises concern over the use of other nanoparticles, especially those with heavy metal composites in antimicrobial therapy. Further studies are required to understand and combat these challenges before carbon nanoparticles could be deployed as antibiotics for the next generation therapeutics.

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Conflict of Interest None to declare.

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Chapter 15
Dendrimeric Entities as Chemical Alternatives Toward Antimicrobial Therapy

Nilotpal Borah, Abhijit Gogoi, and Jiban Saikia

Abstract The intrinsic ability of certain pathogenic microbes to grow resistance toward the beneficial impressions of various antimicrobial medications threatens the efficacious intervention and remedy for mushrooming infections carried out by multiple parasites, fungi, viruses, and bacteria. Thus, antimicrobial resistance has amplified the alarming threat to the public health worldwide, which necessitates novel antimicrobial agents to restrict the severity caused by the multidrug-resistant (MDR) pathogens. In contrast to the conventional approach of expending antibiotics based on small molecules to target the specific bacterial nucleic acids, proteins, or cell wall enzymes, the requirement for efficient antimicrobials has tilted toward polymeric entities. The integration of antimicrobial functional groups into polymeric molecules can get rid of the drawbacks exhibited by low-molecular-weight antimicrobial agents, which include transitory antimicrobial activity and environmental toxicity. Within this framework, dendrimers have revealed itself as a fascinating platform in the recent past for certain biomedical applications owing to their uniform dispersity and nanoscale morphology and controlled core with adjustable branch and surface functionalities. Tuning the functionality of the dendrimeric entity, novel efficient resources can be synthesized which may lead to dendritic scaffold with prospective for the apposite design of certain antimicrobial agents having distinct mechanism of action. This chapter illustrates the limitations confined to the current antimicrobial agents and describes the potentials borne by the different classes of dendrimers including cationic dendrimers, anionic dendrimers, glycodendrimers, peptide-based dendrimers, and organometallic dendrimers with a purpose to supplant several antibiotics. It also attempts to delve into hitherto unknown territory in

N. Borah
Surface Chemistry, Oil and Natural Gas Corporation Ltd., Sivasagar, Assam, India

A. Gogoi
Toxicology Division, Directorate of Forensic Science, Guwahati, Assam, India

J. Saikia (✉)
Department of Chemistry, Dibrugarh University, Dibrugarh, Assam, India
e-mail: jibansaikia@dibru.ac.in

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this field of study with constant consideration of the contemporary possibilities for insightful and fruitful research into the use of dendrimers as antibacterial agents.

Keywords Dendrimers · Antimicrobial · Multidrug-resistant pathogens · Diseases · Glycodendrimers · Cationic dendrimers · Anionic dendrimers · Peptide-based dendrimers · Organometallic dendrimers · Pathogens · Gram-positive bacteria · Gram-negative bacteria · Cytotoxicity · Dendrimeric peptide · Nucleic acids · Monodispersity · Macromolecules · Microbes · Antibacterial

Abbreviation

AMP	Antimicrobial peptide
AMPD	Antimicrobial peptide dendrimer
CTX	Cholera toxin
DMSO	Dimethyl sulfoxide
MAP	Multiple antigen peptide
MDR	Multidrug-resistant
MeI	Methyl iodide
MIC	Minimum inhibitory concentrations
PAMAM	Polyamidoamine
PITC	Phenyl isothiocyanate
PPI	Polypropylenimine
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
STED	Stimulated emission depletion
TEM	Transmission electron microscopy

15.1 Introduction

Dating back to the beginning of twentieth century, with the advancement of scientific notion "magic bullet," Paul Ehrlich coined the term chemotherapy where the fundamental perception lies in the strategic development of a chemical moiety with appropriate recognition activity, facile derivatization of the functional groups, and continuous efforts to ascertain the extent of its activity and effectiveness. This discovery of Arsphenamine (salvarsan) and its chemical derivative neoarsphenamine (Neosalvarsan) by Ehrlich in 1910 marked the initial use of antimicrobial agents in the world as the remedy for syphilis caused by bacterium *Treponema pallidum*. Despite extensive explorations, these remained as the sole magic bullet used in chemotherapeutic treatment for microbial infections until the serendipitous detection of penicillin in 1928 by Alexander Fleming. Penicillin having antibacterial effect toward the *Staphylococci* and other gram-positive pathogens was employed in the

medical practice in the 1940s. Its profound efficacy and benign use had led to save millions of lives during World War II. Concurrent to Fleming's trial for purification of penicillin, sulfonamidochrysoidine (KI-730, Prontosil) was synthesized by Bayer chemists Josef Klarer and Fritz Mietzsch and tested by Gerhard Domagk for antibacterial activity in numerous diseases. However, in 1935, Prontosil was found to be a predecessor to the active drug, since it was converted to sulphanilamide ushering the dawn of the sulphonamide era. Succeeding two decades had witnessed the expansion in categories of new antimicrobial agents and flourished the golden age of antimicrobial chemotherapy. Soil bacteria-based antibiotics came to the limelight in 1944 with the discovery of streptomycin, an aminoglycoside antibiotic derived from the soil bacterium *Streptomyces griseus*. Since then, various antibiotics, including chloramphenicol, tetracycline, macrolide, and glycopeptide (e.g., vancomycin) were discovered from soil bacteria.

In the pursuit of achieving a broader antimicrobial spectrum coupled with superior antimicrobial activity, continuous improvements and modifications are accomplished in each class of the antimicrobial agents. Penicillin, for example, was initially effective for gram-positive organisms such as *S. aureus*. Penicillin-resistant *S. aureus* began harvesting penicillinase enzyme capable of penicillin-hydrolysis and thus resulted the emergence of methicillin. Consequently, amplification of the antimicrobial spectrum resulted in ampicillin for the gram-negative *Enterobacteriaceae*, while piperacillin works befittingly even upon *Pseudomonas aeruginosa* (Gould 2016; Saga and Yamaguchi 2009).

Primarily, antimicrobial agents are categorized based on their particular mode of action utilized in treating bacterial infections. Generally, five major mechanisms of action are apparent: (1) inhibition of the cell wall synthesis or damage to cell wall which includes β-lactams and vancomycin; (2) inhibition of protein synthesis exhibited by erythromycin, tetracyclines, aminoglycosides, and oxazolidinones; (3) interference with nucleic acid synthesis comprising rifampin that inhibits RNA synthesis and fluoroquinolones that inhibits DNA synthesis; (4) inhibition of metabolic pathway shown by sulphonamides and folic acid analogues; and (5) disruption of the bacterial membrane structure or damage to cytoplasmic membrane by polyene and polymyxins (Reygaert 2018; Neu 1992).

Although the diverse range of mechanisms may hint for better control over the organisms, in contrast, microorganisms gradually acquire resistance to antimicrobials within a few months to several years after its introduction into the clinic (Davies 1996). More alarming facts suggest that bacteria can easily collect and exchange antibiotic-resistant genes between other cells and even different species. Prominent factors for developing antimicrobial resistance consist of augmented administration of antimicrobial drugs and inappropriate prescriptions for antimicrobial therapy. Mostly, the resistance strategies adopted by the bacteria comprises (1) effluxing the antibiotic from the microbial cell; (2) inactivation of the antibiotic by attaining deactivating enzymes; and (3) transformation of the antibiotic's target structure on the bacterial cell surface (Walsh 2000). This has led to the absolute requirement of alternative novel approaches to fight and prevent the occurrence of the antimicrobial resistance. Such new classes include dendrimeric antimicrobials

capable of targeting or constraining virulence factors. In the recent past, apart from their potent use in antimicrobial chemotherapy, dendrimers have been explored as bacteriophobic coatings along with its suitable candidature for drug delivery, thus augmenting its prospective for the expansion of novel antimicrobial therapy.

15.2 Dendrimers: A Brief Introduction

The word dendrimer has been derived from the Greek words "dendron," meaning tree, and "meros," meaning part. Dendrimers are well-defined, multivalent molecules consisting of a well-defined tree-like branched structure. These molecules were first reported by Fritz Vögtle et al. as "cascade molecules," while Tomalia et al. gave the term dendrimer. Dendrimers, being nearly monodisperse macromolecules having nanoscopic dimension, possesses discrete molecular architectures (Fig. 15.1) comprising three different domains: (a) a central core: consisting of a single atom or a collection of atoms encompassing at least two chemical functionalities which afford linkage for the branches; (b) branches: originated from the core, these consist of repeating units with a minimum of one junction of branching. The repetition is followed according to geometric progression resulting in an array of radially concentric layers characterized as generations (G) and (c) terminal functional groups: these constitute the peripheral boundary of the dendrimeric molecule dictating its competence during nucleic acid complexation or entrapment of drug molecules (Abbasi et al. 2014; Nimesh 2013).

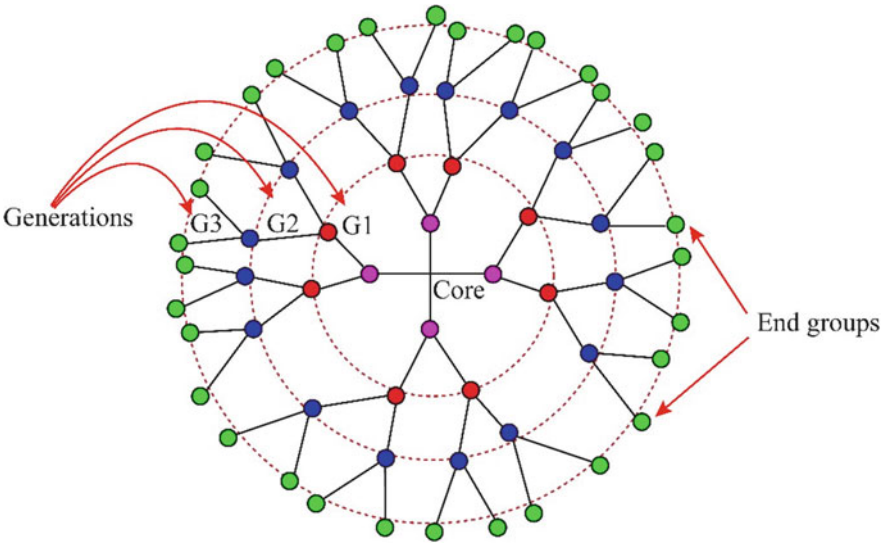

Fig. 15.1 Schematic representation of the structure of a dendrimer

15.3 Why Are Dendrimers Chosen as Antimicrobial Agents?

Dendrimers possess well-defined discrete structure, size monodispersity, and apt stability accompanied by the proficiency for surface functionalization, which in turn enhances its efficacy as drug delivery agents. Alteration of the end or surface group functionalities of dendrimers with biologically active antimicrobial groups may lead to an augmented antimicrobial activity of the same determined by the increase in the high molecular antimicrobial functional group on the surface of the molecule (Chis et al. 2020). In another approach, dendrons of various generations are coupled together with a linear core yielding a branched dendrimer with a non-uniform orthogonal architecture termed as asymmetric dendrimers. The asymmetry is beneficial for fine-tuning the structures and molecular weight of the dendrimeric entity, allowing precise control over the functions of each dendron during interactions with drug molecules, imaging agents, and additional therapeutic moieties. Dendrimers, being globular macromolecules of nanometer size, make themselves ideal for antimicrobial drug delivery on the nanoparticle platform. Unlike small molecule antimicrobials, the presence of quaternary ammonium salts as functional end groups in certain dendrimeric biocides may result in superior antimicrobial properties against certain bacteria due to the high density of active antimicrobials on the dendrimeric surfaces (Karthikeyan et al. 2016). As dendrimers are bulkier macromolecules, their penetration through the cell membrane barrier is seemingly challenging and reaching the target regions of interest for the anticipated antimicrobial action may prove to be difficult to achieve. Hence, for efficacious antimicrobial treatment, the target regions for the antimicrobial activity must be selected carefully (Chen and Cooper 2002).

15.4 Various Classes of Antimicrobial Dendrimers

15.4.1 Glycodendrimers

Recognition of sugar molecules present in the eukaryotic cell surface and multivalent protein- carbohydrate interactions between bacterial cell surface proteins and eukaryotic glycoproteins or glycolipids has enabled the microbes in securing host cell (Remaut and Waksman 2004). These interactions are purely noncovalent, and multivalency is required to achieve effective recognition and binding of bacteria with the host cell. Glycodendrimers are monodisperse branched tree-like structures with multiple carbohydrate units as the end groups. These are developed with a design principle to resemble the surface architecture of the host cell and might be effective in minimizing the attachment of bacteria to the eukaryotic cell at the expense of the former (Hoyos et al. 2021).

Cholera toxin (CTX) and enterotoxin of *Escherichia coli* both bind to eukaryotic cells through the interactions with oligosaccharides of GM1, present on the cell

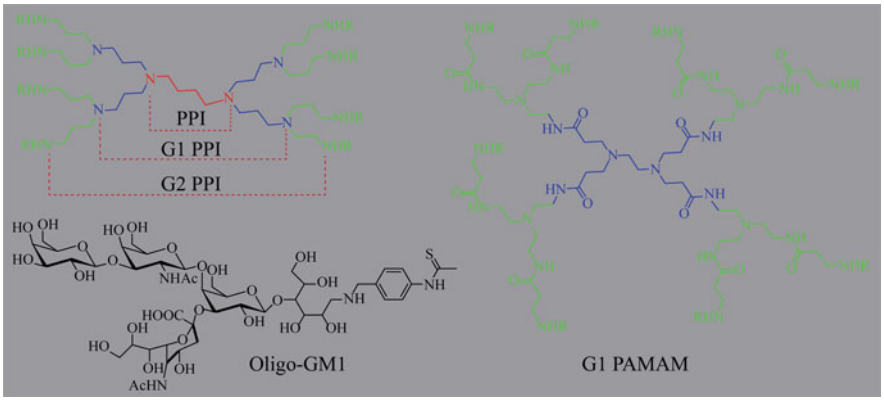

Fig. 15.2 Structures of PPI- and PAMAM-based glycodendrimers with oligo-GM1 sugar appended to the surface

surface (Masserini et al. 1992; Van Heyningen et al. 1971). Oligosaccharide residues coupled with poly-L-lysine molecules were already reported to exhibit antibacterial activities against those bacteria. Therefore, dendrimers with multiple oligosaccharide moieties were attempted to synthesize, expecting to get better antibacterial activities as a result of the increased concentration of saccharide molecules. Accordingly, two polypropylenimine (PPI)-based dendrimers up to second-generation and a first-generation polyamidoamine (PAMAM) dendrimers with phenylisothiocyanate (PITC)-derivatized multivalent oligosaccharide (oligo-GM1) were synthesized (Fig. 15.2) (Thompson and Schengrund 1997). The glycodendrimers were shown to be capable of inhibiting the binding of the choleragenoid and the heat-labile enterotoxin to GM1-coated wells at molar concentrations 5 to 15 times lesser than native GM1 and beyond 1000 times lower than free oligosaccharide. This has proved the importance of dendrimeric framework in enhancing the antibacterial properties.

In the search for suitable replacement of costly oligo-GM1-linked dendrimer, dendrimers linked with polyethylene glycol-modified galactose residues were obtained (Branderhorst et al. 2007). The antibacterial potentials were high, and the IC_{50} values were roughly comparable to the monovalent oligo-GM1 analogue.

Similarly, multivalent lactose-based glycodendrimers were discovered to be efficient against the cholera toxin B component. These glycodendrimers were synthesized by Pieters et al. and consisted of repeating units of 3,5-di-(2-aminoethoxy)benzoic acid with a maximum of eight lactose groups (third generation) in the periphery (Vrasidas et al. 2001). The binding affinities with the toxin were estimated using fluorescence assay of the glycodendrimers and determined to be in the range of 18 mM to 33 μM for monovalent and octavalent lactose dendrimers, respectively. It was further modified with elongated spacer arms, and lactose was replaced by GM1 oligosaccharide ligand (Pukin et al. 2007). The novel oligo-GM1-linked dendrimers demonstrated exceptional binding that was up to 380,000-fold stronger than the monovalent analogue.

15.4.2 Cationic Dendrimers

The antibacterial potential associated with the cationic dendrimers stems from their ability to interact and disrupt the bacterial cell membrane. The interaction and disruption of the bacterial membrane by such dendrimers is strictly connected to their supramolecular structure and charge distribution. Surfaces of cationic dendrimers are frequently functionalized with ammonium groups with different degrees of alkyl substitution and chain lengths. The presence of such groups has increased the electrostatic interactions with the bacterial cell, as their surfaces were negatively charged (Lind et al. 2015; Matos et al. 1991). Therefore, they strongly interact with each other, which resulted in an enhancement in membrane permeability followed by bacterial lysis and cell content release. Such charged molecules are obviously water-soluble, which enable them to explore their antibacterial properties in the real aqueous world. However, antibacterial activity requires a balance of positive charge and generation number; alternatively, it may prove to be cytotoxic to the host cell. Therefore, the search for efficient dendrimers selective to the bacterial cell membrane is going on (Alfei and Schito 2020; Chen et al. 2020).

Quaternary ammonium compounds are well-known antibacterial agents (Chen et al. 2000), and Cooper et al. had used this concept to test a quaternary ammonium functionalized dendrimers as antimicrobial agents (Chen et al. 1999). The newly synthesized dendrimer had 16 quaternary ammonium groups per molecule obtained by amalgamation of dimethyldodecylammonium chloride functionalities on top of poly(propylene imine) dendrimers. As a result, in addition to the cationic charge, there was a significant hydrophobic concentration near the periphery. The antibacterial activities of the reported cationic dendrimers were significantly improved with the inclusion of the quaternized functionalities. However, the antibacterial characteristics of such dendrimers are also influenced by a few other parameters including dendrimer size, hydrophobic chain length, and the type of the counter anions present. Cooper et al. discovered that hydrophobic chains with chain length C_{10} were more efficient against *E. coli* in a structure-activity assay, followed by C_{12} (Chen et al. 2000). Again the effect of generation number too has a particular influence on the antimicrobial activity as G5 > G4 > G1 > G2 > G3.

Ortega et al. synthesized and tested a series of new amine- and ammonium-terminated carbosilane dendrimers for antibacterial activity against gram-positive and gram-negative bacteria (Fig. 15.3). The amine-terminated carbosilane dendrimers were achieved from the reactions of (chloromethyl)silyl-terminated dendrimers with 3-dimethylamine phenol, and it was further quaternized with methyl iodide to obtain the ammonium-terminated carbosilanes (Ortega et al. 2008). The ammonium-terminated dendrimer was insoluble in mixed aqueous solution with less than 1% DMSO. As expected, dendrimers are superior in combating the *Staphylococcus aureus* (gram-positive) and *Escherichia coli* (gram-negative) than their monofunctional counterpart. Similarly, ammonium-terminated carbosilane dendrimers formed by reacting the dendrimer with allydimethylamine and then quaternizing with MeI was also found to be a potent antibacterial agent (Ortega et al. 2011). In water or other protic solvents, quaternized complexes formed by reaction with MeI are soluble

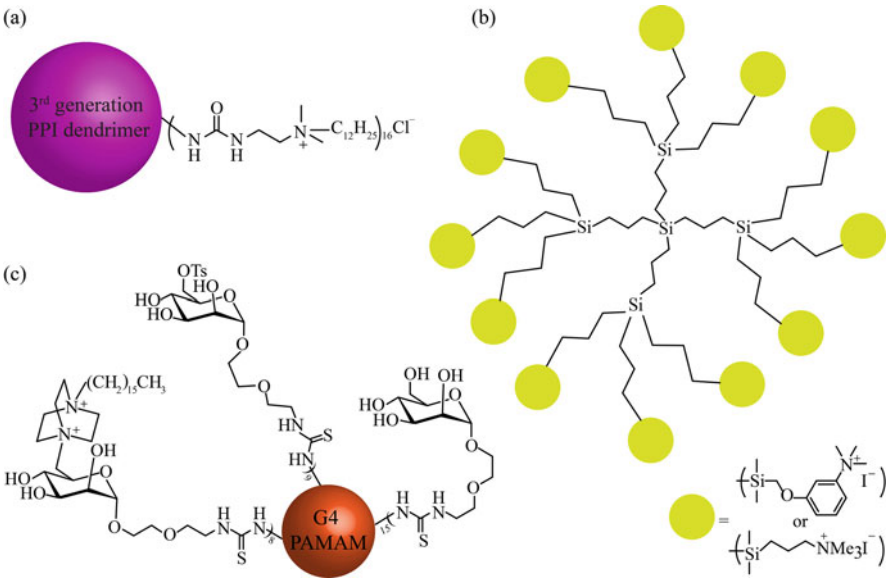

Fig. 15.3 (**a**) Third-generation PPI-based cationic dendrimers with quaternary ammonium group, (**b**) the skeleton of carbosilane-type cationic dendrimers with different end groups, (**c**) fourth-generation PAMAM-based cationic dendrimer

and stable. For both first- and second-generation dendrimers, the MIC values against *Staphylococcus aureus* are slightly higher than those against *E. coli*. Other cationic carbosilane dendrimers of the type G_n-$[Si(CH_2)_3N(Et)CH_2CH_2NMe_2]_x$ and G_n-$\{[Si(CH_2)_3N^+R(Et)CH_2CH_2N^+RMe_2]_x(X^-)_y\}$ (where $n = 1$, 2, and 3; R = H and X = Cl; and R = Me and X = I) (Rasines et al. 2009) and S containing carbosilane dendrimers with terminal $-NH_3^+$,$-NMe_3^+$, and $-[NMe_2(CH_2CH_2OH)]^+$ groups (Paniagua et al. 2014) were also effective antibacterial agents. The replacement of Si atoms with S atoms has a beneficial influence on antibacterial characteristics, whereas increasing the size of substituents on N atoms has a detrimental impact on antibacterial properties. Now, a fourth-generation mannose functionalized poly(amidoamine) dendrimers (G (4)-PAMAMs) has been reported with 4-aza-1-hexadecylazoniabicylo-[2.2.2]octane or C16-DABCO as quaternary ammonium end groups (VanKoten et al. 2016). It was a colossal dendrimer with exceptionally high quaternary ammonium group concentrations at the dendrimer framework. Consequently, the obtained MIC values were nearly ten times as low as the control compound for against *S. oralis*, *S. aureus*, *B. cereus*, *P. aeruginosa*, and *E. coli*. This dendrimer presumably utilizes both the mannose and the quaternary ammonium end groups to interact detrimentally with the bacterial cell wall.

15.4.3 Anionic Dendrimers

Grienstaff et al. were the first to discover antibacterial activities of anionic amphiphilic dendrimers with surface blocks composed of succinic acid, glycerol, and myristic acid possessing various numbers of acid and alkyl functionalities (Meyers et al. 2008). The cytotoxicity of glycerol-based dendrimers (Fig. 15.4) was found to be strikingly selective toward prokaryotic gram-positive bacteria (*Bacillus subtilis* AG174) in comparison to a eukaryotic human cell. The authors also discovered that the commercially available anionic sodium dodecyl sulphate (SDS) amphiphile and nonionic amphiphile Triton X-100, used as control chemicals, were cytotoxic to the bacterial strain.

15.4.4 Peptide-Based Dendrimers

Among the several strategies to replenish our diminishing resources of anti-infective agents, antimicrobial peptides (AMPs) have grasped keen attention in the last few decades (Gan et al. 2021; Li et al. 2021). AMPs being a vital component of the distinct immune system of all metazoans are characterized by the typically cationic, amphipathic structures which can destabilize biological membranes and capable of

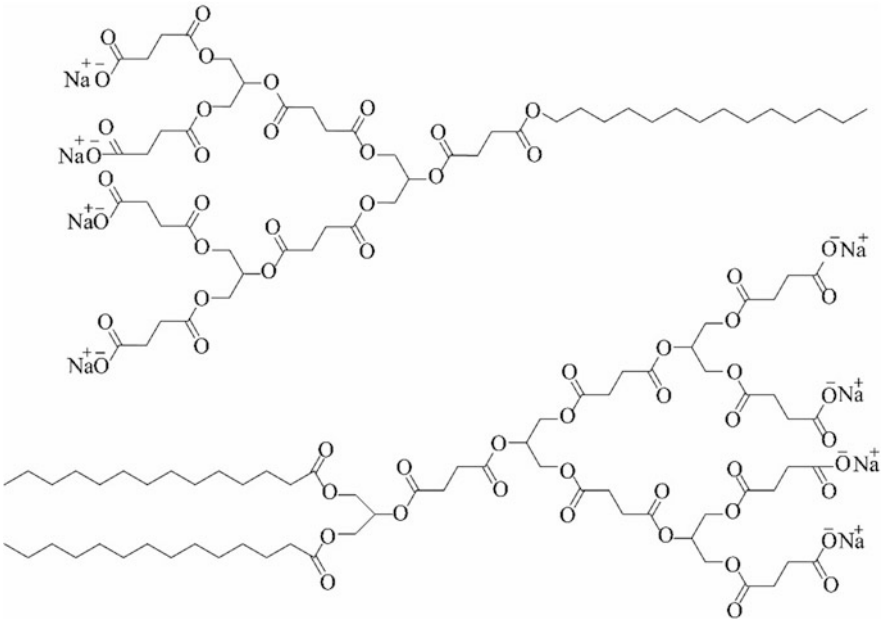

Fig. 15.4 Skeletons of two glycerol-based anionic dendrimer with different numbers of hydrophobic tails

forming transmembrane channels along with functioning as immunomodulators (Scorciapino et al. 2017). The AMPs also exhibit certain disadvantages by virtue of their peptidic nature, occasional bioavailability, and poor proteolytic stability, which have hindered the progressive clinical growth severely. In an attempt to overcome these drawbacks, analogous multimeric synthetic peptides have been developed.

Dendrimeric peptides, which were developed in the decade of 1980, are composed of an array of branching peptides arranged around a multivalent core. These initially resulted in multiple antigen peptide (MAP) systems as immunogens for generating site-specific polyclonal and monoclonal antibodies. The dendrimeric core of a MAP (Fig. 15.5) encompasses a divalent Lys core whose α and ε amines multiply twofold geometrically following each branching generation. Overall, dendrimeric peptides have better antimicrobial activity than monomeric peptides due to their multivalent nature, which is attributed to the higher local concentration of bioactive units in dendrimeric molecules, as well as the reduced vulnerability to the action of proteases and peptidases due to the steric hindrance imposed by the branching chains, which improves the pharmacokinetic properties of the peptides.

Tam and co-workers have reported a peptide-based dendrimer decorated with tetravalent (D4) and octavalent (D8) polylysine cores (Tam et al. 2002). R4 tetrapeptides (Arg-Leu-Tyr- Arg) or R8 octapeptides (Arg-Leu-Tyr-Arg-Lys-Val-Tyr-Gly) adorn the surface of the dendrimeric core (Fig. 15.6). Keeping in mind the antimicrobial action against certain gram- negative and gram-positive bacterial strains, these dendrimeric peptide structures have been synthesized. Irrespective of the peptide sequence (R4 versus R8), the tetra- and octavalent R4 and R8 dendrimers exhibit enhanced broad-spectrum activity under high- and low-salt conditions as compared to the divalent analogues. In terms of potency and activity, tetravalent R4 dendrimers are equivalent to other potent antimicrobial classes consisting of protegrins and tachyplesins. Furthermore, the low toxicity associated with peptide dendrimers is reflected in their minimal hemolytic activity on human erythrocytes.

Pini et al. utilized a phage display tool for selecting suitable peptides or proteins equipped with specific binding properties to construct a dendrimeric peptide. Using phage library selection, a specific peptide sequence (Gln-Lys-Lys-Ile-Arg-Val-Arg-Leu-Ser-Ala) was carefully chosen and conjugated to a tetravalent polylysine dendrimer core (Pini et al. 2005). The antimicrobial activity of the synthesized dendrimeric peptide against *E. coli* was shown to be superior to that of the monomeric version. Low MIC, excellent stability against blood proteases, minimal hemolytic activity in human erythrocytes, and low cytotoxic effects on eukaryotic cells were all apparent in the same dendrimeric peptides, making it a viable candidate for clinical development of a new class of antimicrobials. Bruschi and his group discovered a new dendrimeric peptide (SB041) with a tetra-branched structure and four similar peptides convergent to a lysine core (Fig. 15.7a) (Bruschi et al. 2010). A lipophilic aminovaleric acid chain was introduced to the design principle of the dendrimeric peptide to improve membrane binding, and pyroglutamic acid was utilized instead of glutamine at the N-terminus to give greater stability by circumventing the cyclization process. The dendrimeric peptide outperforms the

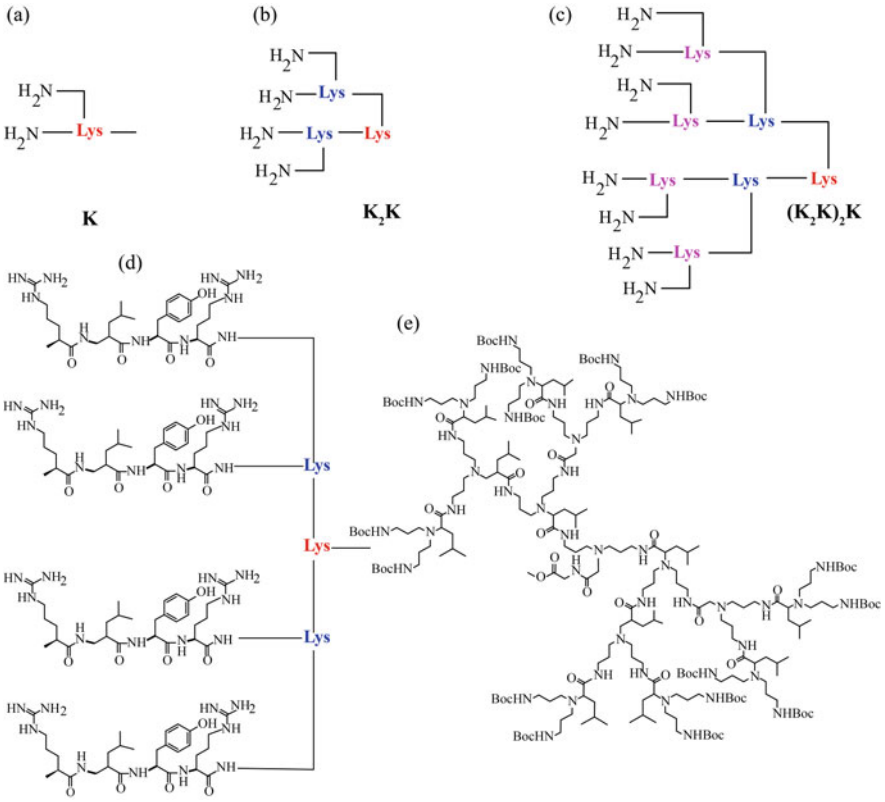

Fig. 15.5 Schematic illustration depicting the three types of dendrimeric cores with three generations of lysines: (**a**) two-branched Lys (red font), (**b**) four-branched (Lys)2Lys (red-blue fonts), (**c**) eight-branched [(Lys)2Lys]2Lys (red-blue-magenta fonts), (**d**) (Lys)2Lys core containing α and ε branches with peptide sequence Arg-Leu-Tyr-Arg, and (**e**) a peptide dendrimer-bearing L-Leucine

lipopeptides colistin and polymixin B in terms of antimicrobial efficacy against gram-negative pathogens. In comparison to the original peptide dendrimer, it also exhibits better solution stability. In another effort, Scorciapino and co-workers have developed another dendrimeric peptide (SB056) as depicted in Fig. 15.7b with hydrophilic and hydrophobic amino acids in an alternating pattern, forming an amphiphilic strands in a lipid environment (Scorciapino et al. 2012). The occurrence of a short lipophilic chain upsurges its membrane activity. The novel lipodimeric dendrimer displays exceptional selectivity toward gram-negative strains, whereas its linear counterpart performs poorly against most of the tested strains.

The antimicrobial activity of dendrimeric peptides is determined by the presence of various functional groups in amino acid residues and their ability to penetrate the cell membrane. Siriwardena et al. have reported a third-generation AMPD (denoted as G3KL, Fig. 15.8) containing repetitive units of lysine (K) and leucine (L) which exhibits excellent activity toward strains of *A. baumannii*, *P. aeruginosa*, *E.coli*, and

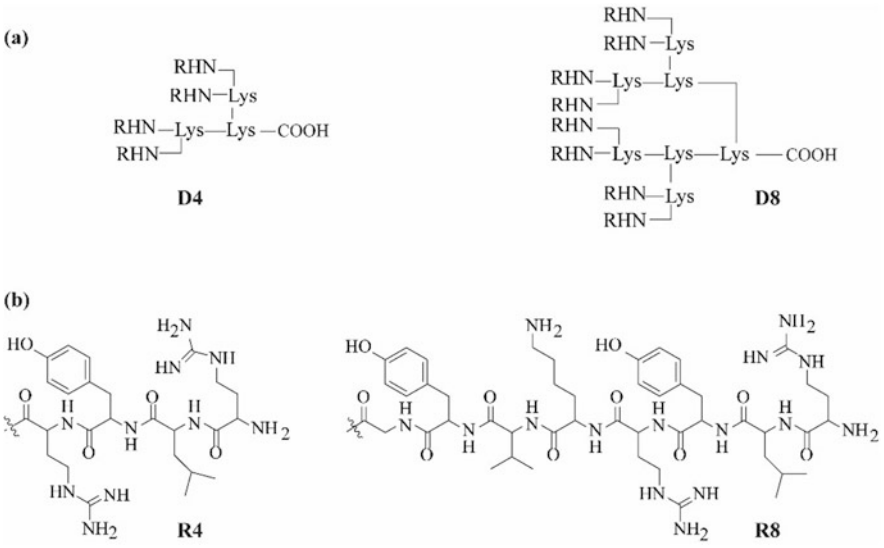

Fig. 15.6 (**a**) Schematic representation of the peptide dendrimer cores D4 and D8. (**b**) Peptide sequence structures denoted by R4 and R8 attached to the surface of the cores

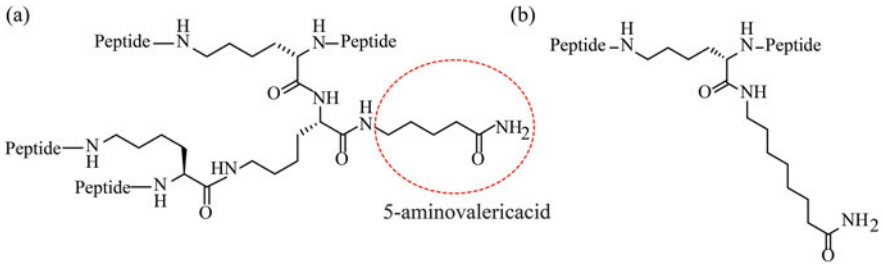

Fig. 15.7 Molecular structure depicting the primary sequence of (**a**) the dendrimeric (tetrameric) antimicrobial peptide SB041 and (**b**) dimeric SB056

K. pneumonia (Pires et al. 2015). G3KL AMPD has been demonstrated inhibiting *P. aeruginosa* biofilm formation by using scanning electron microscopy observation and confocal laser scanning micrographs. The studies reveal that the peptide dendrimer depending upon its dose may lead to the destruction of the biofilm morphological structure and thickness along with a potential to disseminate the biofilm entirely (Han et al. 2019). Fluorescence-labelled analogues of G3KL-bearing fluorescein (G3KL-Fluo) or dansyl (G3KL-Dansyl) by means of super-resolution stimulated emission depletion (STED) microscopy, time-lapse imaging, and transmission electron microscopy (TEM) reveal the mechanism where the localization of dendrimer occurs at the bacterial membrane, thereby initiating the membrane depolarization with consequent permeabilization and destruction of the inner and outer bacterial membrane (Gan et al. 2019).

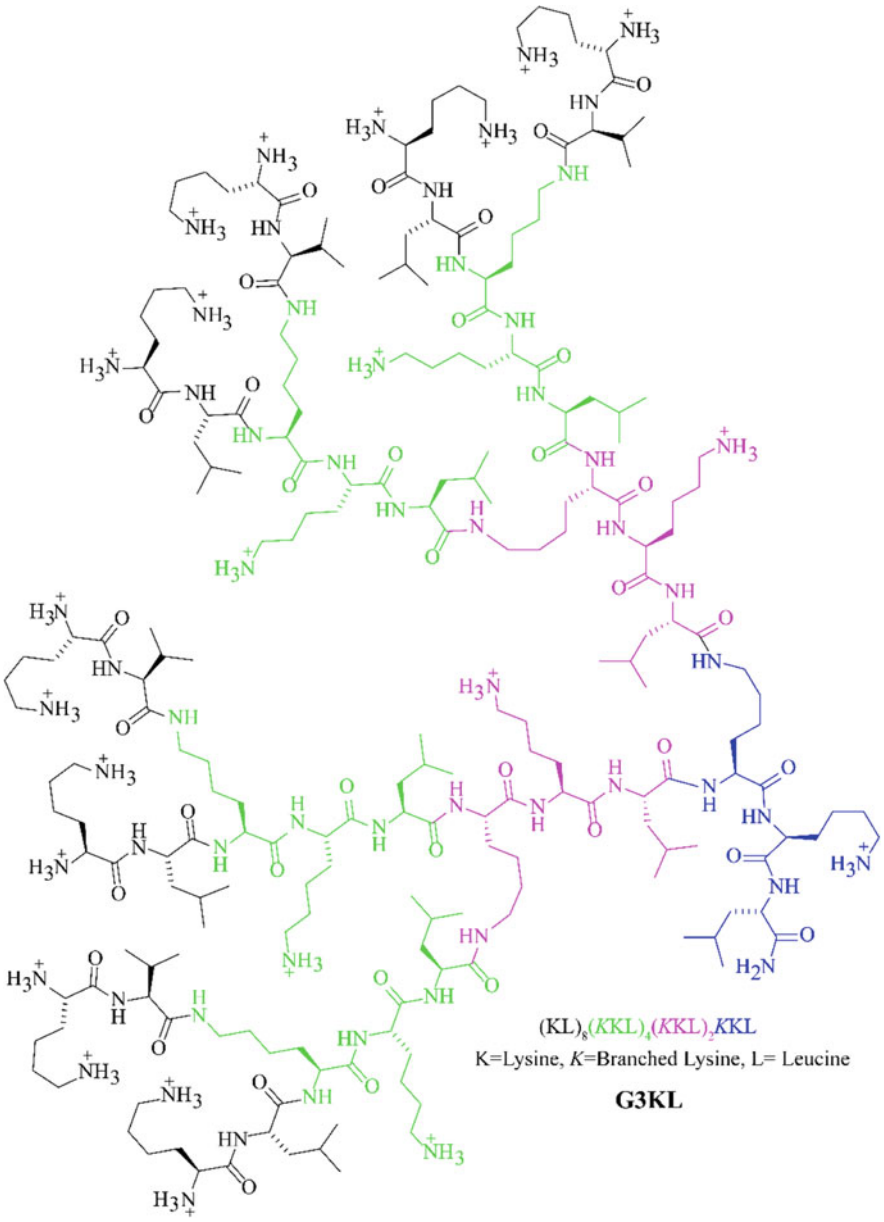

Fig. 15.8 Molecular structure of the novel antimicrobial peptide dendrimer (AMPD) G3KL

Recently, a second-generation (G2) AMPD denoted as TNS18 exhibited similar activity as G3KL against *P. aeruginosa* and an increased activity against *S. aureus* but lesser effectiveness against *K. pneumoniae* than G3KL. A chimeric AMPD named DC5 has also been synthesized by combining the peripheral branches of

G2 (TNS18) decorated with a G3 (T7) core (Fig. 15.9). The resulting DC5 displayed substantial antimicrobial action against several strains of *E. coli*, *K. pneumonia*, MRSA, *A. baumannii*, and *P. aeruginosa* (Siriwardena et al. 2018; Siriwardena et al. 2019).

In a fascinating approach, Kallenbach et al. have established that dendrimers with shorter peptide lengths can also have antibacterial capabilities. As illustrated in Fig. 15.10, the tetravalent dendrimeric peptide consists of a lysine core and a dendrimeric surface of Trp-Arg dipeptides. The peptide dendrimer reveals antimicrobial activities against ampicillin- and streptomycin-resistant *E. coli*, as well as multidrug-resistant *S. aureus*, while exhibiting minimal hemolytic activity. In fact, when compared to linear brush-like peptide and other polymers decorated with the Trp-Arg peptide sequence, the antimicrobial capacity of the tetravalent peptide dendrimer was found to be more efficacious with lesser cytotoxicity (Hou et al. 2009; Liu et al. 2007).

15.4.5 Organometallic Dendrimers

In a novel approach to expand the boundaries of antimicrobial agents, Abd-El-Aziz and co- workers have synthesized a different class of antimicrobial dendrimers incorporating organometallic moiety with a distinct mechanism of action (Fig. 15.11) (Abd-El-Aziz et al. 2015). The newly designed antimicrobial dendrimers (Den 1 to Den 9) integrate cationic and redox-active sandwich complex, η^6-arene-η^5-cyclopentadienyliron(II) (Cp-Fe^{II}-arene). Two distinct mechanisms of action are considered to be in operation. These redox-active organometallic compounds generate reactive oxygen species (ROS), which cause oxidative damage to microbes. Furthermore, the dendrimer's antimicrobial activity has been boosted by the presence of a positive charge on the Cp-Fe^{II}-arene complex, which disrupts the microbial cell membrane. The amalgamation of both mechanisms enhanced the overall antimicrobial activity of the freshly synthesized dendrimers against gram-positive bacterial strains, including methicillin-resistant *S. aureus* and vancomycin-resistant *E. faecium*. Cytocompatibility of the reported dendrimers were achievable in epidermal cell lines and mammalian red blood cells, making them potential tools for antimicrobial therapy.

Modifying the same organometallic dendrimer having cationic η^6-dichlorobenzene-η^5-cyclopentadienyliron(II) complex, Abd-El-Aziz and co-workers have taken a step forward and developed new hybrid antimicrobial macromolecules with superior activity against infectious multidrug-resistant microorganisms (Abd-El-Aziz et al. 2017). The reported hybrid antimicrobial dendrimers were developed by functional group introduction upon the organometallic dendrimers using quaternary ammonium or 2-mercaptobenzothiazole groups (Fig. 15.12c, d). The functionalization has altered the glass transition temperature of the synthesized dendrimers with concomitant improvement in the antimicrobial activities. Electron paramagnetic resonance spectroscopy has revealed the generation of free radicals,

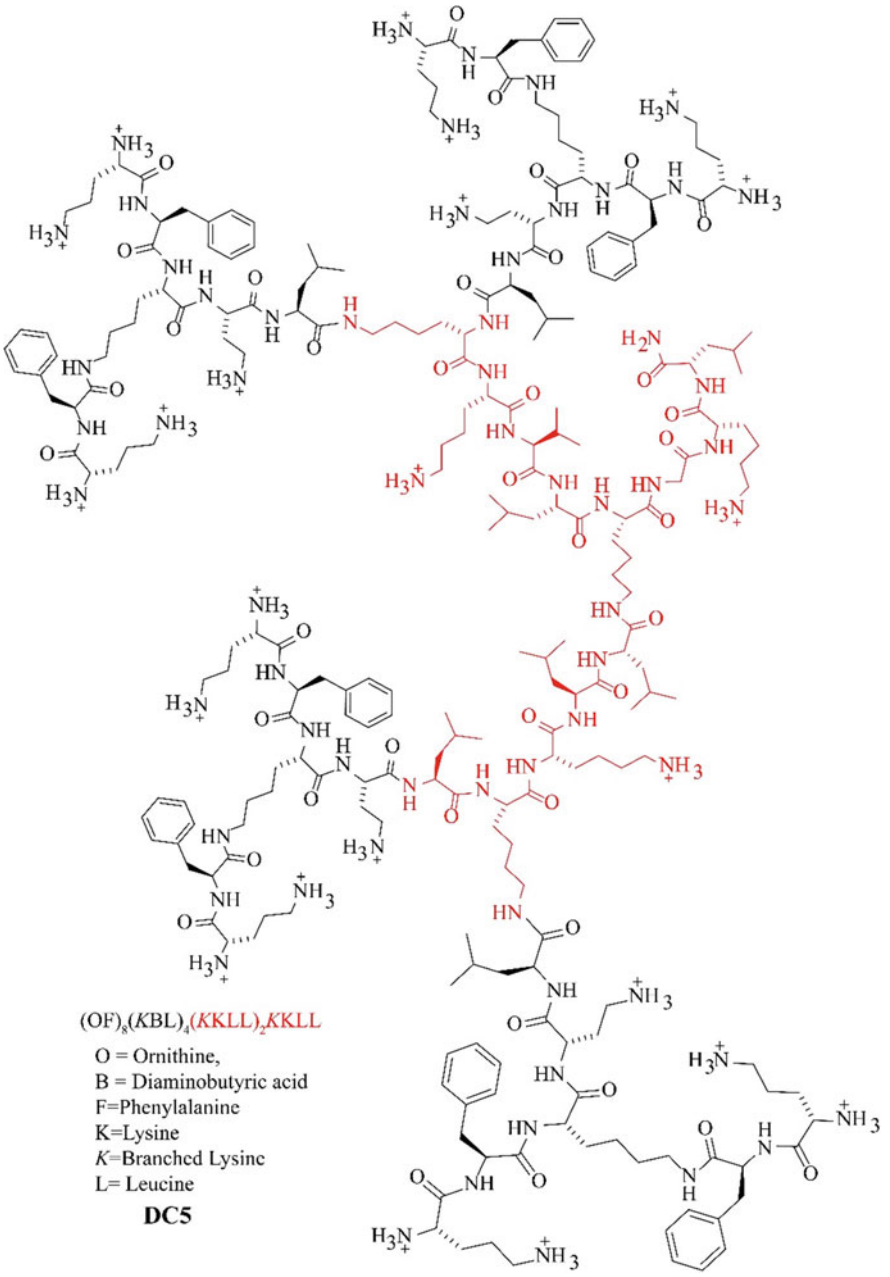

Fig. 15.9 Molecular structure of the antimicrobial peptide dendrimer DC5

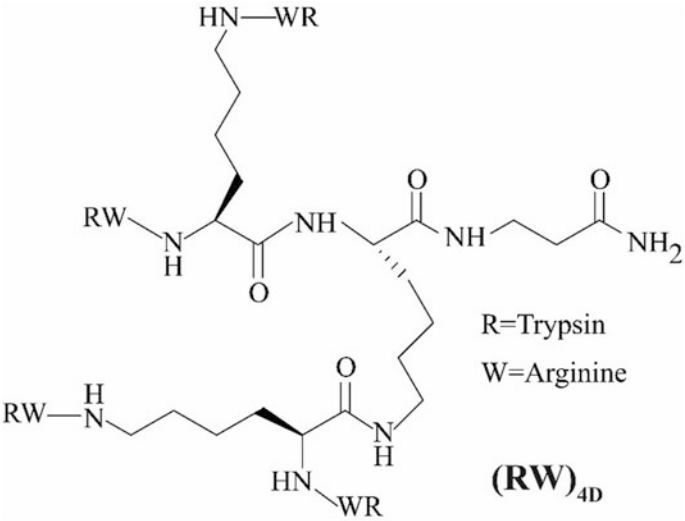

Fig. 15.10 Molecular structure of the antimicrobial peptide dendrimer $(RW)_{4D}$ having shorter peptide length

which carry significant biological and catalytic implications. In vitro antimicrobial assays have revealed that hybrid dendrimers have superior antimicrobial efficacy against multidrug- resistant pathogens like methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium*, as well as other microbes.

Recently, Abd-El-Aziz and his co-workers have efficiently integrated paracetamol moiety to a series of organoiron dendrimers and studied their antimicrobial activities toward certain gram-positive bacterial strains. These dendrimers are shown to possess maximum efficacy against methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus faecium*, and *Staphylococcus warneri*. The findings also suggest that the antimicrobial activity of the organoiron dendrimers is proportional to the size of the redox-active dendrimer and its terminal functionalities (Abd-El-Aziz et al. 2020).

15.5 Future Prospects

The potential of dendrimers as an effective antimicrobial agent is mostly explored with various polyamide-, polyimide-, and polylysine-type dendrimers (Sapra et al. 2019). Therefore, it demands novel approaches in designing various other types of dendrimers to input new aspects in antimicrobial research (Lai et al. 2021; Fan et al. 2021; Mahira et al. 2018; Wang et al. 2018). The structure-activity relationship is needed to be established so that there would be a clear correlation between the generation number of a dendrimer and resulting antimicrobial output. The research

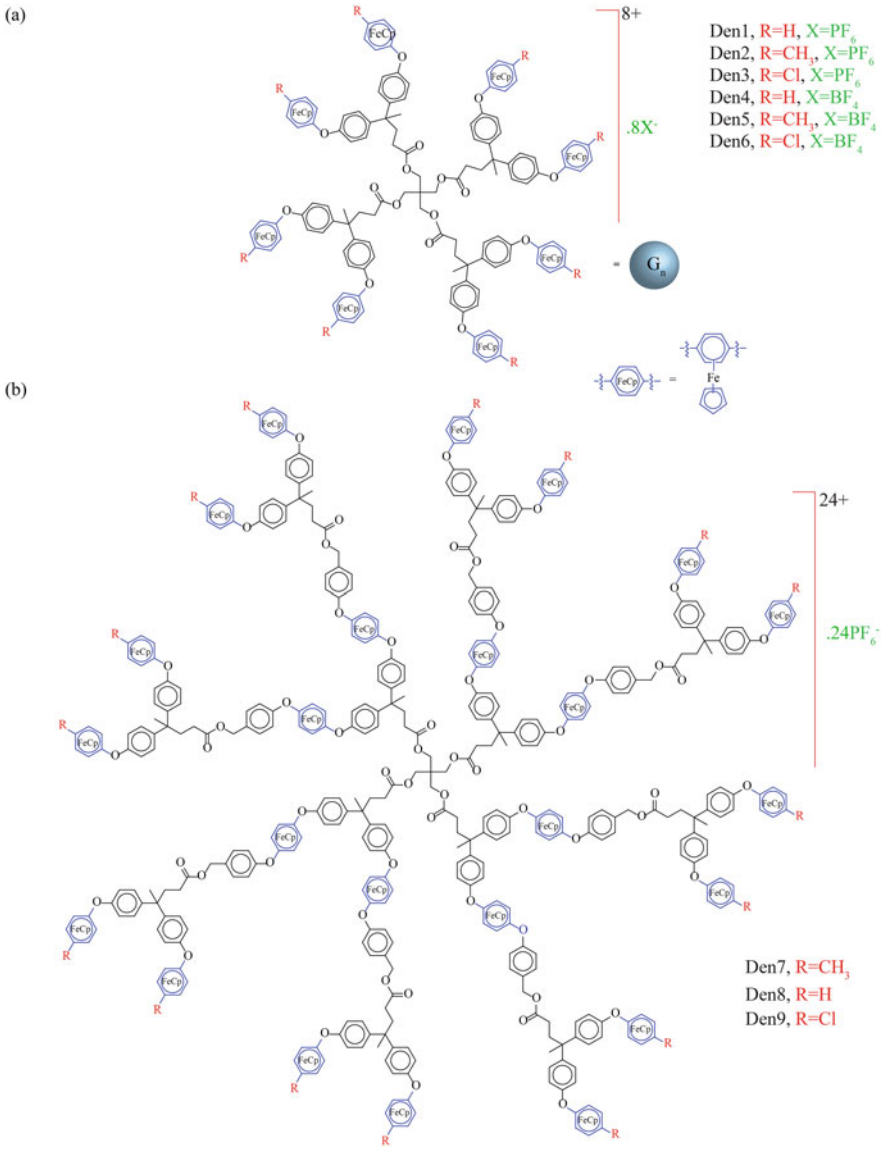

Fig. 15.11 Schematic representation of the molecular structure of (**a**) zeroth generation and (**b**) first generation of the antimicrobial organometallic dendrimers

on the effect of loading various biomacromolecules, such as proteins on the final antimicrobial activity and the synergic effect of an antimicrobial drug on loading with a dendritic scaffold would be interesting to study (Gou et al. 2017; Cui et al. 2021).

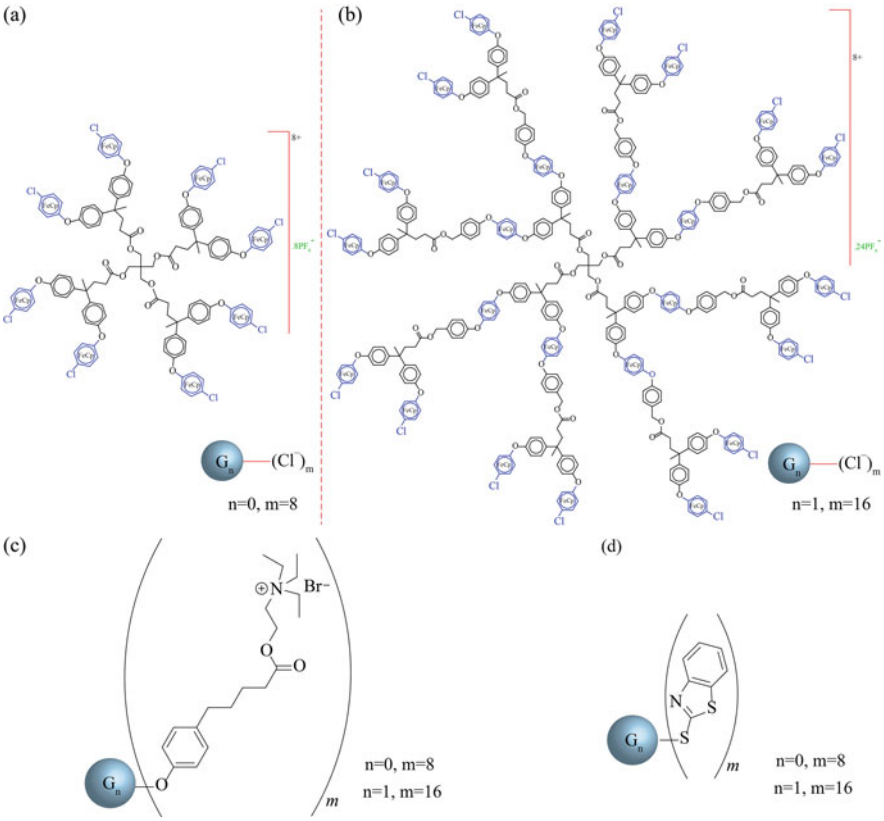

Fig. 15.12 Schematic illustration of the functionalization of the antimicrobial organometallic dendrimers (**a**) and (**b**) with quaternary ammonium groups and 2-mercaptobenzothiazole, respectively, to yield hybrid antimicrobial dendrimers (**c**) and (**d**)

15.6 Conclusion

With the impetus to fight microbes, the era of antimicrobial therapy has evolved progressively on the global scales simultaneously providing a plethora of information about the microbial world. Since most pathogenic microorganisms have a proclivity for developing resistance to certain antimicrobial agents, exploring different antimicrobial drugs to combat these diseases is of utmost significance. Dendrimers having distinctive architecture contain potential possibilities for creating novel antimicrobial agents with superior activity and potency for both industrial and biomedical applications. Dendrimers carry an excellent possibility for antimicrobial agents and biocide delivery systems due to their globular shape, reactive group arrangement at the surface, amplified local concentration of specific functionality, cooperative and polyvalent effects, and polycationic structure. As apparent in the

preceding cases, glycodendrimers provide excellent specificity, whereas cationic dendrimers disrupt the bacterial cell due to their supramolecular structure and charge distribution, making them excellent candidate for antimicrobial therapy. Due to the presence of both cationic and hydrophobic residues in antimicrobial peptide dendrimers, their mechanism of action is based on membrane disruption with enhanced selectivity due to their polypeptide structure. Organometallic dendrimers, on the other hand, integrate the initial association with the cell membrane as well as the oxidative damage caused toward the microbial growth by producing reactive oxygen species (ROS). Thus, using antimicrobial dendrimers against bacterial aggregates on surfaces may result in a diminished trend in the reoccurrence of resistant strains and arrest the keen interest of the researchers for further investigations to generate a fresh and productive class of antimicrobials for industrial as well as biological uses (Staneva and Grabchev 2021).

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Conflict of Interest The authors declare no competing financial interest.

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Chapter 16
Ionic Liquids, Ionic Liquid Nanoparticles, and Nanocomposites: The Future Antibiotics

Susmita Das

Abstract The chapter presents a detailed review of the ionic liquids (ILs) as well as ionic liquid nanoparticles and nanocomposites as antibacterial agents. Frozen or room temperature ionic liquids are organic salts comprised of inappropriately sized cations and anions which results in frustrated packing and reduced melting point. This class of compound has shown tremendous tunability and huge potential as future antibacterial agents. Researchers in this field have reported a series of quaternary ammonium, imidazolium, pyridium, pyrrolidinium, phosphonium, etc. ionic liquids with appreciable antibacterial properties against a broad spectrum of bacterial strains. Detailed study reveals interesting trends between the structure and antibacterial properties for a given family of ILs. In addition to conventional ILs, certain antibiotics have been converted to ILs for increased activity and improved drug efficacy. Antibacterial ILs and their nanocomposites have also demonstrated biofilm-resistant characteristics proposing their potential as coating materials on biomedical surfaces that are prone to biofilm growth.

Keywords Ammonium · Amoxicillin · Ampicillin · Antibacterial · Antimicrobial · Biofilm resistance · Designability · Imidazolium · Ionic liquid nanocomposites · Ionic liquids · Mechanism of action · Morpholinium · Oxonium · Phosphonium · Piperidinium · Poly ionic liquids · Polymorphism · Pyridinium · Pyrrolidinium · Task specific

Abbreviations

ILs	Ionic liquids
PIL	Poly ionic liquid

S. Das (✉)
Department of Chemistry, Amity Institute of Applied Sciences, Amity University Kolkata, Kolkata, India
e-mail: sdas@kol.amity.edu

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PILM Poly ionic liquid membrane
TSIL Task-specific ionic liquid

16.1 Introduction

Ionic liquids (ILs) also known as molten salts are low melting organic salts composed of unsymmetrical cations and anions (Welton 2018; Lei et al. 2017). Generally, inorganic salts like NaCl are constituted of small and symmetrical ions that lead to crystallization into high melting solids. However, big and unsymmetrical ions in ILs result in frustrated packing and thus low melting points. Generally, salts with melting points below 100 °C are denoted as ILs which are further classified into room temperature ionic liquids (RTILs) (Hallett and Welton 2011) and frozen ionic liquid (FILs) (Pang et al. 2017) with melting points below and above 25 °C, respectively. Although, the first ionic liquid, ethylammonium nitrate was developed by Paul Walden in 1912 (Walden 1914), the field remained dormant for years until Hurley and Wier realized the utility of low melting salts and discovered 1-ethylpyridinium bromide-aluminum chloride in 1951 (Hurley and Wier Jr 1951).

IL research has gained tremendous attention in various fields since the last two decades owing to the wide range of properties attainable from numerous cation-anion combinations (Dong et al. 2017; Mai and Koo 2016; Lei et al. 2014; Wickramanayake et al. 2014; Gurkan et al. 2010; Bara et al. 2009). The RTILs have demonstrated huge applications as solvents that are capable of solubilizing highly polar to highly nonpolar solutes (Hallett and Welton 2011; Smith et al. 2014). Additionally, the non-volatility, nonflammability, high thermal stability, and broad electrochemical window of ILs render them more desirable as solvents compared to their organic counterparts. Furthermore, the tunability of these salts permit their applicability in diverse technologies such as catalysis, medicine, separation science, sensors, nanotechnology, lubricants, electrolytic devices, electrochemical devices, and so on (Quijada-Maldonado et al. 2018; Das et al. 2013; Yue et al. 2011; Giernoth 2010; Davis Jr 2004). Such diverse applications render them with the name "task-specific ionic liquids (TSILs)." Generally, imidazolium, phosphonium, ammonium, pyridium, oxonium, etc. cations combine with borates, phosphates, halides, chlorates, and so on to form ILs of different chemical and functional properties (Fig. 16.1).

Designability of ILs have led to further classifications such as task-specific ILs (TSILs) (Quijada-Maldonado et al. 2018; Das et al. 2013; Yue et al. 2011; Giernoth 2010; Davis Jr 2004); poly ILs (PILs) (Smith et al. 2020; Guo et al. 2019; Qin et al. 2017; Guo et al. 2015); and their moderately higher melting counterparts, GUMBOS (Pérez et al. 2020; Kolic et al. 2016; Magut et al. 2013; Jordan et al. 2012; Das et al. 2010; Tesfai et al. 2009). GUMBOS expanded as group of uniform materials based on organic salts and their nanomaterials referred to as nanoGUMBOS were first introduced by Warner and coworkers that possess melting points between 25 and 250 °C and have demonstrated numerous functionalities for applications ranging

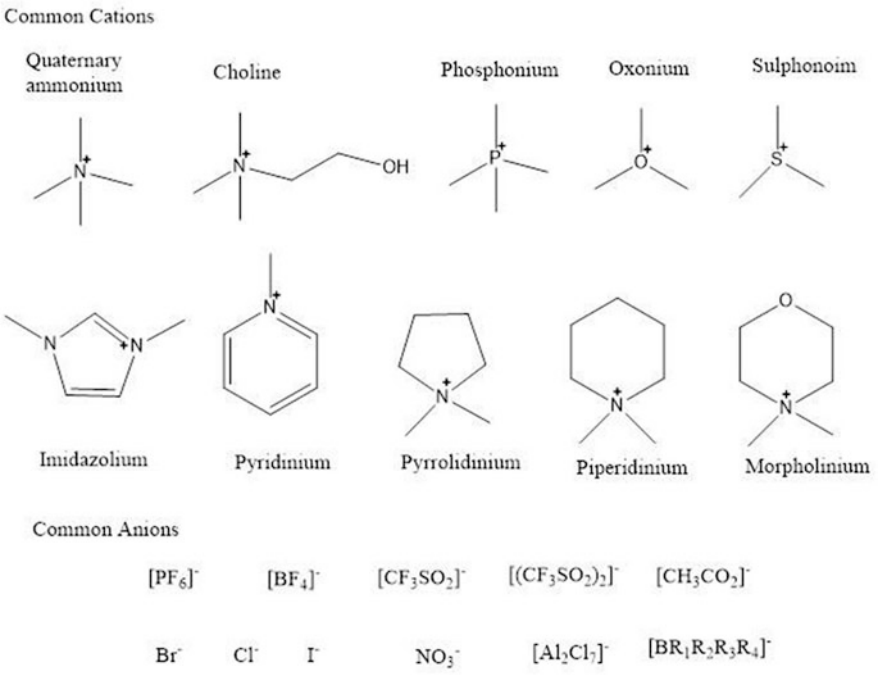

Fig. 16.1 Common cations and anions forming ILs

from separation science, optoelectronics, biomedicine, sensing to therapeutics and drug delivery (Pérez et al. 2020; Kolic et al. 2016; Magut et al. 2013; Jordan et al. 2012; Das et al. 2010; Tesfai et al. 2009). Thus, this highly designable and tunable class of material is found to extend its applicability in numerous domains among which the area exploring its antimicrobial activity is found to be extremely promising. In this chapter, we present the journey of ILs as antimicrobial agents.

16.2 Antimicrobial Activity of Ionic Liquids

The increasing resistance to microbes has put forward the demand for development of novel antimicrobial agents. Over the past one and a half decade, numerous studies have reported the application of ILs as biocidal, bactericidal, biofilm-resistant, and antifouling agents. Several categories such as quaternary ammonium, imidazolium, pyrrolidinium, pyridinium, phosphonium, etc. ILs were examined for their antimicrobial as well as related properties, and the observations suggest tremendous potential of these organic salts to contribute toward the development of promising antimicrobial agents (Saraswat et al. 2020; Bains et al. 2019; Borkowski et al. 2016; Hossain et al. 2011; Tesfai et al. 2009).

16.2.1 Antimicrobial Activity of Quaternary Ammonium ILs

Quaternary ammonium-based ILs were investigated for their antibacterial activity by Rogers and coworkers and was found to demonstrate bactericidal affect against both gram-positive and gram-negative strains (Pernak and Chwała 2003; Pernak et al. 2006). Yin and coworkers have reported a series of hydroxylammonium ILs all of which were active against *Staphylococcus aureus* (*S. aureus*) (Ismail Hossain et al. 2011). Two of the synthesized ILs, viz., 2-hydroxyethylammonium lactate and 2-hydroxy-N-(2-hydroxyethyl)-N-methylethanaminium acetate exhibited antimicrobial activity against a broad range of human pathogens (Hossain et al. 2011). A series of choline-based ILs synthesized by Siopa et al., namely, dimethylethanolamine monoquaternary ammonium salts, diethanolamine, methyl diethanolamine, and triethanolamine monoquaternary ammonium salts, exhibited broad-spectrum antimicrobial activity (Siopa et al. 2016). It is observed that inclusion of hydroxyl group and C14⁻C16 linkers enhance their antimicrobial effect. Chiral ammonium-based ILs were found to not only possess antibacterial but also antifungal properties, and ILs with 11 carbon atoms in the alkyl chain were reported to be most effective (Pernak and Feder-Kubis 2005). It is generally observed that increased alkyl chain length or the number of alkyl groups attached to the quaternary ammonium cation (Pendleton and Gilmore 2015) enhances the bactericidal effect. It is also reported that alteration of anionic counterpart does not significantly affect the IL activity. However, association of alkyl quaternary ammonium cations to various azolate anions, such as dodecylmethylammonium, benzalkonium, hexadecyl trimethyl ammonium cations associated with benzotriazole, 1,2,4-triazolate, etc., have demonstrated significant antibacterial and antifungal activity (Walkiewicz et al. 2010).

16.2.2 Imidazolium Ionic Liquids

Imidazolium ILs and various imidazolium derivative-based ILs are another important category of ILs that are extensively explored for their biocidal and herbicidal activities. Among the several imidazolium derivatives investigated, alkyl imidazolium, alkoxy imidazolium, menthol substituted imidazolium, and certain metal complex-associated imidazolium ILs have demonstrated promising activity against a wide variety of microorganisms such as rods, cocci, fungi, etc. (Forero Doria et al. 2018; Miskiewicz et al. 2018; Pendleton and Gilmore 2015; Pernak et al. 2004). Antimicrobial activities of both alkoxy and alkyl imidazolium ILs show direct correlation between the length of the alkyl chain and the minimum inhibitory concentrations (MICs). Bactericidal effect of an IL depends on a delicate balance in their structure and is found to vary with lipophilicity to certain extent. It is observed that alkyl or alkoxy imidazoliums with chain length between C8 and C16 exhibits significant antimicrobial activity in contrast to their shorter or longer counterparts (Pendleton and Gilmore 2015). Thus, for every category of ILs, there exists an

optimum value of chain length at which they are most active. For example, alkoxy imidazolium ionic liquids demonstrate best activity with a C12 chain, while N-cinnamyl imidazole of varying chain lengths exhibit lowering of MICs with increasing carbons in the alkyl chain with minimum MIC for C10. These ILs demonstrated best activity against gram-positive bacteria compared to the gram-negative strains. Imidazolium-based trigeminal tricationic ionic liquids demonstrate extraordinary antibacterial activity with an octyl substituent which is superior to the corresponding mono-imidazolium chlorides (Pernak et al. 2007). Chiral imidazolium ILs like menthol-substituted alkyl imidazolium ILs show substantial effect of their cationic structure on the antimicrobial activity (Feder-Kubis and Tomczuk 2013; Feder-Kubis et al. 2010). It is also observed that the presence of metals such as selenium in the side chain of imidazolium ILs improves their antimicrobial activity specifically toward algae. Carboxy functionalized imidazolium conjugated to N-terminal of an antibacterial peptide result in an IL with synergistic effect of both imidazolium and peptide. The resulting IL is found to be selective toward bacterial cells and active against drug-resistant strains. Presently, ILs are not only explored as possible antimicrobial drugs but also in wound dressings and water treatment. In a recent study reported by Bain et al., benzimidazolium ILs have been used in self-cleaning wound dressings where the IL chelates to Fe(III) ions, making it unavailable to the bacteria for their survival and growth (Bains et al. 2019).

In addition to the cationic component, the nature of anionic counterpart also contributes to the antibacterial activity of the ILs, although several reports suggest their contribution to be insignificant. It is observed that ILs composed of 1-alkyl-3-methyl imidazolium cations and tetrachlorocuprate (II) or tetrabromoargentate (I) anions containing antibacterial Cu and Ag, respectively, are found to exhibit improved bactericidal activity (Gilmore et al. 2013).

16.2.3 Antimicrobial Activity of Pyridinium Ionic Liquids

Pyridinium ILs are anion significant category of ILs that show noticeable antimicrobial activities. Alkyl pyridiniums are the simple pyridinium ILs that exhibit such properties, and biocidal effect was more prominent for higher alkyl chain lengths (Pernak et al. 2003; Docherty and Kulpa Jr 2005). Although pyridinium ILs with eight carbons (C8PyBr) are the shortest to show the antibacterial activity, C10PyBr is found to be active against gram-positive strains, and C12PyBr and C14PyBr are active against gram-negative bacterial strains. However, among all CnPyBr, the C14PyBr is the only one which is found to be active against fungi. Besides alkyl pyridiniums, alkoxy pyridiniums with more than 8 carbons in the alkyl chain are reported to be effective against both gram-positive and gram-negative bacterial strains, although best activity is obtained for the alkoxy pyridinium containing 12 carbons. The presence of ester group increases the IL adsorption at the air/water interface and its biological activity against fungi. In addition to the above discussed

derivatives, 4-dimethylaminopyridinium derivatives and IL-coated nanoparticles are also found to exhibit antibacterial and antifungal activity (Anwar et al. 2018).

16.2.4 Antibacterial Activity of Phosphonium Ionic Liquids

Phosphonium-based ILs (Phos-ILs) exhibit high thermal stability and offer superior properties as compared to nitrogen-based ILs (Cieniecka-Rosłonkiewicz et al. 2005). Imidazolium cations are generally unstable as the second carbon of the imidazolium makes them somewhat acidic that leads to carbene formation (Parshall et al. 1977). Besides being used as intracellular antioxidants, anticholinesterase inhibitors, tumor imaging agents, and chemotherapeutic agents (Kumar and Malhotra 2009), antimicrobial activities of a few Phos-ILs are also reported (Brunel et al. 2018). Benzyltriphenylphosphonium salts exhibited antitrypanosomal activity against *Trypanosoma brucei*, and it was established that the existence of bulky substituents (alkyl or aryl) surrounding the phosphonium ion result in reduction in MICs of these ILs (Taladriz et al. 2012). Bisphosphonium salt-derived benzophenone exhibit remarkable toxicity to human protozoan parasite *Leishmania* (Luque-Ortega et al. 2010). Very recently Das et al. have reported the development of mono- and bisphosphonium ILs through purely ionic approach, and the resulting ILs demonstrated selective bacterial toxicity towards gram positive *S. aureus* and gram negative *E. coli* based on the number of phosphonium ions present in the salt (Das et al. 2021). Phosphonium salts with single and double alkyl chains were investigated by Endo and coworkers. Herein the effect of the chain length (C10–C18) on antibacterial activity against wide range of pathogens including methicillin-resistant *S. aureus* (MRSA) was examined. The study brings forward a direct correlation between molecular structure and antibacterial activity (Kanazawa et al. 1994). It is observed that higher alkyl chain lengths and the presence of aryl groups improve the antimicrobial activity of Phos-ILs.

16.2.5 Antibacterial Activity of Few Other Ionic Liquids

Among the other ILs with antimicrobial activities, some reports also exist for pyrrolidinium, oxonium, morpholinium, and piperidinium salts (Saraswat et al. 2020; Pernak et al. 2011; Iwai et al. 2011). It was observed that although the antimicrobial effect of these salts was not as significant as ammonium, pyridinium, imidazolim, and phosphonium ILs, in certain conjugation they do exhibit appreciable effect. Saraswat et al. showed that the non-covalent conjugates of a series of pyrrolidinium ILs with an antibacterial peptide, melittin, demonstrate remarkably improved antibacterial activity against *E. coli* and *S. aureus* (Saraswat et al. 2020). Pernak et al. have reported a series of 4-benzyl-4-ethyl morpholinium ILs with various inorganic and organic anion combinations, and their study reveals weak

antibacterial and antifungal activity of these ILs (Pernak et al. 2011). Studies also suggest that in comparison to the monomeric forms, pyrrolidium and oxinium poly ionic liquids are more effective which is discussed in a later section of this chapter.

16.2.6 Antibacterial Activity of Ampicillin and Amoxicillin Ionic Liquids

Ampicillin and amoxicillin molecules have been widely used as antibiotics since decades. However, their wide use has led to the development of numerous drug-resistant strains. Since the development of functional ILs, in recent years various ampicillin (Amp) and amoxicillin (Amx) ILs based on imidazolium (IM) and pyridinium (Pyr) were synthesized, and the study of their antibacterial activity reveals manyfold improved bactericidal effect compared to the precursors.

M. Cole et al. have developed imidazolium ampicillin and pyridinium ampicillin ionic liquids and examined their activity against *Escherichia coli* (*E. coli*) 0167187, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and *Enterococcus faecium*, and the results suggest 43 times improved activity with ampicillin ILs as compared to Na-Amp (Cole et al. 2011). Ferraz et al. reported a series of penicillin [secoPen] and amoxicillin [seco-Amx] ILs with cation hydroxyl salts [C2OHMIM]. [C2OHMIM][secoPen] demonstrates relative decrease in inhibitory concentration (RDIC) in the order of 100 against sensitive *S. aureus* ATCC25923 (Ferraz et al. 2020). On the other hand, [C16Pyr][seco-Amx] showed an RDIC of $>$ 1000 against methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300. In a separate study, [C16Pyr][Amp] was found to exhibit improved activity against clinically resistant gram-negative strains of *E. coli* (Ferraz et al. 2020).

16.2.7 Antibacterial Activities of Poly Ionic Liquids

Poly ionic liquids (PILs) are polymeric ILs that has developed interest among scientists working in the area of antibiotics for their promising and extremely attractive properties. PILs are antibacterial agents that are proved to kill bacterial cells at much lower concentrations causing minimal hemolysis toward human red blood cells (RBC) and low cytotoxicity.

Imidazolium-based ILs have been examined in details for their antibacterial effect, and in the recent decade, it is observed that imidazolium-based poly ILs and poly ionic liquid membranes (PLIM) are more effective in killing bacteria in comparison to the corresponding monomers (Fang et al. 2019; Zheng et al. 2016). Imidazolium-based PILs were synthesized by Zheng et al. using photo-crosslinking followed by anion exchange with amino acids tryptophan (Trp) and L-proline (Pro). The resulting PILs were found to demonstrate strong activity against *E. coli* and

S. aureus, good blood compatibility, low bovine serum albumin adsorption, and low cytotoxicity. The effect of alkyl chain length on PILMs is different from the PILs and the monomeric imidazolium ILs. It is observed that higher alkyl chain length in PILMs demonstrate lower activity against both gram-positive and gram-negative bacteria. Imidazolium PILs coordinated with $CuCl_2$, $FeCl_3$, and $ZnCl_2$ demonstrate strong antimicrobial activity against both *S. aureus* and *E. coli*. These PILMs present low hemolysis toward human RBC and long-term antibacterial stability even after prolonged (~90 days) immersion in aqueous medium. Thioimidazole PILs are found to kill *Pseudomonas aeruginosa* much more effectively than benzalkonium chloride (BAC) generally used as antimicrobial preservatives in pharmaceuticals and personal care products (Smith et al. 2020).

Besides imidazolim, pyrrolidinium PIL homopolymers are also reported to yield greater antibacterial activity against *E. coli* and *S. aureus* compared to the monomer (Qin et al. 2017). Pyrrolidinium PILMs demonstrate exceptional hemocompatibility and low cytotoxicity that impart its excellent potential to be used for topical antibacterial agents. In addition, Guo et al. have also reported chiral amino acid-derived PIL membranes and have investigated the effect of chirality on antibacterial activity. It is observed that the D-enantiomeric amino acid group demonstrated higher antibacterial activity against methicillin-resistant *S. aureus* (MRSA). It is also observed that the ionically bonded PILs are more effective toward MRSA than the covalently linked membrane (Guo et al. 2019).

16.2.8 Antibacterial Activity of Ionic Liquid Composites and Nanocomposites

PILs based on quaternary ammonium salts are extremely cytotoxic, and thus their uses are limited. However, glycopolymer-grafted polymer of quaternary ammonium ILs conjugated with Fe_3O_4 nanoparticles shows excellent performance in killing *E. coli* and have potential application in water treatment (Hong et al. 2020). Ionic liquid nanocomposites have also demonstrated promising application as antimicrobial coatings. Benzimidazolium IL-metal nanocomposite with metals such as Ag, Au, and Cu demonstrates potent activity toward gram-positive and gram-negative pathogens in comparison to the ILs. In particular IL@Ag nanocomposite shows remarkably low MIC values against both *E. coli* and *S. aureus* and has potential application as hydrophobic coating materials (Bains et al. 2020). Benzimidazolium IL coated on ZnO nanoparticles have reportedly revealed improved antibacterial activity (Rojas et al. 2020). On the other hand, ZnO nanoparticles dispersed in ILs like 1-butyl-3-methyl imidazolium chloride and choline acetate show appreciably high antibacterial effect which is attributed to their reduced aggregation in comparison to phosphate buffer saline (PBS). Among the two ILs studied, the dispersion of ZnO in 1-butyl-3-methyl imidazolium chloride was found to be highly effective against skin epidermis (Aditya et al. 2018).

Phosphonium salts incorporated in various polymers have also been investigated for antimicrobial activities such as phosphonium salts incorporated on styrene-divinylbenzene copolymers showed antibacterial activity against *Staphylococcus aureus* (*S. aureus*), *Escherichia coli* (*E. coli*), and *Pseudomonas aeruginosa* (*P. aeruginosa*) (Cakmakci et al. 2019). Triphenyl phosphonium-modified PPO (polyphenylene oxide) polymer also found to have antibacterial activity (Chang et al. 2010), and tetradecyltriphenylphosphonium bromide (P66614Br) functionalized few-layered graphite exerted long-term antimicrobial activity against *E. coli and S. aureus* (Xie et al. 2011).

16.2.9 Biofilm Resistance of ILs and IL Nanocomposites

Most of the hospital-acquired infections are spread through biofilms that adhere to surfaces of medical implants as well as non-implantable surfaces. Biofilms generally exhibit greater tolerance toward antibiotics compared to the planktonic cultures. Some of the ionic liquid-based antimicrobials exhibit commendable antibiofilm activity against both clinically and industrially problematic microbial biofilms. Seddon and coworkers reported a series of 1-alkyl-3- methylimidazolium chloride and quinolinium-based ILs against a wide range of clinically significant pathogens including MRSA (Carson et al. 2009; Busetti et al. 2010). Reddy et al. have demonstrated the antibiofilm activity of a long chain 1-alkyl-3-methyl imidazolium ionic liquids (Reddy et al. 2017). It was observed in their study that the hexadecyl derivative showed phototrophic biofilm resistance against multiple species of bacteria. Among quinolinium-based ILs, 1-alkylquinolium bromides with 12 to 14 carbon show maximum antibiofilm activity against a range of gram-positive and gram-negative bacteria. ILs also prevent growth of natural phototropic biofilm exhibiting their antibiofilm activity against multispecies biofilms (Gilmore 2011). In a recent study, Das and coworkers have reported a novel triarylmethane-based IL-graphene oxide nanocomposite with nearly four times improved antibacterial activity in comparison to graphene oxide that are demonstrated to serve as efficient biofilm-resistant coatings (Prusty et al. 2021). The study suggests bactericidal effect of the nanocomposite as compared to bacteriostatic effect of graphene oxide nanosheets alone.

16.3 Mechanism of Action

Antibacterial activity of ILs is strongly influenced by their structural characteristics, and a general analysis of the reported studies suggests enhanced antibacterial activity with increased lipophilicity. Thus, ILs with more than ten carbons in the alkyl chains exhibit remarkable biocidal effect with optimum activity lying between C12 and C16 depending on the type of IL (Gilmore 2011). Although extensive understanding of

the mechanism of action of ILs in support of their antibacterial activity is rare, certain theories suggest that loss of membrane integrity is a prime cause for antibacterial activity of ILs (Cho et al. 2016). Doria et al. demonstrated from their molecular dynamics study that ILs with longer alkyl chain length exhibits stronger interaction with the lipid surface as evidenced by a passive diffusion toward the surface followed by penetration. Pernak and Chwała (2003) suggested antielectrostatic effect as a possible mechanism of cell death for choline-based ILs. However, the same explanation does not stand well for phosphonium ionic liquids. Besides disrupting the cytoplasmic membrane, quaternary compound are known to coagulate the cytoplasmic constituents leading to denaturation and inhibition of enzymes. In a recent report by Das et al., the probable mechanism of action of a few mono- and bisphosphonium ILs has been examined by the use of molecular docking. It was observed from their studies that the phosphonium ILs primarily exhibit antibacterial activity through strong binding to the bacterial ribonucleic acids (RNA) and cell wall. RNAs play important role in outer membrane protein modulation and other bacterial survival activities. Drug binding to bacterial RNA may thus inhibit protein synthesis and additional survival processes resulting in cell death. Similarly, drug binding to the cell wall may inhibit the peptidoglycan maturation eventually leading to cell wall damage and thus bacterial death (Das et al. 2021). Phosphonium ampicillin ILs were however found to primarily act via interaction with the penicillin-binding proteins, a mechanism established for action of ampicillin. Thus, it is observed that the associated counterion may also determine the mechanism by which an IL will exhibit its bactericidal effect, and thus no general pathway of action can be stated for a given family IL.

16.4 Future Prospect

The enormous designability of ILs enable incorporation of anticipated properties with minimization of undesired aspects. The cytotoxicity of such compounds can be easily tuned by simple variation of the associated counterion (Magut et al. 2013). ILs are also known to exhibit reduced polymorphism unlike the conventional drugs which in turn result in higher drug efficacy and decreased toxicity (Egorova et al. 2017). Additionally, ILs being super-solvents due to their amphiphilic characteristics have recently been demonstrated to exhibit uniform drug delivery besides therapeutic activity (Albadawi et al. 2021). Multidrug-resistant bacteria impose huge global threat and thus demand continuous development of novel antibiotics. The discussed properties of ILs suggest ample scope to be explored as antibacterial as well as other pharmaceutically active agents.

16.5 Conclusion

Our comprehensive review on the prospect of ILs as antimicrobial and antibiofilm coatings has revealed enormous potential of this family of compounds to be used against a wide variety of resistant and nonresistant bacterial strains as well as fungi. Reports suggest that cationic ILs like quaternary ammonium, imidazolium, pyridinium, and phosphonium demonstrate appreciable antibacterial activity against *E. coli*, *S. aureus*, methicillin-resistant *S. aureus* (MRSA), *Enterococcus faecium*, *Klebsiella pneumoniae*, and so on. Researchers have come up with interesting correlation between structure and antibacterial activity of ILs. It is observed that quaternary ammonium ILs with hydroxyl groups and C14–16 spacers exhibit strong antibacterial activity, while ILs with C11 chain demonstrate significant antifungal activity. Alkyl and alkoxy imidazolium and alkyl pyridiniums with C8–C16 carbon chains and C10–C18 chains of alkyl phosphonium ILs are reported to show remarkable antibacterial activity. Antibacterial activity of other cations like pyrrolidinium, morpholinium, oxonium, and piperidinium is not that significant in the monomeric form. However, some of their poly ionic liquids have demonstrated significant bactericidal effect. Besides cations, various anions like tetrachlorocuprate (II) or tetrabromoargentate (I), ampicillin, and amoxicillin are also known to improve the antibacterial activity of ILs. Poly ILs and poly IL membranes (PILMs) derived from imidazolium and pyrrolidinium are reported to be more effective against bacteria in comparison to the corresponding monomeric ILs. In addition, PILs have also shown great hemocompatibility and low cytotoxicity. In recent years, IL-metal/metal oxide nanocomposites have demonstrated great potential as antibacterial and biofilm-resistant agents. These have shown bactericidal effect against various gram-positive and gram-negative pathogens and therefore proposed for use as topical antibiotic as well as antibacterial coatings. Although the mechanism of action for the bactericidal effect have not been thoroughly investigated and reported, still some molecular dynamic simulation studies suggest that ILs of chain length between C12 and C16 can effectively interact with the lipid molecules at the membrane which may affect their growth and metabolic activities.

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Conflict of Interest The author declares that there is no conflict of interest.

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Part V
Probiotics and Other Alternative Approaches

Chapter 17
Prebiotic Immunomodulators to Enhance Mucosal Immunity and to Reduce Mass Use of Antibiotics

Amlan Jyoti Ghosh, Sagar Sarkar, Supriyo Ghosh, and Tilak Saha

Abstract The mucosal lining of the gastrointestinal tract, respiratory tract and urogenital tract exhibits immunological properties like ciliary projection, production of cytokines, and secretion of antibodies. In recent years, mucosal immunity is being explored as one of the most important strategies for disease prevention. Prebiotics on the other hand are a class of nutrients that are mostly consumed by the host's local microbiome. Role of prebiotics on overall health of animal has sparked curiosity of scientific communities. Prebiotics are metabolized by the resident microbiome, which results in the production of biologically essential compounds such as short-chain fatty acids (SCFAs) and various other metabolites, such as fructo-oligosaccharides and galacto-oligosaccharides. Prebiotics and their by products can act as good immunomodulators. But the problem with prebiotics is that they are generally present in a very minuscule amount in our daily diets, which is generally insufficient to produce biologically significant amount of beneficial end-products via bacterial metabolism. Exogenous administration of prebiotics not only improved the microbiome but also the overall immune status of experimental models. The importance of mucosa in the immune system and the properties of prebiotics in modulating the immune health together can be harnessed to provide a beneficial approach for disease management. Hence, prebiotics may possibly be one of the most viable alternatives to minimize the widespread use of antibiotics for infections related to not only respiratory mucosa but also other important mucosal sites like the gastrointestinal system.

A. J. Ghosh · S. Ghosh · T. Saha (✉)
Immunology and Microbiology Laboratory, Department of Zoology, University of North Bengal, Darjeeling, West Bengal, India
e-mail: tilaksaha@nbu.ac.in

S. Sarkar
Immunology and Microbiology Laboratory, Department of Zoology, University of North Bengal, Darjeeling, West Bengal, India

Department of Zoology, Siliguri College, Darjeeling, West Bengal, India

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Abbreviations

ACE	Angiotensin-converting enzyme
AMPK	AMK-activated kinase
AMR	Antimicrobial resistance
APC	Antigen presentation cell
BALT	Bronchitis-associated lymphoid tissue
CFU	Colony forming unit
DC	Dendritic cell
M cell	Microfold cell
MALT	Mucosa-associated lymphoid tissue
MDR	Multidrug resistant
MRSA	Methicillin-resistant *Staphylococcus aureus*
MUC	Mucin
NALT	Nasopharynx-associated lymphoid tissue
NF-κB	Nuclear factor kappa beta
NK cell	Natural killer cell
POS	Pectic oligosaccharide
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RS	Resistant starch
SARS-CoV	severe acute respiratory syndrome coronavirus
SCFA	Short-chain fatty acid
sIgA	Secretory immunoglobulin A
TCR	T-cell receptor
Th cell	T helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg cell	T regulatory cell

17.1 Introduction

Immunity is the potentiality of our body to protect us not only from all the external but also from internal substances that might cause harm to the biological system of our body. The main function of the immune system is to discriminate between self

and non-self (Patchen et al. 1987). The mucosal barrier is an important component of innate immunity and regarded as the initial line of defense. It acts as both a physical and a chemical barrier. The mucosal lining of our body is a defensive system that evolved over time. It is deliberately positioned in places that are getting exposure to the outside environment. The mucosal lining can be found in the respiratory tract, gastrointestinal tract, and urogenital tract. It exhibits properties like ciliary projection, mucus, cytokines, and antibodies generation (Peterson and Artis 2014). Mucosal immunity has become one of the most important targets for disease prevention in recent years. Despite the fact that the human body is naturally equipped with a very sophisticated mucosal lining system, our contemporary lifestyle and environmental influences make this lining less efficient. In the last few decades, overuse of antibiotics for many forms of infectious diseases is creating a global health crisis. It resulted in antibiotic resistance, which has led to more serious infections, complications, longer hospital stays, and even increased fatality. Prebiotics are a class of nutrients that are found to enhance mucosal immune status. These prebiotics can be considered as powerful immune-modulators. Prebiotics are metabolized by the resident microbiome to produce biologically essential compounds such as short-chain fatty acids (SCFAs) and bacterial metabolites like fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) (Davani-Davari et al. 2019). In recent years, their association with overall human health has provoked the curiosity of researchers. Due to the presence of prebiotics in a very minuscule amount in regular meals, scientists are aiming not only to mass-produce them but also to assess their safety doses as a promising contender for improving human health.

From the first cholera pandemic of 1817 and the Spanish flu of 1918 to the very recent COVID-19 pandemic, every time the healthcare system faced great challenge and exhaustion (Piret and Boivin 2020). The prevalence of infectious and communicable diseases has increased exponentially over the past few decades. There are therapeutic strategies available like vaccination, immunomodulation, and antibiotics. But none of them are fully effective during communicable disease scenario. Moreover, therapeutic management strategies for immune system-related complications have also evolved significantly in recent times. It is estimated that clinicians prescribe more than 90% of all antibiotics for respiratory tract illnesses (Llor and Bjerrum 2014). Moreover, the wide usage of drug-resistant pathogens created a situation which is as dangerous as any pandemic. Whereas systematic intake of prebiotics is much safer because it imparts beneficial effects on the gut microbial system of our body. Our gut is colonized with millions of microbes, diverse in taxonomical and functional aspects. Some of the microbes have immense probiotic potentials and their efficiency can be regulated by the application of prebiotics.

In the recent COVID-19 pandemic, it was reported that preexisting antibiotic resistance was causing difficulties in treating COVID-19 patients (Knight et al. 2021). The mucosal layer in the respiratory tract regulates infections. The development of antimicrobial resistance (AMR) and emergence of various multidrug-resistant strains (MDR) superbugs is one of the most dangerous outcomes of unregulated and widespread use of antibiotic. Hence, there is an ongoing rush among the scientific community for developing new alternative therapeutic management

strategies. Usage of prebiotics to enhance the mucosal immunity could be a feasible alternative strategy to cut the extensive usage of antibiotics.

17.2 Immunity and Its Types

The immune system is a sophisticated biological system. Its primary function is to discriminate between self and non-self (Patchen et al. 1987). This non-self might be an infectious organism, a transplanted organ, or an altered endogenous cell. The immunological reactions in vertebrates against any non-self have two hands: innate (or nonspecific) and adaptive (or acquired or specific) (Benny and Vanitha 2004). Cellular and humoral responses both are involved in these two hands. Adaptive immune response is specific, whereas innate immune response is nonspecific as it does not have immunologic memory. Adaptive immunity has immunologic memory against a pathogen, so this hand of immunity is specialized, and immunological responses improve with successive exposure to that particular pathogen. Monocyte macrophage system and complement system are associated with innate cellular and innate humoral immunity respectively. T lymphocytes (T cells) are the major cellular component of acquired immunity, whereas B lymphocytes (B cells) are the humoral component. Generally innate and acquired immune systems work together to control or eliminate infection. Sometimes innate immunity is sufficient enough to combat with the invading disease-causing agents, but in certain scenario, they do so with the help of adaptive immune system. NK cell and antigen-presenting cells (APCs) are the most important part of the innate immunity, whereas B and T cells are the part of adaptive immunity. APC can break down the disease-causing pathogens into smaller fragments, triggering the immune system (acquired) to neutralize or destroy the pathogen. B cells which are very much important part of immune system develop into antibody-producing plasma cells when they are exposed to specific antigen present in the bone marrow. TCRs (T cell receptors) are expressed by T cells when they are migrated toward the thymus. This migration is regulated by certain signaling molecule. T helper cells (with specific protein clusters of differentiation—$CD4^+$) and cytotoxic T cells (with specific protein clusters of differentiation—$CD8^+$) are differentiated from TCR-expressing T cells in the thymus when they are getting exposure of cytokines secreted by APCs (Sharma and Sharma 2007).

17.2.1 Mucosal Immunity and Its Function in Adaptive Immune Response

The interface between an organism and the environment must be permeable to prevent the spread of harmful organisms and foreign agents. This barrier is composed of special epithelial layers that support the defense mechanisms against

invading pathogens. Various cells and their products of inborn immune system can recognize the patterns of pathogenic microbes by recognizing their surface antigens. This system is activated due to certain antigens on the microbes. The characteristic features of mucosal immunity distinguish it from systemic immunity. These features include the presence of distinct populations of lymphocytes, preferential induction of tolerance against non-dangerous antigens and mucosal system defense mechanism (Tlaskalová-Hogenová et al. 2002). There are three main functions of mucosal immunity. These are (a) mucous membranes' protection from colonization by potentially harmful microbes, (b) preventing the uptake of undegraded antigens, and (c) stopping the production of potentially harmful immune activation by several exogenous particles.

The membranes covering the urogenital tract, the digestive tract, and respiratory tracts are equipped with chemical-cleansing mechanisms that effectively remove foreign matter. The human body's internal organs are also protected by a large and highly specialized immune system. This local immune system contributes over 80% of all immunocytes in a healthy human adult. These cells aggregate in or transit through numerous mucosae-associated lymphoid tissues (MALT), which collectively make up the largest lymphoid tissue system in the body (Holmgren and Czerkinsky 2005). The MALT is a highly compartmentalized immunological system that operates independently of the systemic immune system. It consists of anatomically defined lymphoid tissues like the Peyer's patches, mesenteric lymph nodes, appendix and solitary follicles in the gastrointestinal tract (GIT), and tonsils and adenoids at the aerodigestive tract's entrance, which serve as the primary mucosal inductive sites for immune responses (Mowat 2003). The gastrointestinal and respiratory tracts are the major areas where mucosal immunity plays very important role.

17.2.2 Mucosal Immunity in the Gastrointestinal Tract (GIT)

The GIT is one of the most important mucosal barriers not only because it is constantly exposed to exogenous antigens but also it has to provide a place for the colonization of microbes. Microbial colonization poses a risk of infection and inflammation if epithelial or immune cell homeostasis is disrupted. The dynamic stability between the mucosal immune cells and microbial populations required a clear distinction of self and non-self matters. The gut lining consisting of epithelial cells does this by forming a physical and metabolic barrier to both commensal and pathogenic microorganisms. Intestinal epithelial cells (IECs) can also recognize and respond to various microbial stimuli, which aids in strengthening the barrier function and assisting in the coordination of appropriate immune responses. As a result, IECs play an important role in maintaining the balance of mucosal immune cells (Peterson and Artis 2014). Moreover, in the GIT, mucosal immunity and adaptive immunity induced by commensal microbiota work together to protect the host from foreign pathogen invasion and maintain intestinal homeostasis (Gunther et al. 2016; Honda and Littman 2016). Intestinal adaptive immunity generated by intestinal resident

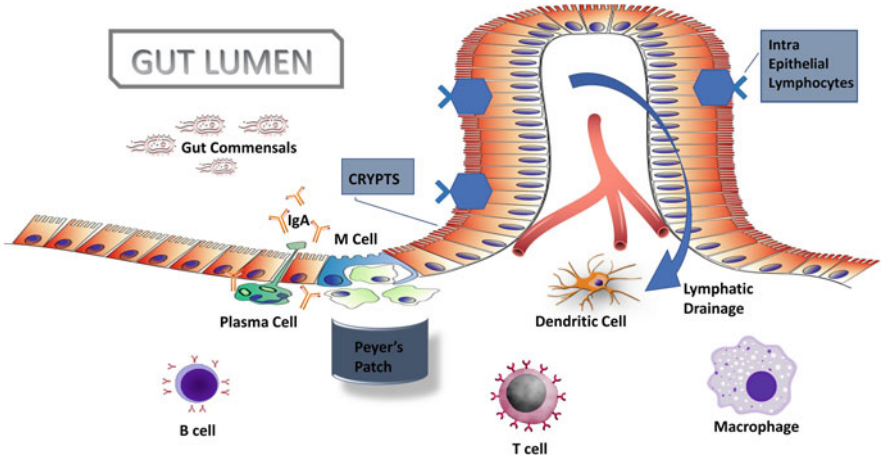

Fig. 17.1 Overview of mucosal immunity in the GI tract

microbiota is very important for maintaining immunological tolerance to symbiotic bacteria, intestine barrier integrity, and gut homeostasis (Agace and McCoy 2017). Another important tissue in the GIT is the gut-associated lymphoid tissue (GALT). It primarily consists of Peyer's patches, effector cells in the lamina propria, intraepithelial lymphocytes, and other molecules. The GALT is known to regulate the gut mucosal immune system. The microfold cells (M cells) that are present in junctional site between adjacent epithelial cells uptake the antigens and pass to further downstream effector immunocompetent cells (Fig. 17.1). For proper immune response against pathogens, there must be a proper balance between effector T cell and T regulatory (T_{reg}) cell population. By binding to commensal and invading microbes, secretory immunoglobulin A (sIgA) helps to maintain the balance of intestinal mucosal immunity and barrier function (Chairatana and Nolan 2017).

17.2.3 Mucosal Immunity in the Respiratory Tract

The mucosal surfaces of the lungs and upper airways are a common location for infection. The immune defense of the airways is vital, difficult, and complex, with the primary aim being infection prevention and elimination. Two main parts of the mammalian respiratory systems are upper (from the nose and oral canals to the throat) and lower (the trachea and lung). The glottis separates the two airway compartments and keeps the lower respiratory tract essentially clean in healthy humans (Sato and Kiyono 2012). When microorganisms colonize the lower respiratory tract, inflammatory reactions are triggered. The upper respiratory tract is the main entrance of inhaled air. As because it is inhaled directly from the environment, this part is constantly bombarded with environmental particles including pathogens

and other antigenic particulates (Broadbent and Subbarao 2011). But thankfully this region is well protected by the epithelial cell layers that protects the respiratory tract from its luminal side. The epithelial cells adhered to each other tightly via the junctional proteins and form a closed epithelial covering (Tsukita et al. 2008). Epithelial cells have well-developed cilia and produce mucus that is predominantly made of polysaccharides like mucin (MUC) (Thornton et al. 2008). The cilia and the mucin work together to keep large foreign particles out of the alveoli. Ciliary movement carries foreign bodies toward the mouth, where they are coughed out. This movement is one of the important steps in phylaxis (Wanner 1986).

The nasopharynx region is the main site where mucosal immune responses in the respiratory tracts are produced, both across the nasal epithelium and via the tonsils and adenoids. These are collectively called as the nasopharynx-associated lymphoid tissue (NALT). The mucosal immune system uses NALT as an inductive site (Russell et al. 2020). Another respiratory-linked lymphoid tissue is the bronchitis-associated lymphoid tissue (BALT), which is missing in adults and only occurs in children and adolescents. It can be induced to form by infections (Tschernig and Pabst 2000). So NALTs are the main respiratory inductive sites for respiratory tract mucosal immunity. As in the GALTs, in the NALTs, there are also the presence of M cells in the junctional sites of adjacent epithelial cells, which are specialized in antigen absorption. M cells' cilia are shorter than those of traditional epithelial cells. The M cell develops a pocket-like structure on its basal surface that can retain immunocompetent cells. Because in the M cells, lysosome presence is weak; most integrated antigens simply pass through the M cells unchanged before being picked up by dendritic cells (DCs). They are subsequently processed and presented to immunocompetent cells by antigen-presenting cells (APCs). The APCs subsequently travel to the NALT's T-cell area, where the degraded antigen is presented to the naive T cells, whereas the germinal center development and antibody class switching take place in the B-cell area of NALTs (Mora et al. 2006). Then using the sphingosine-1-phosphate system, a lipid mediator, activated B cells escape from the NALT through efferent lymph vessels and enter the body circulatory system (Fig. 17.2).

17.3 Strategies for Vaccination Harnessing the Aspects of Mucosal Immunity

Local attacks by microbial infections or toxins, as well as poor immune system component activity, might disrupt mucosal equilibrium. Insufficient systemic immunity frequently results in pathological manifestations of the GIT and respiratory tracts, whereas pathologically increased activity can result in a variety of inflammatory changes in these mucosal surfaces, depending on the type of cells involved and the inflammatory mediators produced by them (Ogra et al. 2001). All of these can trigger a variety of diseases that could arise due to the alterations in mucosal

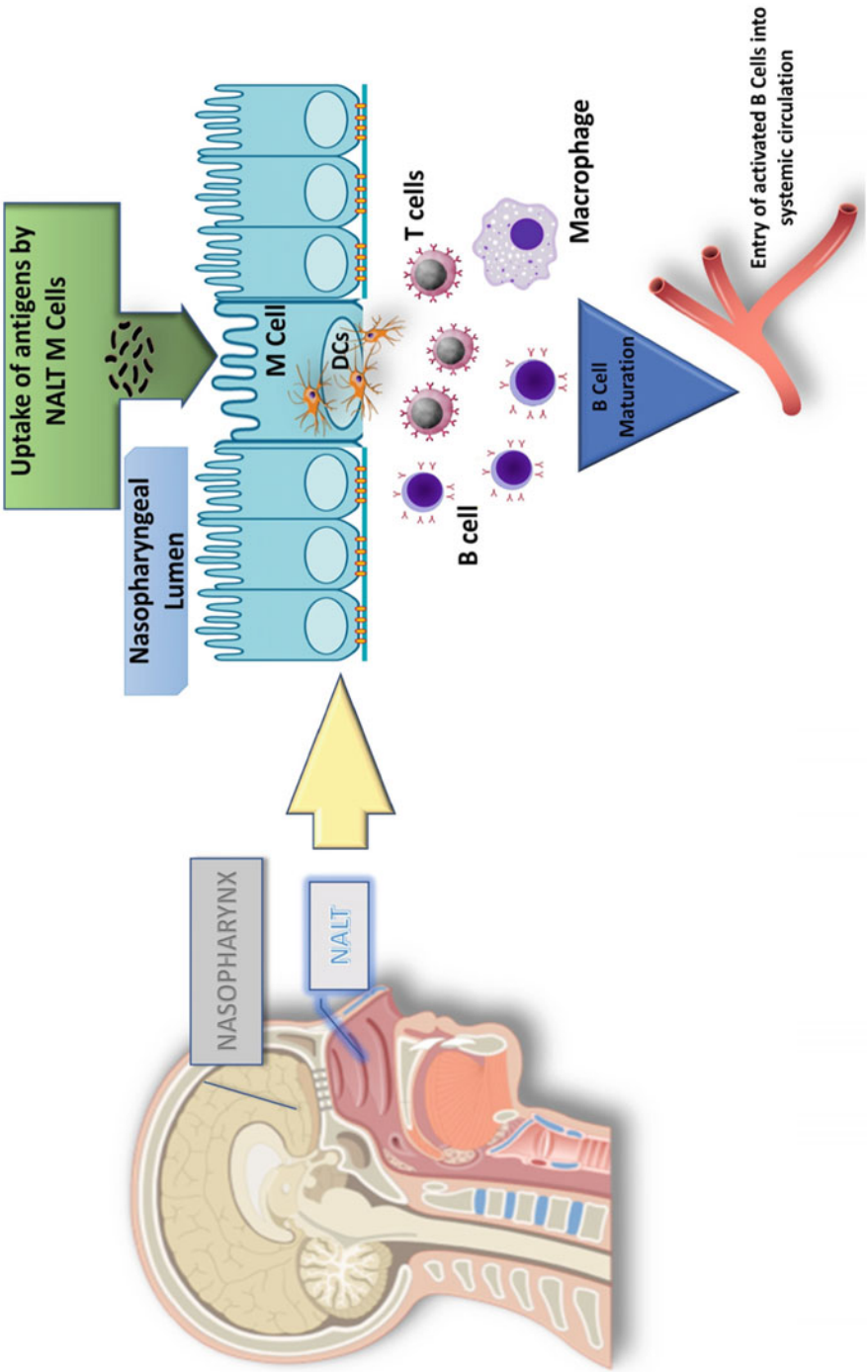

Fig. 17.2 Overview of mucosal immunity in the upper respiratory tract involving the NALT

immunity and tolerance mechanisms that are either genetically fixed or generated by the environment. And this aspect is used by the scientists to develop vaccination strategies involving the aspects of mucosal immunity (Holmgren and Czerkinsky 2005). Moreover, most infectious diseases affect or begin on a mucosal surface, and this is one of the fundamental reasons for utilizing a mucosal vaccination route. But, in the case of anti-infectious vaccinations, the physiological propensity of the mucosa's, i.e., unresponsiveness to non-proliferating antigens, should have to be overcome in order to make more profound vaccination strategy. Antigens passing through the epithelial microfold cells (M cells) induce a mucosal immune response; as a result, antigen carriers include polymeric structures and liposomes, which are carried preferentially through M cells. Furthermore, the ability of certain proteins (microbial adhesion proteins) to adhere to epithelial surfaces is harnessed (e.g., the B subunit of the cholera toxin) (Tlaskalová-Hogenová et al. 2002). New adjuvants are in constant development, and efforts are being made to determine the best immunization techniques and use numerous delivery routes. When we look at the present medical system, the options for mucosal vaccines are really restricted. All nine mucosal vaccination formulations licensed for use in humans (eight oral and one intranasal) are either live attenuated or whole-cell inactivated. These techniques are frequently followed in experimental settings due to the higher acceptability of orally delivered whole-cell killed antigens, whereas the unprotected subunit antigens are susceptible to degradation and clearance, and, most importantly, the lack of established mucosal adjuvants that have to be used with those subunit antigens (Lavelle and Ward 2022).

Very recently the entire world had been shaken up due to the emergence of a global health concern—COVID-19. In late December 2019, the city of Wuhan, China, reported cases of pneumonia with an unclear etiology. The causative agent was identified as the betacoronavirus SARS-CoV-2, which is closely linked to SARS-CoV, the virus that caused the SARS outbreak in 2002–2004. SARS-CoV-2 produced a large COVID-19 epidemic in China, which eventually expanded over the world and was proclaimed a pandemic in March 2020 (Zhou et al. 2020). Coronaviruses are enclosed viruses having a single-stranded, positive-sense RNA-based single-stranded genetic architecture. As soon as it reached the pandemic state, the whole scientific community all around the world started working for development of efficient vaccines (Krammer 2020). Till date there are vaccination strategies already developed and applied to real human populations, but still the efficiency of these vaccines is questioned frequently including the adverse effects.

One of the unique approaches in vaccine development against SARS-CoV-2 is harnessing the features of mucosal immunity. IgG (most commonly IgG1) is the main kind of antibody in serum that is carried into the lungs and is thought to protect the lower respiratory tract. Secretory IgA1 is expected to protect the upper respiratory tract the most (sIgA1) (Krammer 2020). Infection with respiratory viruses causes both a systemic immune response dominated by IgG1 and a mucosal immunological response dominated by sIgA1 in the upper respiratory tract. Many respiratory viruses may be sterilized as a result of this treatment. In a very recent study, it has been seen that a single-dose intranasal administration of ChAd-SARS-CoV-2-S-

inoculated mice, hamsters, and macaques provided better protection against SARS-CoV-2 challenge. In all of these animal models, viral clearance was found after infection with SARS-CoV-2 in both the lower and upper airways. Thus, intranasal immunization with ChAd-SARS-CoV-2-S can trigger an immune response in the nose, which is the virus's point of entry, preventing illness, infection, and transmission (Bricker et al. 2021; Hassan et al. 2020).

17.4 Immunomodulators

Immunomodulators are substances that help to modulate or restore our immunity when the immunological homeostasis has become disrupted. They can be natural or synthetic. Immunomodulators restore equilibrium to immune systems that have become unbalanced. Natural immunomodulators are less powerful but they have less adverse effects. Immunomodulators have several applications, including suppressing immune system (immunosuppressants) and stimulating immune system (immunostimulants). Several of them can have both features (immunosuppressant or immunostimulant) (Patil et al. 2012). Immunostimulants induce immunity and help us to combat with conditions like tumor, infections, etc., whereas immunosuppressive modulators help us to treat situation, e.g., autoimmune diseases.

17.5 Types of Prebiotics

Prebiotics are nondigestible dietary component that benefits the host by selectively promoting the development and/or activity of one or a restricted number of bacteria in the colon and therefore enhances human health. At the sixth meeting of the International Scientific Association of Probiotics and Prebiotics (ISAPP) in 2008, the definition of dietary prebiotics was defined as a selectively fermented products that changes in the composition and activity of the gastrointestinal microbiota, which conferring benefits upon the health of the host. The following are the key criteria for determining a substance is a prebiotic: (a) resistant toward pH of the stomach (acidic), (b) fermented by the bacteria (intestinal bacteria), and (c) this substance can benefit the development and/or activity of gut bacteria selectively, which benefits the host (Gibson et al. 2010).

There are several types of prebiotics (Davani-Davari et al. 2019) which are described below:

(a) *Fructans*.

Oligofructose (FOS fructo-oligosaccharide) and inulin are the examples of fructans. Their structure is a fructose linear chain with a β (2 → 1) linkage. At the

end of the linear chain, they have glucose molecule with β (2 → 1) bond (Louis et al. 2016).

(b) *Galacto-oligosaccharides*.

GOSs are lactose extension products which are classified in two categories: (a) GOS with galactose and (b) GOS without galactose. In the first category, they have extra galactose moieties at carbon number 3, 4, or 6. In the second category, lactose-derived GOS by enzyme mediated transglycosylation process.

Bifidobacteria and *Lactobacilli* can be substantially stimulated by GOSs. In infants, GOS 3 has been demonstrated to be significantly integrated by *Bifidobacteria*. GOS also stimulates other bacteria (Louis et al. 2016).

(c) *Starch and Glucose-Derived Oligosaccharides*.

There are some starches which are resistant to digestion in the upper intestine, known as resistant starch (RS). RS produces abundant amount of butyrate. So, it is considered as prebiotic (Fuentes-Zaragoza et al. 2011). Glucan-containing polydextrose is an example of this kind of prebiotic. Certain studies speculated that it has potential to improve *Bifidobacteria* composition in the gut (Costabile et al. 2012).

(d) *Other Oligosaccharides*.

Pectin, more specifically the pectic oligosaccharides, is very important. This kind of pectic oligosaccharides (POS) are based on galacturonic acid or rhamnose extension. Carboxyl groups are replaced by methyl esterification process, and carbon position number 2 or 3 gets acylated. Various sugars or ferulic acid moieties are attached to side chains (Yoo et al. 2012).

(e) *Noncarbohydrate Oligosaccharides*.

It is speculated that cocoa-derived flavenols have potential to be a prebiotic (Tzounis et al. 2011).

Below is the summarized list of certain prebiotics and their effects on the host physiology (Table 17.1).

17.6 Prebiotics as Potent Immunomodulatory Agents

Prebiotics accelerate the growth of some commensal bacteria. Those commensals inhibit pathogen adhesion and invasion to the intestinal epithelial barrier. Several mechanisms are involved such as pH change, enhancing barrier function, inducing mucus formation, generating SCFA, and triggering cytokine production to avoid pathogenic adhesion and invasion (Korzenik and Podolsky 2006; Walker 2000).

It has been observed that supplementation with oligofructose and inulin-rich diet in mice resulted in significantly higher activity in natural killer (NK) cells (particularly splenic NK cells) and increased phagocytic activity in macrophages (specifically peritoneal) (Kelly-Quagliana et al. 2003). Inulin intake enhances macrophage phagocytic capability and secretory immunoglobulin A (IgA-s) synthesis; both of them is crucial in the gastrointestinal tract's defense (Van Loo 2004). The

Table 17.1 List of prebiotics their sources, effects, and limitations

Type of prebiotic	Sources	Effects	Associated synbiotic organisms	Limitations	References
FOS (fructo-oligosaccharides)	Blue agave, yacon root, garlic, onion, leeks, chicory root, Jerusalem artichokes, asparagus, bananas	Increment of beneficial microbiota levels and reduction of colonic pH	*Lactobacillus* genus bacteria	Excessive levels sometimes can cause diarrhea, stomach cramps, and digestion problems	Scholtens et al. (2006), Benjamin et al. (2011)
GOS (galacto-oligosaccharides)	Dairy products, beans, and certain root vegetables	Reduction of the growth of harmful bacteria and immunomodulation	*Lactobacillus* and *Bifidobacterium* genus bacteria	Can cause mild side effects like gas (flatulence), bloating, stomach cramps, and diarrhea	Macfarlane et al. (2008), Saavedra and Tschernia (2002)
Soluble fiber (guar gum, pectin)	Leafy vegetables and majority of fruits containing high plant fibers	Anti-inflammatory function, lipid profile enhancement, epithelial cell integrity	NA	Not reported	Guslandi et al. (2000), Guslandi et al. (2003)
Inulin	Leeks, asparagus, onions, wheat, garlic, chicory, oats, soybeans, and Jerusalem artichokes	Enhancement of innate immune response	*Lactobacillus* and *Bifidobacterium* genus bacteria	Frequent bowel movements and can act as allergens for some peoples	Emilia et al. (2013), Macfarlane et al. (2008)
Lactoferrin	Cow milk and human milk	Enhancement of genital tract microbiota	*Lactobacillus* and *Bifidobacterium* genus bacteria	Can cause diarrhea. In the case of high dosage, it can cause skin rash, loss of appetite, fatigue, chills, and constipation	Artym and Zimecki (2021)

proportional number of fecal *Lactobacilli* and *Bifidobacteria* increased in rats supplemented with a mixture of GOS/FOS (ratio 9:1; optimum at 5% w/w of total diet), while FOS/inulin (2% w/w of total diet) enhanced the specific response of delayed type hypersensitivity (DTH) but had not any effect on fecal *Lactobacilli* and *Bifidobacterial* proportion (Vos et al. 2006). Moreover, severe colitis can also be managed when the host fed a combination of commensal bacteria and inulin (Schultz et al. 2004).

A reduced expression and production of interleukin-6 by peripheral blood mononuclear cells while examining the effects of FOS in older individuals is observed. It is found that supplementing the diet of elderly people with GOS (5.5 g/day) reduced the proinflammatory cytokine (IL-6, IL-1, and TNF-α) production, while increasing the NK cell activity and anti-inflammatory cytokine (IL-10) production (Vulevic et al. 2008). Mucosal immune system (restricted in the gut) was triggered and stimulated by a meal enriched with a FOS:inulin mix (5%) and supports the concept of utilizing food supplements containing a FOS:inulin mix in the improvement of oral vaccine effectiveness. There were no evidence of immunological suppression or other potential adverse effects in the trial when fed the FOS combination (Benyacoub et al. 2008).

A trend for a rise in endogenous secretory IgA in the feces was found in babies who did not get protective maternal SIgA from breast milk and were given conventional formula containing 0.6 g GOS + FOS (9:1)/100 mL till the eighth month (Bakker-Zierikzee et al. 2006). It is also seen that in 259 babies at risk for atopy, oligosaccharides were associated with postnatal immune system development. The cumulative occurrence of atopic dermatitis was considerably decreased in babies who swallowed GOS and FOS combination during the first few months of life (Moro et al. 2006). The newborn's digestive system gets colonized by bacteria due to breastfeeding. The occurrence of allergy disorders in children is generally associated with the absence or inadequate colonization of mucosa (particularly intestinal one). Human milk's oligosaccharides and bifidogenic nucleotides interact with the newborns' gut, increasing *Bifidobacteria* predominance (Ly et al. 2011; Macfarlane et al. 2007). Synthesis and secretion of polymeric IgA, as well as balanced T helper cell response and regulation of hypersensitive reactions, all need sufficient colonization of the intestines (Fanaro and Vigi 2008). Allergic children have an aberrant "adult-type" *Bifidobacterium* flora, with higher levels of *Bifidobacterium adolescentis* strain instead of the conventional baby flora, which is dominated by *Bifidobacterium bifidum* (He et al. 2001). Long-term intake of orally given lactulose (1% or 3%) had an immunomodulatory influence on the makeup of T-cell subsets in GALT, secondary lymphoid tissues, and peripheral blood of probiotic-fed calves, which might have an impact on calf health (Fleige et al. 2009).

Lactobacillus helveticus M92 combined with inulin, lactulose, or raffinose (10 g/l) resulted in immunomodulation, as these synbiotics enabled to activate the immune (mucosal and humoral) response in mice. Both secretory and total serum IgA concentrations were higher in inoculated animals than in controls (Frece et al. 2009).

A vast proportion of communicable diseases occur via the breach of mucosal immune system. The respiratory mucosa, especially NALTs, is the main region

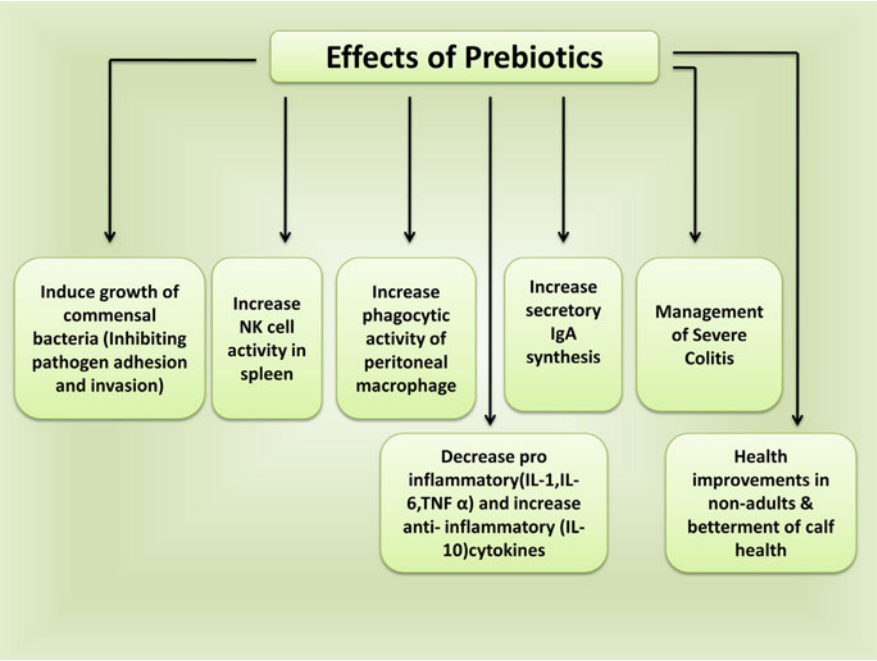

Fig. 17.3 Schematic diagram showing various physiological effects of prebiotics

which is constantly bombarded with exogenous particulate matters and pathogenic substances. Moreover, strategies of vaccination involving respiratory mucosa had shown positive effects. It has been reported that prebiotics also can modulate respiratory mucosal immunity. Prebiotics modulate systemic immune function, but the specific mechanism is uncertain. By modulating T_h1/T_h2 responses in the lungs, they may alter host innate and T-cell responses during a respiratory viral infection. Prebiotics may help to boost the gut's innate immune system (Schijf et al. 2012).

Overall, in a broader perspective, prebiotics not only modulates the microbial composition, but also it has positive effects on various aspects of mucosal immunity as well as immunomodulatory functions (Fig. 17.3).

17.7 Fructan: A Promising Prebiotic

Fructans are not digestible (Van den Ende et al. 2011); however, they may be exposed to mild hydrolysis in the stomach due to acid sensitivity (Di Bartolomeo et al. 2013). The human digestive system's inability to hydrolyze fructans is owing to a lack of hydrolytic enzymes capable of breaking down β-linked bonds. The colonic bacteria can destroy these bonds. Fructans reach to the colon in its intact

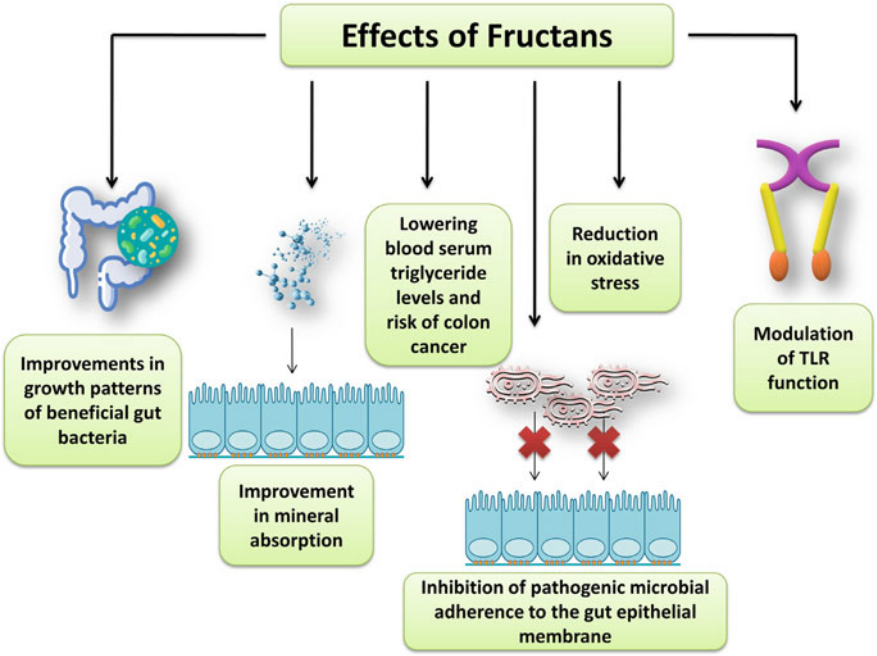

Fig. 17.4 Schematic diagram showing various physiological effects of fructans

form where colonic bacterial enzyme (GH32 family) reacts with them (Van den Ende et al. 2011).

The fermentation of fructans (such as inulin and FOS) and a lower pH modify the intestinal environment, resulting in improved growth patterns of beneficial bacteria such as *Bifidobacteria* and *Lactobacilli* (Tarini and Wolever 2010; Moro et al. 2002). During the process, there is an increase in the synthesis of SCFAs, lactic acid, and gases (hydrogen and carbon dioxide gases). Inulin and FOS ingestion enhances mineral absorption in the intestine (Scholz-Ahrens et al. 2007; van den Heuvel et al. 1999) and reduces blood serum triacylglycerol levels (Brighenti 2007). Inulin/FOS consumption has been linked to a lower risk of colon cancer (Allsopp et al. 2013; Verma and Shukla 2013; Sauer et al. 2007). The immune system regulation by inulin-type fructans with variable DPs (Van den Ende 2013; Lomax and Calder 2009) has now been backed up by concrete evidence on their mechanism of action (Vogt et al. 2013). In addition to their prebiotic effects via indirect processes that involve bacteria and SCFA production, fructans are thought to have more direct impacts. Fructans as well as other sugars are now recognized as antioxidants that may scavenge ROS in plant studies (Keunen et al. 2013; Peshev et al. 2013; Hernandez-Marin and Martínez 2012; Chen et al. 2009). Similar mechanisms in humans may occur in foods and during the intestinal interphase (Van den Ende et al. 2011). Fructans, according to this viewpoint, may be helpful to

prevent/treat illnesses by lowering ROS levels. The association between oxidative stress and a number of gastrointestinal problems is well established (Bhattacharyya et al. 2014; Van den Ende et al. 2011). Furthermore, soluble gut oligosaccharides have been shown to imitate the sugar chains on the glycoproteins and glycolipids found on gut epithelial cells, inhibiting harmful microbe adherence (Dai et al. 2000). Recent research has suggested that FOS and inulins have direct antibacterial properties (Ortega-González et al. 2014). Scientific studies also reveal that fructans (particularly inulin-type) bind to TLR (Toll-like receptor) 2 and TLR 4 as ligands offer a molecular mechanism for their immunomodulatory effects. Some of the prebiotics (e.g., fructans) as well as their fermentation products (e.g., SCFA) may serve as signaling molecules and/or impacting AMPK (AMK-activated kinase) and/or NF-κB signaling pathways in various different ways (Peshev and Van den Ende 2014). The overall beneficial effects of fructans are shown in the following schematic diagram (Fig. 17.4).

17.8 Adverse Consequences of Synthetic Immunomodulators

It has been seen that the synthetic immunomodulatory medications may raise the risk of infection by acting adversely on the immune system. Infections, including those that are caused by opportunistic pathogens, and all of these infections can become severe even though they are normally mild and risk-free. In certain conditions it has been reported that reactivation of latent tuberculosis is occurring in patients after initial recovery, and this is supposed to be a side effect of newer immunomodulatory medications (Miller and Ernst 2009). Moreover, in transplantation surgeries, it has been seen that due to the side effects of synthetic immunomodulators, the patient's quality of life is degraded, and also death in some of the transplant recipients was reported (Schonder et al. 2010). Azathioprine and 6-mercaptopurine—a potent and widely used immunomodulator—can produce medullar suppression, so patients taking these medicines must have a blood count done on a regular basis. Pathological manifestations like pancreatitis, hepatitis, myalgia, and dizziness are some of the adverse side effects of azathioprine and 6-mercaptopurin. Adverse effects of these synthetic immunomodulators can be separated into dose-independent (like fever, rash, alopecia) and dose-dependent effects (nausea and hepatotoxicity) (Andrews et al. 2009). The dose-dependent effects are the most severe and are also called toxic which can lead to fatalities. Bacterial infections, tuberculosis, atypical mycobacterial infection, and pneumocystis have been reported after using these immunomodulatory drugs for prolonged durations (Schuna and Megeff 2000). Another drug methotrexate can be harmful if used in pregnant or breastfeeding women. Another widely used immunomodulatory drug cyclosporine has been reported to cause epilepsy, neurotoxicity, hypertension, headache, and gingival hyperplasia (Toscano et al. 2010). Some drugs like mycophenolate are used as alternatives to cyclosporine,

but these drugs can also increase the risk of diabetes (mainly type 2 diabetes) (Prokai et al. 2012). Sometimes monoclonal antibodies are also used for immunomodulatory regimes, but they can also have other side effects. Various widely used monoclonal antibodies like infliximab, adalimumab, and golimumab can cause reoccurrence of tuberculosis after initial treatment. Immunomodulation using steroids can also have its negative consequences. High dosages of glucocorticoids can cause hepatic steatosis by boosting fat mobilization and redistribution, increasing plasma-free fatty acids by blocking fatty acid esterification in the liver and causing hepatic steatosis (Ben Dhaou et al. 2012).

17.9 Adverse Consequences of Antibiotics

Antibiotics are widely used to treat bacterial illnesses in humans and animals. They work by either killing the bacteria or making it difficult for them to grow and multiply. Nowadays, extensive and unnecessary use of antibiotics created a phenomenon of AMR. AMR is becoming a global health concern, and due to this AMR, various MDRs of known pathogenic microorganisms are emerging and creating a havoc among healthcare professionals. Antimicrobial side effects reveal themselves as adverse drug reactions that affect one or more organ systems. Despite the fact that most antibiotics are safe due to their widespread use, some antimicrobials can cause life-threatening side effects. Effects of antibiotic have been found in different organs of our body; among them hematologic side effects are common with a wide variety of antimicrobial agents. It has been seen that methicillin-resistant *Staphylococcus aureus* (MRSA) killed more people in the United States each year than emphysema, HIV/AIDS, Parkinson's disease, and homicide combined (Llor and Bjerrum 2014). Moreover, isoniazid- and rifampicin-resistant tuberculosis strains are predicted to be responsible for 3.7% of new cases and 20% of previously treated cases of tuberculosis worldwide. These antibiotics have been effective against tuberculosis for decades; however, their effectiveness is now insufficient. Only half of MDR tuberculosis can now be adequately treated with currently available medicines. In total, 84 nations have been identified as having extensive MDR tuberculosis (defined as multidrug-resistant tuberculosis plus resistance to any fluoroquinolone and any second-line injectable medication) (World Health Organization 2020). In recent years, carbapenem-resistant *Enterobacteriaceae* spp. that produce extended-spectrum beta-lactamase have been isolated (Ho et al. 2010). New medications that are effective against these multidrug-resistant gram-negative bacteria, particularly those that produce carbapenems, are in short supply (Boucher et al. 2013). Isolated cytopenias which can manifest as neutropenia, anemia, or thrombocytopenia are common in clinical practice. The most prevalent hematologic adverse effects of antimicrobial therapy are leukopenia and thrombocytopenia (Cunha 1995). The most prevalent causes of isolated leukopenia or thrombocytopenia are β-lactam

antibiotics and the sulfamethoxazole component of trimethoprim-sulfamethoxazole (TMPSMX) (Wong-Beringer 1997). Antimicrobials are to blame for a wide range of neurologic side effects also where encephalitis, seizures, neuromuscular obstruction, and muscle stiffness are the most significant neurologic adverse effects. Encephalopathy is a common side effect of trovafloxacin medication, and it has also been linked to clarithromycin (Ball 1997). Antibiotic-induced pulmonary side effects are a flu-like disease that commonly appears soon after starting rifampin therapy (Aquinas et al. 1972). It has been seen in studies that antibiotic can elicit adverse cardiovascular complications. Ventricular arrhythmias can be caused by the drug's direct effect on myocardial excitability. Ventricular arrhythmias can be caused by a prolonged QTc interval (e.g., torsades de pointes); IV erythromycin, terbinafine, and certain quinolones are the antimicrobials most usually linked to QT interval prolongation. After an antibiotic is given, QTc prolongation occurs quickly, and the QT interval returns to normal after the medicine is taken away (Viskin 1999).

Another system-specific adverse effect of antibiotic is urinogenital complications. For example, nephrotoxicity can take the form of glomerular or tubular toxicity and can be caused by a number of antibiotic drugs. Tubular toxicity is more common and has been linked to multiple-dose administration of aminoglycosides (Yano et al. 2009). Moreover, overuse of some antibiotics has the potential to induce metabolic problems. Ketoconazole can cause gonadal and adrenal dysfunction. Lactic acidosis is a side effect of abacavir treatment, and pentamidine administration is frequently associated with hyperglycemia (Carr et al. 1999).

According to a recent report from the World Health Organization, AMR is no longer a phenomenon exclusive to poor or developing countries; it now affects people all around the world (Thomas et al. 2014). AMR acquired in the community is of particular concern because these illnesses are widespread and can be easily communicated. Moreover, the issue of resistance impacts not just the community but also the individual. Individual resistance to antibiotics can last up to 12 months after treatment, according to a new assessment of patients with bacterial urinary tract and respiratory tract infections who were treated with antibiotics (Costelloe et al. 2010). Infection with antibiotic-resistant bacteria can result in serious disease, higher fatality rates, and a higher risk of complications and longer hospitalization (Livermore 2012; Paul et al. 2010; Kollef 2008). Antibiotic resistance translates into higher healthcare expenses and was predicted that it costs €9 billion each year in Europe (Oxford and Kozlov 2013). If we summarize the whole scenario of AMR and associated emergence of MDR strains, it can be saying that the widespread usage of antibiotics should be stopped immediately in order to avoid an emerging new pandemic situation. As discussed earlier uncontrolled and unscientific use of antibiotics can not only cause adverse effects in individual's systems, but also on a wider perspective, it can cause AMR and associated emergence of MDR strains that can be easily transmitted in communities predisposing them to pandemics. Scientists are now searching for alternative therapeutic strategies that are less harmful and more efficient than that of the available antibiotics.

17.10 Prebiotics as Promising Alternatives to Antibiotics

AMR is now been identified as one of the most serious worldwide public health problems of this century and has aroused the scientific community's interest in recent years (Prestinaci et al. 2015). Most multilateral organizations focusing on global health now recognize antibiotic use in animals used for food production as one of the main drivers of AMR infections in humans, establishing national action plans that commit their members to minimize the indiscriminate use of antibiotics (Asherson et al. 2008). In this context the World Health Organization (WHO) has published a number of guidelines and resolutions, among those that stand out being the overall reduction. In the use of all classes of antimicrobials, with the conditional recommendation not to use those defined as critically important for human medicine, as well as the entire restriction for growth promotion and prevention of infectious diseases that have not yet been clinically detected (Steven et al. 2010). The state of AMR is much more drastic in animal husbandry, and this extensive use of antibiotics can indirectly impose a greater threat of AMR in general populations via the procedure of biomagnification (Manyi-Loh et al. 2018). As a result, certain government regulatory authorities have taken action to prohibit the extensive use of antibiotics in animal agriculture, thus banning them as growth promoters (Preiss and Zeitz 2010). The entire prohibition of the use of antibiotics as growth promoters is likely to result in a reduction in antibiotic resistance (Miller and Ernst 2009). Furthermore, few farming practices must be implemented to lessen the use of antibiotics in animal manufacture, such as adequate animal vaccination, good hygiene and husbandry practices, higher animal welfare, and enhanced breeding programs, all of which imply an increase in production costs, and it is still not enough to completely reduce infection risks (Bradford and Shih 2011). Poultry is one of the most widely exploited species in the world, with a growing and industrialized sector in many regions of the globe (Andrews et al. 2009). Antibiotics have been utilized in poultry production for medicinal, preventive, and growth promotion objectives, particularly in broiler chickens, resulting in enormous profits for poultry producers (Prokai et al. 2012). Although it is true that reducing or eliminating the use of antibiotics in poultry production would have a good impact on AMR control and public health, it would also have negative economic consequences, since production costs and, consequently, the prices of the final products, as well as the international trade of poultry products, would be affected (López-Jornet et al. 2010). Furthermore, antibiotic-free poultry production could result in foodborne infections with *Clostridium perfringens*, *E. coli*, *S. aureus*, *Campylobacter* spp., or *Salmonella* spp., causing public health issues (Srinivas and Meier-Kriesche 2008). While this will harm the health of the birds and decrease of the productive parameters, it will also increase production costs. As a result, the ban on the use of antibiotics in poultry production, as well as other growing trends such as the expansion of the organic products market, has driven poultry producers to identify viable alternatives to antibiotics. Many particular alternatives, such as enzymes, prebiotics, probiotics, organic acids, dietary fiber, highly accessible nutrients, herbs, spices, essential oils,

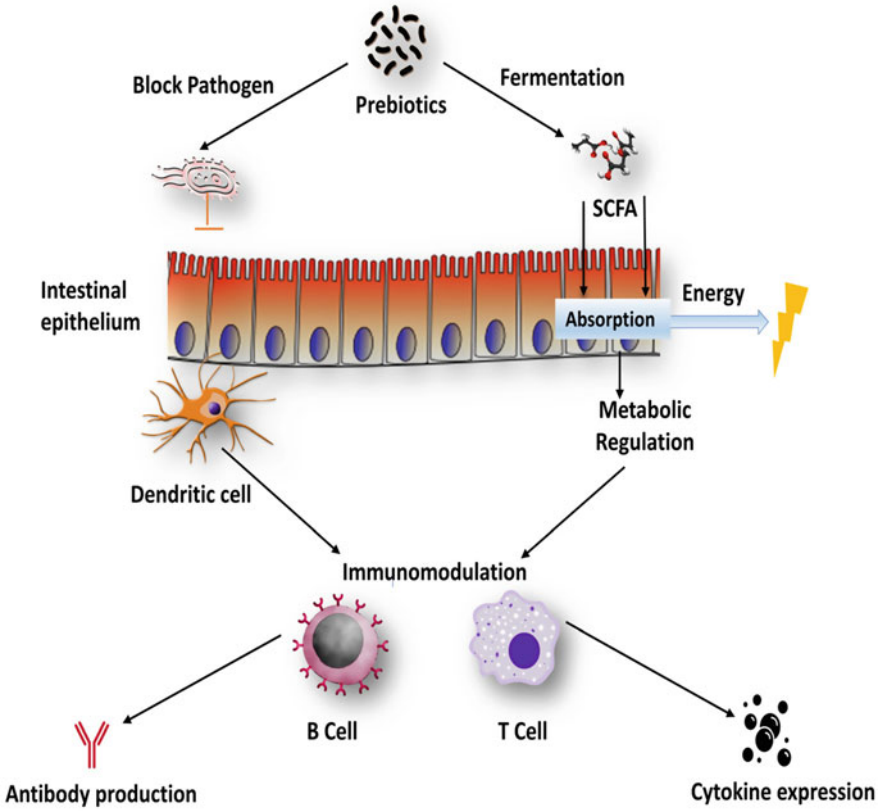

Fig. 17.5 The probable mechanism of the effects of prebiotic in the host immune system

plant components, and vaccinations, have been investigated and marketed for this purpose (Toscano et al. 2010). Prebiotics have proven to be potential alternatives for the poultry sector since they can pass through the digestive tract, facilitating and supporting the symbiotic relationship between the host and the gastrointestinal tract (GIT) bacteria and providing health benefits to the birds (Ramos-e-Silva et al. 2011). Prebiotics as feed additives in chicken, with particular emphasis on their beneficial effects on microbiota composition, ability to control pathogenic infections, positive changes in intestinal shape, enhanced productivity indicators, and immunomodulatory effects as potential modes of action, making them viable alternatives to antibiotics as growth promoters in the chicken industry as well as in human welfare. Overall, it can be concluded that prebiotics can modulate the whole immune system by not only enhancing the resident microbiome but also directly exerting its effects on the immune ecosystem of the host (Fig. 17.5). The change in the interest toward the application of prebiotics can help to manage the emerging problems of AMR.

17.11 Effects of Prebiotics and Probiotics in Pulmonary Health: In the Context of Current COVID-19 Pandemic

As discussed above, prebiotics can help to regulate the modulation of beneficial probiotic strains, and both the prebiotic and probiotic can create a sustainable system in our body so that the overall immune homeostasis can be maintained. COVID-19 is one of the deadliest global health concerns that the entire world is facing right now. The causative agent of COVID-19 is SARS-CoV-2 virus which was first reported during 2019 in China. The target for this virus spike proteins is the receptor angiotensin-converting enzyme (ACE) 2. The beneficial effect of probiotics on ACE enzymes, whether directly or indirectly, has been thoroughly investigated. Probiotic strains can produce bioactive metabolites by the degradation of existing prebiotics. Many of these metabolites has been reported to inhibit ACE enzymes by blocking their active sites (Ayyash et al. 2012). These findings imply that prebiotics and probiotics could function as a blocker for the ACE receptor, which allows SARS-CoV-2 to infect the cells. The idea of employing medicines to block ACE receptors as a COVID-19 therapy method was also evaluated. By boosting probiotic development and survivability, prebiotics may have a great potential effect against COVID-19. Furthermore, via inhibiting the ACE enzymes, prebiotics may have a direct influence on gastrointestinal complications induced by COVID-19. Yeh et al. (2018) conducted a systematic evaluation of 12 researches on the effects of prebiotic and probiotic supplements on influenza illness. The authors found that supplementing with probiotics and prebiotics after an influenza vaccine could improve hemagglutination inhibition and antibody titers. SARS-CoV-2 is a newly emerging virus for which there are presently limited strategies for combating. Several trials investigating the efficacy of probiotics in treating COVID-19 patients are now underway (Infusino et al. 2020). Low numbers of several probiotic species such as *Bifidobacterium* and *Lactobacillus* were found in some COVID-19 patients, indicating gut microbial dysbiosis during COVID-19 pathogenesis. This could be a sign of weakened immunity, so it's been advised that these individuals need dietary support as well as prebiotic or probiotic treatment to restore intestinal flora balance and reduce infection risk (Xu et al. 2020). COVID-19 is a new disease against which humans have not developed immunity. Meanwhile, a patient's food pattern is a critical influence in microbiota diversity in GIT. As a result, a well-balanced diet rich in probiotics and immunity-boosting micronutrients such polyphenols; vitamins A, C, and D; and minerals (especially selenium and zinc) may help to reduce the risk of COVID-19 infection and disease severity (Gasmi et al. 2020). Probiotic-rich foods, such as fermented foods, have a high potential for preventing COVID-19. Consumption of fermented milk containing probiotic microorganisms has been shown in earlier studies to lower the occurrence of upper respiratory tract infections in healthy newborns, children, adults, and the elderly (Shida et al. 2017). Given previous studies of probiotics' potential use in preventing and treating a variety of viral infections, probiotic supplementation appears to be a viable technique combating

COVID-19. Previous research used a broad range of probiotic species and strains, with strain-specific immunomodulatory effects (Wu et al. 2019). To provide preventative and therapeutic advantages against respiratory tract infections, specifically COVID-19, probiotics must be taken in appropriate quantity (>7 log CFU). It has been proven that the quantity and diversity of gastrointestinal microorganisms declines with age and antibiotic medication. Such dysbiosis is relatable with a variety of infectious, metabolic, and inflammatory illnesses (Landete et al. 2017).

17.12 Future Prospect

The availability of prebiotics in daily dietary feed is the key concern related with their usage. But nowadays the commercial application and mass production of prebiotics and synbiotics as dietary supplements are gradually becoming a new norm for modern diet plans. Products like GroBiotic®-A and INLIFE Prebiotics and Probiotics® (González-Félix et al. 2018) are made available to the general public for commercial use. Although probiotics have mostly good benefits, there are a few disadvantages for being aware of before utilizing them for medicinal purposes. The most significant side effects of probiotic usage include bacteremia, fungemia, and sepsis. As a result, utilizing prebiotics as well as probiotics in immunocompromised individuals might result in serious health issues (Theodorakopoulou et al. 2013). The length of a prebiotic's chain plays an important role in the development of its negative consequences. Shorter-chain-length prebiotics may have greater harmful effects. This trend might be explained by the fact that short-chain molecules are digested and fermented relatively quickly in the proximal colon, while longer-chain molecules get fermented in the distal colon in a slow manner. In addition to chain length, the prebiotic dosage can affect its safety profile. 2.5–10 g/day dose of prebiotics may cause flatulence; however, 40–50 g/day dose of prebiotics may cause osmotic diarrhea. It's worth noting that prebiotics need to be consumed in amounts of 2.5–10 g per day to have a positive impact on human health. These possible negative effects haven't been investigated or, at the very least, haven't been included in prebiotic-specific clinical trials (Tsai et al. 2019). There are numerous studies regarding the beneficial effects of prebiotics, but in order to make the prebiotic as general health supplement, we need larger clinical trial. The ability of prebiotic dietary elements to restore the intestinal microbiota is a promising strategy for the treatment and recovery of a variety of severe disorders. This idea has the potential to end the long-running prebiotic controversies, and it might be included in future FAO and/or WHO prebiotic recommendations (Davani-Davari et al. 2019).

17.13 Conclusion

Mucosal lining is a defense system of higher animals that has evolved over time. From the medical point of view, various clinical management approaches are available that are not only used to strengthen the immune system but also for providing prophylactic immune-boosting effects via the processes of vaccination. Moreover, the most widely used therapy for infectious diseases management is antibiotics. Although different alternative therapeutic approaches are available, they also come with limitations. It is obvious that neither antibiotics nor immunomodulation singly can resist communicable drug-resistant microbes. Even more dangerously, synthetic immunomodulatory drugs and antibiotics have their own perilous consequences. AMR is one of the most serious health concerns of modern decade. Due to AMR, various MDR strains are emerging and plaguing the general population and creating havoc among the healthcare system. In this context, prebiotics and associated probiotics can help in managing the overall immune homeostasis in a more natural manner. There are various beneficial aspects of prebiotics which not only help to enhance the potential of mucosal innate immunity but also various metabolic aspects of the host. Prebiotics seems to be more safer and realistic alternative than the traditional therapeutic approaches, not only for the simple mass manufacturing process but also for the ease in respect with storage and transportation. Therefore, as a standardized strategy, creating population-specific prebiotics with consideration to the resident gut microbiota distinct to each community, may eventually help to the decrease of certain illnesses in each community. Finding community-specific, effective, and diverse probiotics for the maintainance of microbiota homeostasis can determine the clinical wellness of the new generation.

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Conflict of Interest None to declare.

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Chapter 18
The Use of Probiotics, Prebiotics, and Synbiotics as an Alternative to Antibiotics

Amit Ranjan

Abstract Antimicrobial resistance stands up as one of the major healthcare concerns throughout the globe and is included in the top ten priorities of the World Health Organization (WHO). The evolution of antimicrobial resistance is linked with various factors, including the underuse/overuse/abuse of antibiotics in clinical cases. Also, antibiotics are an essential component of animal farming and poultry production and are considered one of the important contributors escalating antimicrobial resistance in the environment and foodborne pathogens. The global burden of bacterial infections is expected to increase drastically if a complete ban is employed on antibiotic uses and would create health havoc and substantial economic loss worldwide but its impact on developing and underdeveloped countries would be even heavy. Under these limiting and challenging conditions, probiotics, prebiotics, and synbiotic offer one of the most sustainable and non-alarming alternatives for antibiotics. The probiotic species such as *Lactobacillus*, *Bifidobacterium*, etc. produce a variety of antimicrobial compounds/acids/bacteriocins that act on antimicrobial-resistant bacteria and limit their growth. In contrast, prebiotics such as inulin/oligosaccharides act directly in the intestine and help to enrich beneficial microorganisms. Synbiotics are the combinational use of probiotics and prebiotics to provide similar effects through the growth of healthy microflora. Besides controlling the growth of antimicrobial-resistant/multiresistant pathogens, these provide other immunomodulatory functions which would be even more beneficial to health. In this chapter, we will focus on why these probiotics, prebiotics, and synbiotics are considered as one of the most attractive alternatives for antibiotics, their uses in human health and livestock production, and the various mechanisms which are exploited by these agents that render these effects. We will elaborately explain the multiple methods used to evaluate a probable probiotic before it can be used. With specific examples of probiotics, prebiotics, and synbiotics, we will understand the mechanism such as competitive inhibition, production of inhibitory acids/toxins/antioxidants, and host immune modulation.

A. Ranjan (✉)
Center for infection and immunity, Mailman School of Public Health, Columbia University, New York City, NY, USA

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Abbreviations

CDC	Centers for Disease Control and Prevention
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
FOS	Fructooligosaccharides
MOS	Mannan-oligosaccharides
SCFA	Short-chain fatty acid
WHO	World Health Organization

18.1 Introduction

The first antibiotic resistance was observed in the early 1940s, almost soon after introducing antibiotics as a significant healthcare advancement in treating bacterial diseases (Abraham and Chain 1988). With time the more antibiotics were introduced, leading to the development of higher antibiotic resistance. With the increase of antibiotic resistance, the pathogens/bacterial species acquired more traits and developed multiple resistance (Watanabe 1966). Apart from human health, antibiotics became essential components of the animal farmhouses and poultry as they help limit losses due to infection and are also used as growth enhancers in some instances (Butaye et al. 2003). In the period 2000–2015, global antibiotic consumption has increased from 11.3 to 15.7 DDDs per 1000 people (39%) every day, and these rates were mainly driven by low- and middle-income countries (Klein et al. 2018). If this rate continues with no policy amendments, the rate will increase to another 200% by 2030 (Klein et al. 2018). Thus, antibiotic consumption needs to be curtailed at one end, and there also needs to find out better alternatives for these as their use is inevitable in many of the conditions related to human and animal health.

As the alarming situation is still escalating, antibiotic resistance has been classified as one of the major health concerns by many global health agencies such as the Center for Disease Control and Prevention (CDC), Infectious Disease Society of America, World Economic Forum, and World Health Organization (WHO) (Aslam et al. 2018). Alternatives to antibiotics are being looked up to achieve medical security and a sustainable environment that is maintained. Probiotics, which are mostly the commensal and beneficial bacteria; prebiotic compounds that enrich the growth of specific microbes; and synbiotics (prebiotic and probiotic combination) present one of the most attractive and safe alternatives to antibiotics (Reid 2006;

Trafalska and Grzybowska 2004; Hume 2011). This chapter will discuss how these alternatives are characterized as antimicrobial agents and the molecular mechanism through which they render this activity. We would also discuss some of the other beneficial activities of these alternatives.

18.2 Probiotics

The term probiotic is derived from the Latin word "pro" and German word "bio," which means "for life." The concept of probiotic is ancient and is one of the pioneers' thoughts from Nobel Laurette Elie Metchnikoff for aging. He believed that the colonic microorganisms are proteolytic and produce toxins which are the reason behind aging. However, such conditions can be decreased by using fermented milk through the reduction of intestinal pH (Mackowiak 2013). It was defined as "microbial preparations or any component of microbial cells which impart beneficial effects and well-being" (Marteau et al. 2002). Since, the concept of probiotics has evolved over time, and at present, the expert panel of FAO (Food and Agriculture Organization) and WHO defined probiotics as "live microorganisms" which, when administered in adequate amounts, confers a health benefit (Hill et al. 2014).

18.3 Methods to Determine Probiotics as an Antimicrobial Against Another Organism

There are different ways that are used to determine a probiotic microorganism as an antimicrobial against other pathogens. The methods can be broadly classified into (a) in vitro methods and (b) in vivo methods.

18.3.1 In Vitro Methods

The in vitro methods are similar to how any compound is determined to be antimicrobial, but certain modifications are employed to it. One of the most ancient methods is the agar spot test (Soomro et al. 2007). The agar spot assay is performed simply by spotting the probiotic organism on the lawn of indicator organism on agar plates. The plates contain the defined/selective medium for the indicator organism. If the tested organism possesses any antimicrobial activity, a clear zone around the spot known as the zone of inhibition would be observed after incubation, and the antimicrobial activity would be expressed in the arbitrary units per ml (Au/ml) or the diameter of the zone of inhibition. This method has been modified into various forms such as agar spot assay in which spots of probiotic strains are first prepared

and then overlayed by indicator organism within soft agar; spot-on-lawn assay in which spots of probiotic/testing organism are made within a sealed bottom on agar bore and the plates are incubated for certain period for setting the culture after which the indicator organism is overlayed and incubated; and cross streak assay in which the probiotic strain is streak parallel onto agar plates followed by perpendicular streaking of indicator organism and inhibition at the intersection of lines represent the antimicrobial property (Choi and Chang 2015; Soomro et al. 2007; Parente et al. 1995). Another method is agar well diffusion assay method. In this method, cell-free supernatant from probiotic strains is used. The plates are inoculated with a standard amount of indicator organism, and the probiotic strain is then inoculated with dilutions of cell-free extract in bored wells (6–7 mm). The zone of inhibition is observed after incubation and expressed as the diameter of the zone of inhibition or Au/ml. In its modification, the bored wells are replaced by a paper disc, and clear zones around them are measured to determine antimicrobial activity. This modified method is called paper-disc diffusion assay. Another in vitro method is co-culturing assays. As the name suggests, in the co-culturing method, both the probiotic and indicator organisms are mixed together, incubated in an ideal environment, and then enumerated for the indicator organism. The results are expressed as colony-forming units per ml (CFU/ml). The microdilution method is one of the most common and reproducible methods in co-culture assay (Millette et al. 2007). In the microdilution method, the cell-free extracts at different dilutions are mixed with an equal amount of indicator organism in a 96-well microtiter plate and incubated for the required time. After incubation, the indicator organism is counted, and the lowest concentration at which the growth of the indicator organism is inhibited is specified as MIC (minimum inhibitory concentration). Another important method of testing in a co-culture condition is co-culturing with cell lines. Most of the studies use intestinal cells of human and other mammalian hosts such as HT-29, IPEC-J2, Cac0-2. HIEC-6, and BALB/c3T3 (Presti et al. 2015; Abdel-Daim et al. 2013; Cencic and Langerholc 2010). These help to evaluate the antimicrobial activities of probiotics and also evaluate the cell toxicity if induced by them in the cellular environment. As most of these methods are in vitro, it does not provide very strong evidence to be used directly as antimicrobial agents directly in humans as many factors such as cellular immune environment, biofilm capabilities, etc. are not considered in these. However, still, these provide the first evidence of the probable probiotic as an antimicrobial agent, which could be tested further in animal models.

18.3.2 In Vivo Methods

In vivo methods include the use of animal models. The animal models include invertebrate models, fish, rodents (mice, rats), birds (chicken), and higher mammals (hamsters, rabbits, pigs, etc.) (Feng et al. 2016; Mazaya et al. 2015; Zhou et al. 2014; Bendali et al. 2011). In all models for evaluation of the probiotics, there is the requirement of at least two groups of animals. The first group include the

pathogen-infected animals, while the second one should have probiotic-treated pathogen-infected animals. However, it is encouraged to have additional control groups for better evaluation of results. The outcomes in survival, infection, and several other factors associated with risks are evaluated. Nevertheless, the last requirement of any probiotics before approval is randomized double-blinded, placebo-controlled trials in humans to assess both the safety and efficacy of any of the probiotic agents to be administered to control the specific pathogen.

18.4 Mechanisms of Action in Probiotics

The probiotics act on the pathogen or drug-resistant organisms by employing a variety of mechanisms. These mechanisms counteract the growth of invading pathogens, minimize the use of external antimicrobial compounds, and thus provide an alternative for antibiotics. These mechanisms include competitive inhibition, production of antimicrobial compounds, and immunomodulation.

18.4.1 Competitive Inhibition

Competitive inhibition is the phenomenon in which the probiotic competes with the incoming pathogen for sites of attachment to intestinal flora or competes for the nutrients. This phenomenon is often observed in probiotic strains of *Lactobacillus* and *Bifidobacterium* species. These *Lactobacillus* and *Bifidobacteria* share carbohydrate-binding specificities with some enteropathogens, making it possible to compete for the receptor site on host cells for some pathogens (Neeser et al. 2000). For example, the probiotic strains *Lactobacillus plantarum 299v* and *Lactobacillus rhamnosus GG* have been shown to inhibit the attachment of enteropathogenic *Escherichia coli* (*E. coli*) on intestinal epithelial cells and increase mucin production (Mack et al. 1999). Also, *Clostridium difficile*, a pathogen of the gut, is not able to utilize oligosaccharides. It depends mostly on monosaccharides for its energy source, so when the flora is rich in bacterial population that uses monomeric glucose molecules, this pathogen is unable to thrive and gets reduced (Wilson and Perini 1988).

18.4.2 Production of Antimicrobial Compounds

Many probiotic strains produce certain compounds that inhibit the growth of other/pathogenic organisms in their immediate microenvironment, thus limiting the use of antibiotics in treating such pathogens. These compounds include organic acids, hydrogen peroxide, antimicrobial compounds such as bacteriocins, colicins, and

short-chain fatty acids (Maggi et al. 2000; Mishra and Lambert 1996; Thomas et al. 1994). The probiotics of *Lactobacillus* species provide examples for some of these compounds. The *Lactobacillus* species in the female reproductive tract produce hydrogen peroxide, which reduces the pH and bacteriocin, such as lactocin 160, which can act on pathogens such as *Gardnerella vaginalis*, and eliminate it from the tract (Maggi et al. 2000; Turovskiy et al. 2009). Also, commensal *E. coli* are known to produce a variety of colicins, which helps check the attachment of incoming pathogens in the host microflora (Micenkova et al. 2019).

18.4.3 Immunomodulation

Probiotics are capable of inducing immune responses within the host cells, and thus they may enhance many functions in the clearance of pathogens. These immune functions could be in many forms, such as the production of pro−/anti-inflammatory cytokines, production of IgA, and enhanced immune cell populations (Lammers et al. 2002; Haller et al. 2000; Park et al. 2002; Ogawa et al. 2006). For example, *Bifidobacterium* species can help in reducing the impacts of inflammations in the conditions of infections due to *S. aureus* or *Klebsiella pneumoniae* through the production of anti-inflammatory cytokines (Vieira et al. 2016).

18.5 Probiotics Classified to Be Used as Antimicrobial Agents

Probiotics are used as antimicrobial agents against many pathogens. To date, most of the probiotics are from the genus *Lactobacillus*, followed by *Bifidobacterium* and other organisms. Table 18.1 contains the list of all probiotic organisms which are either used or have been potentially classified to be used against antibiotic-resistant pathogens.

18.6 Prebiotics

Prebiotic is one of the recent concept introduced and refers to the nondigestible component of food having a beneficial impact on the host's health. The definition of prebiotics has been revisited several times over the years. Still, currently, it can be defined as the component of food which are resistant to digestion by mammalian enzymes of the gut but could be readily fermented by the intestinal microorganism and render selective benefits to the host either by stimulation of growth or activity or both of intestinal bacteria (Roberfroid 2007; Gibson et al. 2004). Prebiotics consist

Table 18.1 List of commonly used probiotics, prebiotics, and synbiotics in humans and animals

A. Probiotics	
Genus	**Species**
1. *Lactobacillus*	*acidophilus*, *amylovorus*, *casei*, *gasseri*, *helveticus*, *johnsonii*, *pentosus*, *plantarum*, *reuteri*, *rhamnosus*, *iners*, *paracasei*, *fermentum, brevis*, *fermentum*, *paraplantarum*, *viridescens*
2. *Bifidobacterium*	*adolescentis*, *animalis*, *bifidum*, *breve*, *infantis*, *longum*, *thermophilum*, *lactis*
3. *Enterococcus*	*faecium*
4. *Lactococcus*	*lactis*
5. *Escherichia*	*coli Nissle* (*1917*)
6. *Bacillus*	*clausii*, *coagulans*, *pumilus*, *mojavensis*, *subtilis*, *amyloliquefaciens*, *aerophilus*
7. *Streptococcus*	*thermophilus*
8. *Saccharomyces*	*cerevisiae*
B. Prebiotics	**C. Synbiotics**
1. Fructooligosaccharides (FOS)	1. *Lactobacillus* + *inulin*
2. Galactooligosaccharides (GOS)	2. *Lactobacillus*, *Bifidobacterium*, and *Enterococcus* + FOS
3. Xylooligosaccharides (XOS)	3. *Lactobacillus* and *Bifidobacterium* + oligofructose
4. Soy-oligosaccharides (SOS)	4. *Lactobacillus*, *Bifidobacterium*, and *Enterococcus* + inulin
5. Transgalactooligosaccharides (TOS)	
6. Isomaltoligosaccharides (IMO)	
7. Maltooligosaccharides (MOS)	
8. Lactitol	
9. Lactosucrose	
10. Lactulose	
11. Inulin	

of an oligosaccharide group of molecules within food additives and are generally derived from plants or animal sources. These include molecules such as fructooligosaccharides (FOS), galactooligosaccharides (GOS), soy-oligosaccharides (SOS), xylooligosaccharides (XOS), pyrodextrins, lactulose, isomaltooligosaccharides (IMO), and inulin (Hume 2011).

Prebiotic is one of the best choice to use in poultry as antibiotics are used in the poultry industry for various purposes such as growth promoters, metaphylaxis, and treatment of various infections (Mehdi et al. 2018). The overall poultry production and consumption of poultry products result in a large amount of antibiotic use annually, for which the data from many countries are not available. With the growing awareness of the global problem of antimicrobial resistance, agencies like the Food and Drug Administration (FDA), European Food Safety Authority, and the European Center for Disease Prevention and Control have made several guidelines that mandate reducing the use of antibiotics in poultry as the poultry industry would

have to face major economic losses if a complete antibiotic ban is imposed (Roth et al. 2019; Siekkinen et al. 2012). This would also result in a massive influx of foodborne pathogens such as *Salmonella* and *E. coli* into the food chain (Cox and Popken 2004; Cox and Popken 2006). Among the various alternatives to antimicrobial agents, prebiotics is one of the better alternatives in poultry as it improves gut health through the symbiotic growth of beneficial microorganisms naturally.

18.7 Mechanism of Action of Prebiotics

Prebiotics are the molecules that are used to promote the growth of beneficial bacteria in the colonic microenvironment. The complete mechanism for rendering these beneficial effects is not yet understood. Still, it is believed to be a cumulative effect of many processes such as the bifidogenic effect, competitive inhibition, and immunomodulation. Most prebiotics such as inulin increase the colonic relative abundances of *Bifidobacterium* species which then ferment these compounds into molecules such as short-chain fatty acids and antimicrobial peptides (Gibson and Roberfroid 1995). The increase of *Bifidobacterium* abundances could compete with the receptor binding of incoming pathogens and decrease the pH, making a difficult microenvironment for the pathogen's survival (Xu et al. 2003). Additionally, the fermentation products such as SCFA are used by colonic cells as energy sources and act as immunomodulatory molecules for the production of cytokines through various immune cells. Some of the mechanisms such as how prebiotics functions can be summarized below.

18.7.1 Bifidogenic Effect

As most of the prebiotic molecules increase the beneficial bacterial population of *Bifidobacterium* and *Lactobacillus*, the first effect which is observed is the bifidogenic effect. The prebiotic compounds such as FOS, MOS, and inulin have been shown to improve the health of chickens as the increased population of *Bifidobacterium* and *Lactobacillus* is observed after prebiotic supplementation and decreased population in pathogenic bacteria such as *Escherichia coli*, *C. perfringens*, and coliform bacteria (Slawinska et al. 2019; Kim et al. 2011; Shang et al. 2010). *Bifidobacterium* and *Lactobacillus* compete for the sites within the microflora, and hence other pathogens cannot attach as these are present in higher numbers.

18.7.2 Colonization Inhibition

The prebiotic compounds also increase the production of short-chain fatty acids such as butyrate and propionate, which could induce the higher expression of genes for mucin production and hence provide a greater physical barrier in the colonization of pathogens on colonic cells besides being the readily available source for colonocytes (Willemsen et al. 2003). SCFA also impacts the virulence of pathogens such as *Salmonella* through the downregulation of genes encoding for invasion in the pathogen (Van Immerseel et al. 2006). Another important pathogenic mechanism for adherence of pathogens such as *Salmonella*, *Vibrio*, and *E. coli* is the type 1 fimbriae which are mannose-specific lectins (Thomas et al. 2004). The prebiotic molecules such as MOS can inhibit this adherence phenomenon through binding to these lectins leading to blockage or nonavailability of binding sites for these pathogens (Baurhoo et al. 2007).

18.7.3 Healthy Gut Maintenance

Prebiotic is known to improve gut health in a variety of ways. The use of prebiotic improves the colonic length as well as various cells in the colonic microenvironment (Rehman et al. 2007; Xu et al. 2003). The goblet cell population and villi density are increased, due to which the production of mucin and absorption of nutrients from the intestine are improved (Johansson et al. 2008).

18.7.4 Immunomodulatory Effects

The basic effect of prebiotic compounds is increased bacterial density of microorganisms such as *Bifidobacterium* and *Lactobacillus*. These microorganisms modulate the expression of various intestinal genes and immune cells within the immediate environment. These include the expression of genes related to antimicrobial peptides and cytokines (Vamanu and Vamanu 2010; Vinolo et al. 2011). Thus, the impact of these expressions result in better immune clearance through phagocytosis, neutralization of various oxidative stress, and prevention of inflammation (Slawinska et al. 2019; Babu et al. 2012).

18.8 Prebiotics Classified to Be Used as Antimicrobial Agents

Prebiotics are generally obtained from plant or animal products and consist of oligomeric units of carbohydrate molecules. Depending on the number of units of monomers, the prebiotics can be monomeric, oligomeric, or polymeric. One of the important characteristics of prebiotic is that a specialized group of microorganisms strictly ferments it. Table 18.1 contains the list of prebiotic compounds which are used in human and poultry nutrition.

18.9 Synbiotics

Synbiotics are another interesting alternative for antibiotic uses, most importantly in the case of poultry farms. Synbiotics comprise a prebiotic fiber such as fructooligosaccharides and a probiotic strain such as *Bifidobacterium*, which grows better as the prebiotic fiber is easily fermented and is readily available to the probiotic organism resulting in advantages to the health of the host. The synbiotic (combination of prebiotic and probiotic) seems one the most promising alternative for uses in hosts as the combination could impact wide mechanisms to restore health benefits (Gourbeyre et al. 2011). However, there needs to be a careful investigation in selecting the combination of prebiotic and probiotic combination. Some of the prebiotic compounds that have been used in synbiotic formulations are FOS, GOS, XOS, and inulin, while probiotics belong to species of *Lactobacillus*, *Bifidobacterium*, *Bacillus*, and *Saccharomyces* genera.

18.10 Mechanism of Action of Synbiotics

As synbiotic consists of both prebiotic compound and probiotic organism, synbiotic's possible mechanism of action becomes even more complex. It employs the mechanisms of both probiotic and prebiotic independently and in combination to maintain a healthy cellular environment within the host. The overall phenomenon that is most likely to be observed is the maintenance of a healthy gut microenvironment through competitive inhibition; production of specific compounds such as SCFA, organic acids, etc.; and modulation of the immune system for better cellular growth and elimination of pathogens (Gourbeyre et al. 2011; Rastall et al. 2005). These mechanisms have been discussed in detail in sections on probiotics and prebiotics.

18.11 Synbiotics Classified to Be Used in Hosts

The synbiotic formulation is a combination of prebiotic and probiotic organisms that can act independently or combinatorially to provide health benefits to host cells. Table 18.1 contains the list of probiotics that have been administered in humans and poultry for this purpose.

18.12 Present Status of the Clinical Status of Probiotics, Prebiotics, and Synbiotics

This section will discuss some of the studies that suggest the clinical uses of prebiotics, probiotics, and synbiotics as alternatives for antibiotic-treated diseases. There are several diseases caused by bacterial infections in which the first choice of treatment is antibiotics. These antibiotics often have harmful effects on the gut microbiome and lead to diarrhea, referred to as antibiotic-associated diarrhea. The recent studies from various groups suggest that the use of probiotic preparations of *Bacillus*, *Streptococcus*, *Lactococcus*, *Bifidobacterium*, and *Leuconostoc* either singly or in combinations helps to overcome the problems of antibiotic-associated diarrhea (Esposito et al. 2018; Alberda et al. 2018; Blaabjerg et al. 2017; Syngai et al. 2016). Apart from antibiotic-associated diarrhea, the traveller's diarrhea, irritable bowel syndrome, inflammatory bowel disease, urinary tract infections, and infections from *Helicobacter pylori* and cariogenic bacteria are benefited from the use of probiotics. Traveller's diarrhea which is often caused by Gram-negative multiresistant pathogens, such as *Escherichia coli*, *Shigella*, and *Salmonella*, had been shown to be a better outcome if probiotics such as *Saccharomyces boulardii* and *Lactobacillus* were used in the treatment (Bae 2018; Sniffen et al. 2018). IBD is one of the most studied diseases where probiotic treatments are being advocated as this is often the result of microbiome disturbance within host leading to several complications and adverse effects. The multi-combinatorial recipes of several probiotics such as *VSL#3*, *E. coli Nissle 1917*, *Bifidobacterium*, *Lactobacillus*, *Streptococcus*, and *Lactococcus* have been shown to have beneficial effects by modulating immune responses as well as microbiome restoration in IBD (Guandalini and Sansotta 2019; Jia et al. 2018; Hod et al. 2018; Jonkers et al. 2012). *Helicobacter pylori* is a chronic pathogen that causes various gastric diseases, and associated malignancies is often treated by triple antibiotic treatment but reemerge. The probiotic strains such as *Saccharomyces boulardii* and *Lactobacillus* have shown some benefits over the traditional treatment (Homan and Orel 2015; Sheu et al. 2006). Another infection that tends to be more recurrent is urinary tract infections. The recurrent infections are often due to multiresistant pathogens such as *Escherichia coli*, *Klebsiella*, and *Proteus*. As *Lactobacilli* are the normal inhabitant of the urinary tract, several *Lactobacilli* and *Bifidobacterium* were found to be useful in decreasing the rate of infections (Sadeghi-Bojd et al. 2020; Wolff et al. 2019; Grin et al. 2013).

Another most common form of infection is dental caries. These are often treated with antibiotics, and alternatives in treatment would be beneficial in many aspects. The probiotic strains of *Lactobacillus* and *Bifidobacterium* and some of the normal oral inhabitants such as *Streptococci oligofermentans* and *Streptococci salivarius* have been shown to be beneficial. They are advised to be used as probiotics in treating these infections (Humphreys and McBain 2019; Schwendicke et al. 2017; Baca-Castanon et al. 2015; Samot and Badet 2013). Prebiotics have been used in most animal-based studies. Some of the examples where prebiotics and synbiotic have also been shown to have a beneficial impact on antibiotic-treated abnormalities include necrotizing enterocolitis, antibiotic-associated diarrhea, and irritable bowel syndrome (Guridi et al. 2020; Ford et al. 2018; Nandhini et al. 2016; Pallav et al. 2014).

18.13 Future Prospects

With the current advancement in characterization methods and technological development, the scientific arena of prebiotic, probiotic, and synbiotic needs to be expanded into system-level understanding. There is a need for studies to concentrate on specific areas such as safety, efficacy, delivery, and mechanistic details of each component that could deliver their advantageous effects. Currently, it appears to be a challenging task as the treatment doses, duration, and systemic impact on interpersonal health are not yet fully understood. When administered, the use of multi-omics-based technologies and a systemic approach in understanding the roles of effector molecules of probiotics and synbiotics needs to be pinpointed. Also, using these agents in conjunction with the traditional therapeutic agents needs to be even more explored. Nevertheless, the uses of these agents as an alternative to antibiotics will be a promising and exciting field to be examined for the upcoming decades as these provide one of the most efficient and indigenous substitutes for antibiotics.

18.14 Conclusion

In the present global increase in antibiotic resistance, it is quite evident that the total ban on antibiotics would result in significant economic and health-associated losses in both human and animal care. So for sustainable environmental and human safety, alternatives such as probiotics, prebiotics, and synbiotics offer one of the most efficient replacements of antibiotic uses. However, many studies still need to elaborate on the safety concerns, multilevel species organization, and system-wide implications of these agents in humans and animals. Overall, the probiotics, prebiotics, and the recent advent of synbiotics remain popular and natural alternatives to antibiotics for future research and development as therapeutics.

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Conflict of Interest None to declare.

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Chapter 19
The Implication of Antimicrobial Peptides Against Bacteria and Their Clinical Aspects

Suhrid Ranjan Dutta and Keshab Chandra Mondal

Abstract Antimicrobial peptides (AMPs), also designated as the host's protective peptide molecule, are short and usually positively charged peptides found extensively in life forms in this globe which range from microorganisms to humans. The continuous and rapid manifestation of multidrug-resistant bacteria is nowadays an alarming threat to human beings and becomes a global problem. In this scenario, AMPs have recently drawn substantial significance as impending preferences in respect to usual antibiotics having an undeniable wide range of antimicrobial activity. More than 3283 antimicrobial peptides (AMPs) have been acknowledged to date according to the antimicrobial peptide database (aps.unmc.edu), and among them (3036 AMPs) are directly acting in opposition against numerous bacteria. Several antimicrobial peptides have got an endorsement by the US Food and Drug Administration (FDA). About a few decades ago, such peptides have drawn considerable implications among scientists, health professionals, and pharmaceutical companies as therapeutics. Besides having antimicrobial efficacy, most AMPs even modulate the immunological synchronization of the host. Interestingly, the majority of AMPs are identified in soilborne gram-positive bacteria and the cutaneous secretion or body fluids of various animals. Furthermore, AMPs exhibit high selectivity, strong efficacy, decreased drug interaction, lower toxicity, and biological diversity. In this chapter, we provide an insight into the classification, biological role, mode of action, and clinical applications along with the prospects of antimicrobial peptides especially useful against bacteria.

Keywords Antimicrobial peptides · Classification · Clinical application · Future medicine as peptides · Molecular targets of antimicrobial peptides · Lipopolysaccharides · Pathogen-associated molecular patterns · Human host defense

S. R. Dutta
Department of Microbiology, Midnapore College (Autonomous), Midnapore, West Bengal, India

K. C. Mondal (✉)
Department of Microbiology, Vidyasagar University, Midnapore, West Bengal, India
e-mail: kcmondal@mail.vidyasagar.ac.in

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peptides · B-Defensin · Biofilms · Ambicin (Nisin) · Polymixin B · Gramicidin S · Parasitic diseases · Anti-gram-negative activity · Cecropin · Pyrrhocoricin · Diptercin · α-Defensins

Abbreviations

AMPs	Antimicrobial peptides
APD	Antimicrobial peptide database
LPS	Lipopolysaccharides
PAMP	Pathogen-associated molecular patterns
STAMPs	Selectively targeted AMPs
WHO	World Health Organization

19.1 Introduction

All living creatures on this planet earth possess a self-defense system to sustain themselves against various odds in a multitudinous environment. Antimicrobial peptide represents one of the most important defense molecules that has evolved in the late Silurian age and still exists today and is found in live creatures including bacteria and humans. In invertebrates, AMPs are the most effective and principal components of the inherent immune system in contrast to the adaptive immune system of vertebrates where it acts as a modulator. The first identified AMP is gramicidin, which has a detrimental effect on gram-positive bacteria, was identified in the year of 1939 from *Bacillus brevis*, and has an immense therapeutic implication at present also. In the exploration of AMP, groundbreaking research was performed by Boman and his team in 1981 by discovering cationic AMP from the *Hyalophora cecropia* moth. About 3036 peptides with known antibacterial functions are reported in the peptide database (APD) (https://aps.unmc.edu/). At the end of 1960, Alexander Fleming discovered lysozyme, and few people accepted this as the first reported AMP. Later on, Fleming discovered penicillin with potent antimicrobial activity and shared the noble prize in 1940 along with two other scientists Howard Florey and Ernst Chain, who brought the therapeutic uses of penicillin and streptomycin front to the people in the year of 1943. This discovery threw back the essentiality of lysozyme in the golden era of antibiotics (Gaynes 2017). Later on, purothionin from wheat was identified in 1942, an effectual growth inhibitor of *Ralstonia solanacearum* and *Xanthomonas campestris*, also detected across all plant kingdoms (de Caleya et al. 1972). Coming out of drug-resistant bacteria is reported in early 1960 which elevated the interest in looking into the host defense molecules and their actions. In this context, two studies were very important in the discovery of AMP: One is lactoferrin from milk, and another is a small molecular antimicrobial peptide

in the hemolymph of wax moth challenged with *Pseudomonas aeruginosa* (Sanchez et al. 1992; Brown et al. 2009).

Meanwhile, from 1970 to 1980, several researchers have reported various α-defensins in humans and rabbits. But the groundbreaking research carried out by Hultmark et al. in 1980 on bacteria injected silkmoth *H. cecropia* and and two inducible antimicrobial peptides were observed, namely, P9A and P9B present in the hemolymph in this insect having anti-gram-negative activity. Immediate to this discovery people have discovered cecropin (Hultmark et al. 1980). The detection of cationic AMP, magainin, is another remarkable finding by Zasloff 1987 from clawed frog *Xenopus laevis* (Zasloff 1987).

Numerous AMPs are explored to date, and approximately 50% of them are reported from invertebrates, specifically from insects. Based on sequence and three-dimensional structural characteristics, these peptides are categorized into three major classes: (1) linear peptides forming α-helices without having cysteine residues, (2) cyclic peptides containing cysteine residues, and (3) proline- or glycine-enriched peptides (Yi et al. 2014). AMPs are approaching to replace conventional drugs and may gain an important market share globally (Silveira et al. 2021). The collegial action of AMPs with traditional antibiotics and their wide spectrum of activities with rarer resistance development likelihood make AMP an attractive candidate for future medicine, especially for microbial diseases and immunomodulation purposes (Mahlapuu et al. 2020).

Pathogenic microbes are accountable for thousands of deadly infectious diseases in different animals including human beings. In accordance to the World Health Organization (WHO), it is truly alarming throughout the globe that infectious diseases take the lives of over 17 million people every year (Bloom and Cadarette 2019). The majority of the infections can be controlled in their initial state by the foremost weapons of the immune system of the respective host, termed innate immunity, which works while the host's protected system is overwhelmed by the enormous number of highly pathogenic microbial invaders having proficient virulence mechanisms. The host's instinctive defending system is comprised of several soluble effector molecules, of which antimicrobial peptides are notable, which act against intruder microbes (Diamond et al. 2009).

In mammals, AMPs are remaining within granules of neutrophils and also in the secretions of epithelial cells which cover the mucosal surfaces and skin (Hancock and Chapple 1999; Boman 1995). Mostly, AMPs are encoded in a cluster and expressed cumulatively resulting in the accumulation of multiple AMPs at a single site (Lai and Gallo 2009). Remarkably, several AMPs are formed as inactive naive peptides which require proteolysis for becoming active peptides (Bals 2000). Therefore their fate not only is dependent on their expression but also necessitates some suitable proteases (Lai and Gallo 2009). In the case of multicellular organisms, some AMPs are articulated constitutively and stored as inactive precursors in a high concentration within the intracellular granules. Such AMPs are released locally upon infection and reach the inflammation sites quickly. On the other hand, the expression of other AMPs is tempted in response to pathogen-associated molecular patterns (PAMPs) or fungal infections or by employing cytokines (Lai and Gallo

2009; Hancock and Diamond 2000). This book chapter extensively represents the origin, structural characteristics, molecular targets, functional mechanism, and clinical promises and prospects of AMPs especially effective on bacteria for providing the comprehensive contemporary knowledge and understanding of AMPs.

19.2 Classification of Antimicrobial Peptides

There are multiplicity approaches for classifying AMPs. A number of these methods are summarized on the classification page of the APD website (aps.unmc.edu/AP/class.php). In these databases, 3033 antimicrobial peptides are reported effective against bacteria, their biofilm, MRSA (methicillin-resistant *Staphylococcus aureus*), and tubercle bacilli only (Fig. 19.1). For instance, the peptides may be classified based on the biosynthesis machinery. Some peptides are created through multiple enzyme systems, whereas most of the AMPs are coded by gene(s). The expression and lysis of gene-encoded AMPs are regulated sophisticatedly as overexpression of AMPs is unsafe. Furthermore, AMPs can be classified on basis of their molecular targets (e.g., membrane-targeting and cell-penetrating peptides) (Pirtskhalava et al. 2021). G. Wang has described the numerous ways to classify AMPs in the years 2015 and 2017 as follows.

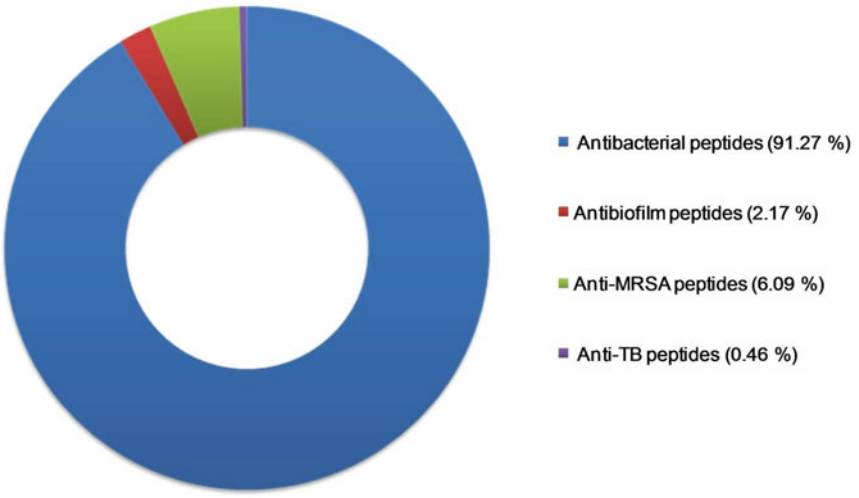

Fig. 19.1 The doughnut plot represents the distribution of all kinds of antibacterial peptides only effective against bacteria (91.27%), bacterial biofilm (2.17%,), MRSA (6.09%), and tubercle bacilli (0.60%)

19.2.1 Classification of AMPs Based on Biosynthetic Machines

AMPs can either be produced by ribosomes from mRNA or by nonribosomal synthetic pathways (Hancock and Chapple 1999). Cecropin, pyrrhocoricin, diptericin, cathelicidin LL37, and human β-defensin are all examples of gene-encoded AMPs (Wu et al. 2018; Wang et al. 2015). A major proportion of nonribosomally synthesized AMPs are usually produced by bacteria in contrast to genetically encoded AMP, an ancient defense molecule of all life forms (Hancock and Chapple 1999). The secondary metabolites of bacteria from nonribosomal origin like polymyxins, gramicidin S, daptomycin, bleomycin, and bacitracin are well-known antibiotics (Hancock 2000; Hancock and Chapple 1999). Peptides articulated by nonribosomal means are very common, and many of them are in use for medicinal purposes compared to ribosomally synthesized AMPs whose function in innate immunity and therapeutic capabilities in the future have been recognized recently.

19.2.2 Based on Biological Sources

In the updated antimicrobial peptide database (APD) in the year 2021, the presence of 3273 antimicrobial peptides is reported to date from six kingdoms (369 bacteriocins/peptide antibiotics from bacteria, 5 from archaea, 8 from protists, 22 from fungi, 361 from plants, and 2424 from animals, including some synthetic peptides. Thus, the widely held AMPs (approximately 80%) originate from animals, especially from amphibians and insects, which is nearing 50% (Wang et al. 2016).

Animal AMPs can additionally be divided in accordance to their sources such as insect AMPs, amphibian AMPs, fish AMPs, reptile AMPs, and mammalian AMPs. The major and well-studied AMP families in the animal kingdom are cathelicidins, defensins, and histatins. A giant share (1127 peptides from amphibians) of antimicrobial peptides has been identified and reported in amphibians. Antimicrobial peptides of amphibians are crucial for their protection against bacteria and important for their striving adaptation in pathogen-enriched niches (Zasloff 1987); Mangoni and Casciaro 2020). In comparison with toads, frogs are also the principal resource of amphibian AMPs of which magainin has gained extreme popularity and was isolated from the epidermal discharging of numerous frog genera like *Xenopus*, *Silurana*, *Hymenochirus*, and *Pseudhymenochirus* (Aisenbrey et al. 2020; Conlon and Mechkarska 2014). Furthermore, cancrin was reported as the first AMP of *Rana cancrivora*, a sea amphibian (Lu et al. 2008). This indicates a much-enriched repertoire of AMPs of amphibians.

19.2.3 Insect's Antimicrobial Peptides

Insects are very copious and one of the largest sources of AMPs that are well-thought-out as nonresponsive to natural bacterial resistance (Wu et al. 2018; Dutta et al. 2017). To date 325 functionally active insect AMPs have been reported in the APD database. The primary site of the synthesis of antimicrobial peptides of insects is fat body tissue and hemocytes, which can contribute to strong adaptability against pathogenic threats (Vilcinskas 2013). The cecropin is the first and eminent antimicrobial peptide of insect, namely, *Hyalophora cecropia*, discovered in 1980 (Hultmark et al. 1980). Cecropins and their homologs are found in *Drosophila*, silkworm, and bees also (Feng et al. 2020).

The mainstream insect AMPs, such as insect cecropins, lebocin, defensins, proline-rich peptides, and attacins, have been found in various orders of insects, effective against numerous gram-positive and gram-negative bacteria. Specifically, peptide fraction II, moricin, and gloverin have been identified only in *Lepidoptera* (Dutta et al. 2016; Yi et al. 2014). Functional gloverins and attacins are large antimicrobial proteins having a higher molecular weight which ranges from 14 to 20 kDa. Cecropin, attacin, defensin, drosomycin, diptericin, drosocin, and metchnikowin have been identified in *Drosophila melanogaster* (Hanson et al. 2019). These classes of antimicrobial peptides' synthetic pathways also have been revealed at the genetic level (de Gregorio 2002). *Drosophila* AMP genes by the Toll and immune deficiency (IMD) signaling pathways have also been well documented (Tanji et al. 2007). Review on AMPs is abundant, but very few reviews are available on insect AMPs (Wu et al. 2018; Li et al. 2006; Imler and Bulet 2005). Numerous literature related to insect AMPs are mainly from *Drosophila* and *Manduca sexta*, which mainly states about the activation of AMPs in response to various infection and their genetic regulation through Toll and IMD signaling pathways (Cao et al. 2015; Fullaondo and Lee 2012). Surprisingly, the pea aphid (*Acyrthosiphon pisum*) does not have any antimicrobial peptides (Shelomi et al. 2020). Jellein, a peptide imitative to the royal jelly of bees, exhibits potential negative effects against several pathogenic bacteria (Zahedifard et al. 2020).

19.2.4 Antimicrobial Peptides from Microorganisms

Some of the well-known antimicrobial peptides, namely, nisin, gramicidin, polymyxin, colicin, and mupirocin have therapeutic implications articulated by bacteria and fungi mainly. Some famous peptides like nisin and gramicidin from *Lactococcus lactis*, *Bacillus subtilis*, *Bacillus brevis*, *Bacillus polymyxa*, and *Pseudomonas fluorescens* are also reported (Micenkova et al. 2019; Cao et al. 2018; Thomas et al. 2010). The chemical synthesis of AMPs is costly; hence the recombinant or biological means of expression has become attractive. The choice of the organism for the biological synthesis of insect-derived AMPs are mainly *Escherichia coli*,

B. subtilis, Pichia pastoris, and *Saccharomyces cerevisiae* (Parachin et al. 2012). Due to the toxicity, proteolytic degradation, and purification, AMPs are tough to produce in *Escherichia coli* and need to produce with fusion tag partners of other proteins (Yu et al. 2015).

Moreover, antimicrobial peptides like defensins, thionins, and snakins have also been identified in the stems, seeds, and leaves of various plants (Tang et al. 2018). Marine microbes especially *Myxococcus fulvus* and *Vibrio* sp. and invertebrates like lugworm *Arenicola marina* are also an enormous source of ribosomally and nonribosomally antimicrobial peptides like andrimid, myxothiazol, and NZ17074 (N1) (Komal 2021; Semreen et al. 2018). The efficacy of these marine AMPs has earlier been tested in the laboratory, and few of them have shown their activities in vivo conditions, for example, As-CATH4 triggers immunity in vivo and can heighten the anti-infective ability of medications utilized in amalgamation with it (van Harten et al. 2018). Myticusin-β is an immunity-related AMP of *Mytilus coruscus* and a hopeful substitute for antibiotics (Oh et al. 2020). Additionally, epinecidin-1, an extremely powerful antimicrobial peptide, has detrimental action against *Staphylococcus aureus* and also acts in opposition against *Helicobacter pylori* with wound healing properties (Neshani et al. 2019).

19.2.5 Antimicrobial Peptides of Mammals

Human host defense peptides (HDPs) can defend human beings from microbial infections with different levels of expression in every stage of human growth. Granules of neutrophils and the secretions from epithelial cells are the primary sources of AMPs in mammals (Hancock and Chapple 1999; Boman 1995). In many cases, AMP genes remain in a cluster, and consecutive co-expression happens, resulting in an accumulation of multiple AMPs (Mahlapuu et al. 2016). Remarkably, many AMPs in mammals are produced as inactive originator peptides or proteins requiring proteolytic lysis to turn into active (Bals 2000). Their regulation is therefore dependent not only on their expression but also on the abundance of appropriate proteases (Lai and Gallo 2009). The expression of these peptides is triggered in response to pathogen-associated molecular patterns (PAMPs) or cytokines (Lai and Gallo 2009; Hancock and Diamond 2000). Cathelicidin LL-37, a famous AMP derived from the human body, is usually found in the infant's skin, whereas human β-defensin 2 (hBD-2) is frequently articulated in the late ages (Gschwandtner et al. 2014). HDPs are usually found in many parts of the body such as the skin, eyes, ears, mouth, respiratory tract, lung, intestine, and urethra. Besides, AMPs such as lactoferrin in human breast milk also play an important role in controlling various pathogens in infants (Field 2005).

19.3 Generalized Mechanism of Action of AMPs

19.3.1 Transmembrane Pore Models

The killing action of AMPs of bacteria through poration requires three principal steps: attachment to the bacterial membrane, accumulation within the membrane, and the formation of channels. The channel formation leads to the seepage of cytosolic materials followed by cell death. An AMP has to cross the negatively charged lipopolysaccharides (LPS) of gram-negative bacteria, or murine layer accompanied by acidic polysaccharides of gram-positive bacteria (Hancock 1997). Furthermore, human neutrophil peptide 1 (HNP-1) plays a significant task in the membrane perforation of *E. coli*, indicating the microbial metabolic activity associated with the poration process (Lehrer et al. 1989; Lichtenstein et al. 1988). However, mainly three well-known models of perforation are barrel-stave pore (Ehrenstein and Lecar 1977), toroidal pore (Brogden 2005), and the carpet model mediated poration (Pouny et al. 1992) as described below (Fig. 19.2).

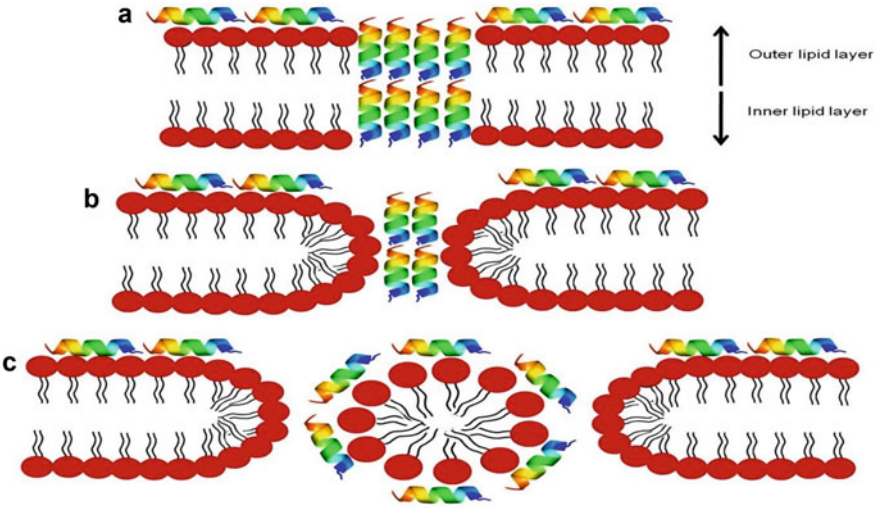

Fig. 19.2 Schematic outline of the possible interactions of the antimicrobial peptide with the bacterial membrane (lipid bilayer) followed by membrane permeabilization (**a-c**). The polar (phospholipid) head group is linked to black colored fatty acyl chain, whereas peptides are shown as alpha-helix. (**a**) Insertion of peptide molecules perpendicularly into the lipid bilayer plane is typically found in the case of the "barrel-stave" state of membrane attacking mechanism. (**b**) In the "toroidal pore" model, peptides are inserted vertically into the plane of the bilayer, and the interactions are established between the hydrophilic portions of the peptides with the polar head groups. The hydrophobic regions remain within the core of the lipid bilayer. (**c**) The "carpet" model proposes that the peptides remain associated with the lipid bilayer as aggregates and the interaction of hydrophilic regions of peptides is often found with the lipid bilayer as a "carpet" like way

19.3.2 Barrel-Stave Pore Model

In the barrel-stave pore model, AMP is structured as dimers or multimers upon adsorbing with the negatively charged bacterial membrane. The peptide assemblage is crucial for poration (Ben-Efraim and Shai 1997). AMP multimers traverse the bacterial cell membrane by penetrating its hydrophobic tail into the lipid bilayer while its hydrophilic part faces toward the pore's lumen. The amassed peptides thus form a barrel-like conduit similar to staves (Yang et al. 2001).

19.3.3 Toroidal Pore Model

The toroidal pore model shares common characteristics with the barrel-stave model of membrane perforation mechanistically, but the AMP forms a monolayer and connects the outer and inner lipid bilayers of the pore (Mor and Nicolas 1994).

19.3.4 Carpet Model

This model describes that peptides first wrap the outer membrane similar to a carpet and then act like detergents followed by the disruption of the lipid bilayer after attainment of a critical concentration. In this situation, the optimum concentration can be reached when the membrane surface is fully or locally enclosed with AMPs and pores are created from micelle-like units (Oren and Shai 1998).

19.3.5 Molecular Electroporation

Some cationic AMP peptides can craft electrostatic charges across the lipid bilayer which is enough for pore generation, and this phenomenon is mainly accredited due to the high content of positively charged amino acid residues (Chan et al. 2006). AMPs make transitory pores which are lethal for microbes.

19.3.6 Sinking Raft Model

Amphipathic peptides are responsible for disparity and shrinkages in the lipid membrane structure, and they may make transitory pores which are lethal for microbes (Dawson and Liu 2008).

19.4 Categorization of AMPs Based on Their Properties

AMPs can be classified on basis of peptide properties like charge, hydrophobicity, amino acid composition, and length (Lei et al. 2019). Based on amino acid composition, numerous AMPs are enriched with specific amino acids. Examples are glycine-rich, tryptophan-rich, histidine-rich, proline-rich, arginine-rich, lysine-rich, aspartic acid-rich, and Ala-rich AMPs (Mishra et al. 2018). Additionally, based on net charge, there are cationic, neutral, and anionic AMPs that exist. On the nature of hydrophobicity, AMPs can be categorized mainly as amphipathic or amphiphilic, hydrophobic, and hydrophilic (Lei et al. 2019). However, natural AMPs are usually more than 15 amino acids in length, even though active synthetic peptides are less than ten residues long and have also been reported (Strom et al. 2003). The synthesis of short peptides is cost-friendly; shorter peptides typically have reduced antimicrobial efficacies in contrast to longer AMPs. Notably, AMPs having 100 amino acids are called antimicrobial proteins like lysozyme, histones, attacins, gloverins, and RNase 7 (Wang 2014).

19.4.1 Categorization of AMPs on Basis of 3D Structure

According to the antimicrobial peptide database (APD), only a little population of AMPs (13%) has a known three-dimensional structure, determined through nuclear magnetic resonance (NMR) spectroscopy or crystallography (Wang and Wang 2004). In addition, the X-ray diffraction study is useful to solve the structures of some AMPs with a folded conformation in the solvent. The structural information is well explained in the APD database, including structural class, the method for structural determination, structural regions, key residues, and membrane-mimetic models for structural determination (Wang et al. 2016). Antimicrobial peptides are mainly classified into four major groups according to their three-dimensional (3D) structure which includes α, β, αβ, and non-αβ peptides (Bulet and Stocklin 2005; Huan et al. 2020) (Fig. 19.3). The AMP family consists of α-helical structures (e.g., magainins and LL-37) which are well known for their multidimensional spectrum of activity. Cecropin is a well-known AMP that also has an α-helical structure (Fu et al. 2004). This family is the largest consisting of 328 entries, while the non-αβ family is the smallest with nine entries (Wang et al. 2016). The β family of AMPs is only composed of β-strands (e.g., human β-defensins). And the αβ family of AMPs consists of both α-helical and β-strands in their 3D structure (e.g., β-defensins). However, the non-αβ family contains neither helical nor β-strands (e.g., indolicidin) (Huan et al. 2020; Wang 2014).

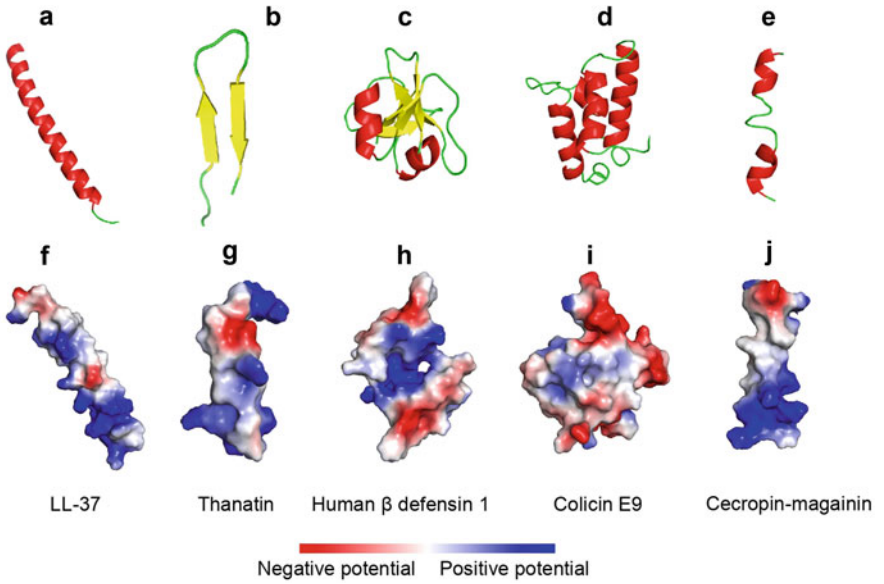

Fig. 19.3 The secondary structures (**a-e**) and respective surface electrostatic potentials (**f-j**) of various antimicrobial peptides, (**a**) LL-37 (PDB: 5NMN), (**b**) thanatin (PDB: 5X03), (**c**) human β defensin 1 (PDB:1IJV), (**d**) colicin E9 (1IMQ), and cecropin-magainin hybrid (**e**) are shown here. The α-helix and β-sheet are in red and yellow color respectively. Surface structure representations of negatively and positively charged residues of the stated antimicrobial peptides are indicated by blue and red color respectively. All of these structural presentations are generated and viewed in PyMol from their PDB file

19.5 Categorization of AMPs Based on Molecular Targets

The effects of AMPs on bacteria are mainly either bactericidal or bacteriostatic (Cardoso et al. 2021). These functions are solely dependent on the interaction of AMPs with the cell wall/cell membrane externally or by the interaction with intracellular materials upon internalization of AMPs (Mookherjee et al. 2020; Li et al. 2017). Therefore, the interactions of AMPs especially happen at the molecular level in various microorganisms mainly rely on the array of targets represented by the microorganism like gram-positive and gram-negative bacteria (Malanovic and Lohner 2016). AMPs can roughly be classified into two categories: cell surface targeting peptides (e.g., nisins and temporins) and intracellular molecule targeting peptides (e.g., Pro-rich peptides). The cell surface attacking peptide includes both membrane-selective and non-membrane-targeting peptides and can further be divided on basis of specific targets such as cell wall or cell wall adherent carbohydrates, lipids, and cell membrane-bound proteins/ligands. Equally, intracellular active AMPs target mainly cytosolic proteins, DNA, and RNA (Shah and Chen 2021). Remarkably, it is established that unlike the single target of antibiotics, AMPs may target multiple intracellular molecules and exert a multi-target mechanism of

action (Shah et al. 2016), Almost one-third of such peptides either interrupt the synthesis of the bacterial murine layer or directly disrupt the bacterial membrane by their insignificant resistance development propensities (Kennedy and Read 2018).

19.5.1 AMPs Acting on the Synthesis of the Cell Wall of Bacteria

It is well known that AMPs necessitate dispersing transversely in the peptidoglycan layer before exerting their activity (Li et al. 2017). HNP1 was reported to enter the outer and inner membranes of *E. coli* and decline the synthesis of DNA, RNA, and protein (Zhang et al. 2021). Particularly, the permeabilization of the inner membrane is crucial for exhibiting the lethal effects of AMPs. The antibacterial attributes of cycloserine happen by hindering the activity of alanine racemase and D-Ala-D-Ala ligase, which is responsible for the amalgamation of D-Ala-D-Ala dipeptide of lipid II of the peptidoglycan precursor (de Leeuw et al. 2010). Teixobactin inhibits the synthesis of the bacterial cell wall by attaching to the highly conserved motif of lipid II and III, which are active precursors of teichoic acid of the cell wall (Jin et al. 2016). The D-form KLKLLLLLKLK-NH2 peptide is known to improve the membrane-penetrating capabilities of *S. aureus* by amalgamating with cell wall components specifically (including peptidoglycan) and results in higher antibacterial activity in comparison to its L isomer (Manabe and Kawasaki 2017).

19.5.2 Interaction with Bacterial Membrane

Many AMPs display a direct and rapid antimicrobial activity by disrupting the physical coordination of the microbial membrane by translocation across the membrane into the cytosol of the bacteria prior to targeting several intracellular organelles (Hancock and Sahl 2006). It is widely accepted that the membrane interaction is a key factor for exerting direct antimicrobial activity of AMPs (Malmsten 2016; Nguyen et al. 2011; Yeung et al. 2011; Jenssen et al. 2006). The earliest AMP, cecropin A, forms holes in the bacterial membrane of uropathogenic *E. coli* which can easily build up biofilms (Kalsy et al. 2020). Peptide fraction II of *Antheraea mylitta* is reported to kill *E. coli* very effectively as well by aggregating with its membrane by forming a porthole-like structure (Dutta et al. 2016). In these circumstances, the electrostatic attractions within the hydrophobic tail containing cationic AMPs and negatively charged peripheral portion of the bacteria are fundamental determinants for such kind of interactions (Ebenhan et al. 2014; Yeung et al. 2011; Giuliani et al. 2007; Yeaman and Yount 2003). Bacteria are commonly divided into gram-positive and gram-negative following their peptidoglycan content. In gram-positive bacteria, the cytosolic membrane is encircled by a thick peptidoglycan layer

in contrast to gram-negative bacteria, surrounded by a thin peptidoglycan coating (Lin and Weibel 2016). The cytoplasmic membranes of both gram-positive and gram-negative bacteria have profuse contents of phospholipids, phosphatidylglycerol, cardiolipin, and phosphatidylserine, which have negatively charged head groups and therefore attract positively charged AMPs more efficiently (Ebenhan et al. 2014; Yeaman and Yount 2003). Additional electronegative charge to the bacterial surface is also contributed by teichoic acids in the cell wall of gram-positive bacteria and lipopolysaccharides (LPS) in the outer membrane of gram-negative bacteria (Ebenhan et al. 2014; Lai and Gallo 2009). Notably, bacterial cells have an inside-negative transmembrane potential between −130 and −150 mV typically in contrast to mammalian cells which bear the potential ranging from −90 to −110 mV (Ebenhan et al. 2014; Matsuzaki 2009; Yeaman and Yount 2003). The strong negative membrane potential in bacteria contributes to the selectivity of AMPs plausibly for exhibiting their action (Bechinger and Gorr 2016).

19.5.3 Inhibition of Nucleic Acid Biosynthesis

Antimicrobial peptides exert their action by inhibiting some key enzymes or inducing the degradation of the nucleic acid of bacteria and thereby inhibiting the biosynthesis of nucleic acid. Buforins were reported as a DNA inhibitor for the first time and was isolated from the stomach of *Bufo bufo gargarizans* (Park et al. 1996). It has a high affinity for DNA binding and acts as a detrimental agent AMP plausibly inhibiting DNA or RNA metabolism of *E. coli* (Park et al. 1998). Indolicidin, a cathelicidin class of AMP, particularly interacts with the purine or pyrimidine bases of DNA and triggers cross-linking of single- or double-stranded DNA along with it downregulating the function of topoisomerase I but does not inhibit RNA (Subbalakshmi and Sitaram 1998). Pleurocidin also inhibits the synthesis of DNA and RNA by lowering the intake of histidine, thymidine, and uridine (Patrzykat et al. 2002). HNP-1 and pyrrhocoricin can make DNA or RNA biosynthesis and inhibit DnaK (Kragol et al. 2001; Park et al. 1998). The defensins from ostrich, ostricacin-1 (Osp-1) and ostricacin-2 (Osp-2) have the capability of binding and inhibiting the DNA of *E. coli* (Sugiarto and Yu 2007). Similarly, tachyplesin from horseshoe crab can bind DNA also and inhibit its synthesis (Nakamura et al. 1988). Therefore, all categories of Enterobacteria-derived microcin inhibit topoisomerase II of bacteria and hinder the process of making negative supercoiling, a crucial factor of replication (Moreno et al. 1995). 1-1TC24, a tissue factor pathway inhibitor, which is an AMP from tongues, enters the cytoplasm of target cells after the rupture of the cell membrane and then degrades DNA and RNA (He et al. 2017). Lactoferricin B (LfcinB) has multiple molecular targets at its sublethal dose. DNA and RNA syntheses become decreased due to LfcinB treatment in *E. coli* and *Bacillus subtilis* which trigger SOS response as well (Tu et al. 2011; Ulvatne et al. 2004). *Hermetia illucens* derived DLP2/4 has the binding and distortion aptitude toward genomic DNA of methicillin-resistant *S. aureus* and is also able to inhibit

RNA synthesis (Li et al. 2020). Coprisin induces severe damage to the genomic DNA of *E. coli* and induces apoptosis-like cell death (Choi et al. 2015).

19.5.4 AMPs Acting on Protein Synthesis

PR-39 is a cathelicidin peptide of porcine that protects the host from numerous bacterial attacks (Huang et al. 1997). Human cathelicidin also restricts the growth of invasive uropathogenic bacteria (van Harten et al. 2018). It particularly stimulates ATP leakage followed by altering membrane potential in *Bacillus globigii* and *Escherichia coli* (Veldhuizen et al. 2014). Thus it inhibits the initial translation step in bacteria (*E. coli*) and also blocks the peptide outlet tunnel in the 70S ribosome (Mardirossian et al. 2014; Gagnon et al. 2016). Besides this once becomes entered into the cytoplasm, PR-39 inhibits protein synthesis and causes the degradation of proteins required for DNA synthesis, which in turn disrupts DNA synthesis. Typically, the proline-enriched AMPs interfere with protein synthesis via binding to ribosomes (Zhang et al. 2021). Pleurocidin is usually found in winter flounder (*Pseudopleuronectes americanus*) which inhibits protein synthesis in *E. coli* (Patrzykat et al. 2002). Tur1A also inhibits bacterial protein synthesis by binding to the ribosome and blocking the transition from the initiation to the elongation phase (Mardirossian et al. 2018). Bac71-35 binds with the whole ribosome (Mardirossian et al. 2014). Surprisingly, the 70S ribosome trapping of oncocin-type peptide inhibits mRNA translation although apidaecin blocks the 50S subunit assembly (Zhang et al. 2021). Another peptide, namely, Api137, usually binds with the *E. coli* ribosomes and captures the release factor (RF1/RF2) which results in termination of the translation (Florin et al. 2017). Another study showed that the N-terminal fragments (1–25) and (1–31) of nonlytic proline-rich AMP (PrAMP) Bac5 inhibit bacterial protein synthesis by binding the tunnel of the ribosome and preventing the transition from the initial stage to the elongation stage of translation (Mardirossian et al. 2018). Besides this, pyrrhocoricin and drosocin can prevent DnaK from refolding misfolded proteins by inducing a permanent closure of the DnaK peptide-binding cavity (Wronska and Bogus 2020; Le et al. 2017; Kragol et al. 2001).

19.6 Clinical Applications

Cumulated pieces of evidence imply that the long-drawn-out usage of antibiotics effectuates the drug resistance of microbes. Various drug-resistant virulent microorganisms have been recognized to resemble each of these outmoded antibiotics and require a mixture of antimicrobial peptides (Amso and Hayouka 2019). Antimicrobial peptide-mediated healing may range from plain mouthwashes to dealing with severe bacterial sepsis. Nowadays, it is becoming substantially more difficult to

prepare a new antibiotic from natural resources. AMPs exhibited a key role as a broad antimicrobial spectrum and high bactericidal activities, and in this context, AMPs have attracted attention as budding candidates that can be extracted from natural sources and applied against antibiotic-resistant bacteria (Anaya-Lopez et al. 2012). Consequently, engendering novel clinically used antibiotics unconventionally for treating the infections triggered in the course of these pathogenic threats is indispensable (Mohammadi et al. 2017). The crucial requirement to achieve new antimicrobials has been dynamic AMP research. With swift growth in associated understanding and lead compounds, more AMPs may enter clinical tests and treatment shortly. However, the anti-infectivity property of AMP is still slowed down by several challenges including high manufacturer cost, low specificity, lack of a robust guideline for rational design, and potential toxicity to animal cells. Specified the factor such as elevated effectiveness and selectivity, the broad range of targets, lower toxicity, slower accumulation within tissues, and rare resistance development, AMPs have been considered promising anti-infective candidates, especially for polymicrobial infections as found in case of skin and soft tissue infections (SSTI) (Pfalzgraff et al. 2018; Mahlapuu et al. 2016). Static, despite the myriad of AMP-related patents filed, only a very narrow proportion of the AMPs have previously received the endorsement, either through the US Food and Drug Administration (FDA) or the European Medicines Agency (EMA), mandatory for the endorsement into the market for clinical use (Costa et al. 2019). As there are merely insufficient novel antibiotics in the pharmaceutical manufacturing pipelines, it is imperative to unearth new therapeutic classes to tackle the growing menace of multidrug-resistant microorganisms (Coates et al. 2011). The suitable extraordinary quantity of promising AMPs is invented in several patents, and fiction reports fail in the translation into clinical trials typically since the preclinical revisions are regularly executed in the absence of physiological conditions. This means that the influence of, for example, (1) physiological levels of relevant ions, (2) high ionic strength (due to high salt concentration), and (3) presence of proteases and physiological barriers are commonly disregarded (Mahlapuu et al. 2016; Fox 2013). Moreover, a few paradoxical results sometimes originated. High-throughput analysis of the AMP library has revealed AMP with poor in vitro recital is effective in vivo conditions in amounts below their bactericidal concentrations, which was attributed to their immunomodulatory properties promoting bacterial killing by stimulating the immune system of the host (Hancock and Sahl 2006). Also, variability associated with different animal models used for in vivo testing of AMPs may perhaps create results with poor translation into humans.

One of the greatest obstacles on the road to effective clinical applications of AMPs is their minor oral bioavailability. Peptides are unstable in the gastrointestinal tract due to the harsh lower gastric pH and presence of proteolytic enzymes, which is further aggravated by their poor penetration across the intestinal epithelium (Lau and Dunn 2018; Lundquist and Artursson 2016; Di 2015). Hence, the benefits that AMPs represent as a possible new class of antimicrobial agents require efficient strategies for enabling them to step out of their current almost exclusive confinement to topical applications. Some of the approaches that are being devised to tackle these challenges are subsequently being to be addressed.

The mechanistic stroke and the organization of the structure-activity relationship of these antimicrobial peptides are important for scientists to develop new cationic peptide drugs as well. For clinical usage, ambicin (nisin), polymyxin B, and gramicidin S are the prime AMPs (Dijksteel et al. 2021). Daptomycin, a cyclic lipopeptide of *Streptomyces roseosporus*, has a success rate of about 71.5% in phase IV trial when applied to the patient, and in conjunction with beta-lactam antibiotics, daptomycin affects pathogens more promptly (Ball et al. 2004; Arbeit et al. 2004). Dalbavancin, oritavancin, and telavancin are all derivatives of vancomycin and are also permitted by the FDA for oral application to treat vancomycin-resistant pathogens efficiently (Chen and Lu 2020) against vancomycin-resistant bacteria by disfiguring the structure of bacterial cell wall and membrane (Saravolatz et al. 2009; Chen et al. 2007). Moreover, telavancin and oritavancin act in opposition against skin infection microbes by disrupting lipid membrane via attaching with membrane-active peptides (MAP) of bacterial cell membranes and affecting molecular transportation through the membrane (Rosenthal et al. 2018; Higgins et al. 2005). Mupirocin is very effective and used as a topical antibiotic against skin infection or folliculitis (Bork et al. 1989). Oritavancin and cubicin are important drugs, approved by FDA (USA) and also very effective against gram-positive bacteria-mediated skin and skin structure infections (Chen and Lu 2020)). Dalbavancin (once known as BI-397), and telavancin are derivatives of vancomycin are small lipoglycopeptide-type antimicrobial agents (approved by the FDA as an oral solution in 1983) even destroy the cell wall and membrane of vancomycin-resistant pathogens more effectively (Zhanel et al. 2010; Higgins et al. 2005). Some of the clinically useful antimicrobial peptides are enlisted in table 19.1 (Law et al. 2013).

There are abundant AMPs at present under scientific improvement aiming for the healing proposes to confront various bacterial pathogens. The antimicrobial peptides under various clinical trial phases are shown in Table 19.2. The treatment for diabetic foot ulcers can be controlled by topical use of pexiganan, and a magainin-derived synthetic peptide is also in phase III clinical trials (Greber and Dawgul 2017). Meanwhile, Nal-P, an antimicrobial peptide restrains bacterial biofilm formations proficiently which is not easily alleviated by conventional antibiotics and could be a good choice as therapeutics (Luo and Song 2021). Thus it is needed to unveil more such AMPs against biofilm-forming bacteria. The mechanistic approaches of Nal-P-113 are still unclear like another AMP, namely, LL-37 (human cathelicidin) as well. But the topical application of the aforementioned AMPs in diabetic foot ulcers causes re-epithelialization and new blood vessel formation surprisingly (Wiesner and Vilcinskas 2010). Melamine is effective to control keratitis and other ocular infections, besides killing *P. aeruginosa* very promptly, although it has not to get approval from FDA (Yasir et al. 2019).

Several attempts have been taken for developing peptides to make them more suitable for clinical usage. The design of novel antimicrobial peptides would necessitate the optimization of multiple parameters, a problem that has proved difficult to solve. The hydrophobicity of AMP is the crucial determinant of AMP for membranes (Kumar et al. 2018). Different peptides are likely to perforate lipid membrane

Table 19.1 Summary of some FDA-approved antimicrobial peptides as therapeutics. The data was collected from (drugs@FDA http://www.fda.gov/drugssatfda) and confirmed in Drug Bank (Law et al. 2013)

Generic name	Active ingredient (s) (application route)	Applicant/Pharma (production year)	Source	Target organism
Vancomycin	Vancomycin hydrochloride USP (intravenous)	Lilly Research Laboratories, A Division of Eli Lilly and Company (1983)	*Amycolatopsis orientalis*	Enterocolitis causing *Staphylococcus aureus* (including methicillin-resistant strains MRSA) and antibiotic-associated pseudomembranous colitis causing *Clostridium. difficile*, *Staphylococcus aureus* (MRSA)
Bacitracin	Bacitracin zinc (opthalmic)	Akorn ointment, 1972	*Bacillus subtilis*, *B. licheniformis*	Gram-positive bacteria (*Staphylococcus* species, *Listeria monocytogenes*, and *Enterococcus faecalis*
Teicoplanin	Teicoplanin (intravenous or intramuscular)	Sanofi-Aventis	*Actinoplanes teichomyceticus*	Psedumembrananos colitis causing *Clostridioides difficile*
VIBATIV	Telavancin hydrochloride (intravenous)	Cumerlan and Pharms, 2013	Semisynthetic product of vancomycin	MRSA and other gram-positive bacteria
Cubicin	Daptomycin (intravenous)	Cubist Pharmaceuticals LLC, 2016	*Streptomyces roseosporus*	Complicated skin and skin structure infections (cSSSI) causing *Staphylococcus aureus* bloodstream infections (bacteremia) causing *Staphylococcus* sp., *Streptococcus* sp., *Enterococcus* sp., right-sided endocarditis infection causing pathogens, and vancomycin resistant *Enterococci* (VRE)
Dalvance	Dalbavancin hydrochloride (intravenous)	Durata Therapeutics, Inc., 2014	Semisynthetic form of	Treatment of complicated skin and skin structure

(continued)

Table 19.1 (continued)

Generic name	Active ingredient (s) (application route)	Applicant/Pharma (production year)	Source	Target organism
			Actinomadura sp.	infections (cSSSI) caused by gram-positive bacteria acute bacterial skin and skin structure infections (ABSSSI) caused by the following gram- positive microorganisms: *Staphylococcus aureus* (including methicillin-susceptible and methicillin-resistant strains), *Streptococcus pyogenes*, *S. agalactiae*, *S. dysgalactiae*, *S. anginosus* group (including *S. anginosus*, *S. intermedius*, and *S. constellatus*), and *Enterococcus faecalis* (vancomycin-susceptible strains)
Mupirocin	Mupirocin calcium (topical)	Taro Pharmaceutical Industries Ltd., 2020	*Pseudomonas fluorescens*	Staphylococci and Streptococci and against specific gram-negative bacteria, including *Haemophilus influenzae* and *Neisseria gonorrhoeae*
Colistin	Colistimethate sodium (intravenous)	Endo Pharmaceuticals, 1962	*Bacillus colistinus*	*Pseudomonas aeruginosa*
EC5	Synthetic (intravenous)	Chintamani Atreya, Shilpakala Sainath Rao, Krishna Ketha, 2013	Synthetic	*Escherichia coli* and *Pseudomonas aeruginosa*

(continued)

Table 19.1 (continued)

Generic name	Active ingredient (s) (application route)	Applicant/Pharma (production year)	Source	Target organism
Neomycin and polymyxin B sulfate and gramicidin ophthalmic solutions USP	Gramicidin, neomycin, and polymyxin B sulfate (ophthalmic)	BAUSCH AND LOMB, 1996	*Streptomyces fradiae*, *Brevibacillus brevis*, *Paenibacillus polymyxa*	Aerobic gram-negative and gram-positive bacteria, including *E. coli* and *P. aeruginosa*
Neosporin	Bacitracin zinc, neomycin sulfate, and polymyxin B sulfate (topical)	Casper Pharma, LLC, 1971	*Brevibacillus brevis*, *Streptomyces fradiae*, *Paenibacillus polymyxa*	Aerobic gram-negative and gram-positive bacteria including *E. coli* and *P. aeruginosa*
Gramicidin D	Gramicidin D (topical)	Monarch Pharmaceuticals LLC, 1982	*Brevibacillus brevis*	*Bacillus subtilis* and *Staphylococcus aureus*

Table 19.2 List of some antimicrobial peptides and their trials in various clinical states

Name	Source	Tentative medical uses	Phase	Manufacturer
Plectasin	*Pseudoplectania nigrella*	Pneumococcal diseases	Preclinical trial in mice	Novozymes A/S
Iseganan	Synthetic protegrin of porcine	Gram-negative and gram-positive bacteria, oral mucositis	Phase III failed	Intrabiotics Pharma
Pexiganan (MSI-78)	African clawed frog	Mild-to-moderate diabetic foot ulcer infections	Phase III trial	Magainin Pharmaceuticals
Neuprex	Recombinant peptide	Anti-infective properties and is a potent neutralizer of endotoxin	Failed in Phase III	Xoma Pharma
MX-594AN	Cytoplasmic granules of bovine neutrophils	Catheter infection treatment other related acne	Phase IIb	Migenix
EA-230	Human chorionic gonadotropin hormone (hCG)	Sepsis	Phase II	Xoma Pharma
MX-594AN	Synthetic	Topical treatment for acne vulgaris	Phase II	MIgenix Inc
Glutoxim	Synthetic	Tuberculosis	Phase II	Pharma BAM

differentially on basis of their constituents and thus disintegrate both the lipid layer of bacteria and RBC (Lei et al. 2019). Hence, it is resilient to design appropriate antimicrobial peptides without hemolytic activity (Moravej et al. 2018). Besides this, the contemporary delinquent parasitic diseases around the world have fascinated researchers to study the efficacy of natural and synthetic peptides against parasitic

infections. In this context, all sources of AMPs would be promising sources for acquiring antiparasitic and antibacterial peptides as well. For example, several protozoa such as *Leishmania* spp., *Plasmodium* spp., *Trypanosomes* spp., and *Schistosoma* spp. are the candidates that can be treated with antiparasitic peptides (Pretzel et al. 2013). So, it is needless to say that such AMPs have huge possibilities as biochemical therapeutics, mostly copious, especially in insects, and perturb the homeostasis of various microbial pathogens by upsetting their cell wall, membranes, and intracellular materials followed by interfering with the host's metabolic processes (Torrent et al. 2012). The increasing resistance toward antibiotics, less vulnerability to becoming resistant development, and inaccessibility of vaccines have brought attention to look at the usefulness of various AMPs.

Furthermore, different AMP acts differently with the microbial membrane and relies on different means. Most of the investigators have hypothesized that disturbing the actual complexity of the cell membrane is the prime factor for such peptides anticipated for microbiocidal deeds, other than obstructing several vital intracellular processes like nucleic acid and protein synthesis along with the inhibition of numerous decisive enzymes essential for the survivability of microbes (Moghaddam et al. 2014). Predominantly, the composition, three-dimensional structures, and types of amino acids of the AMPs along with the structural configuration of membranes of target microbial cells resolve their interactions. Markedly, scientists are perpetually endeavoring to discover new peptides and trying to solve their mode of action intensely. Therefore, the search for the superlative peptide is a prolonged trial and development activity that requires high expenditure with less success rate, although there are enormous prospects and an optimistic outlook of antimicrobial peptides as potential upcoming remedial agents for human beings (Dutta et al. 2017).

19.7 Future Perspective

Antimicrobial peptides are a highly heterogeneous family of small proteins that usually consists of 12–50 numbers of amino acids and contribute an immense function to the immune system of the host (Bulet et al. 1999). Their function is a kind of pluripotential and pleiotropic besides bactericidal actions. As an optional alternative to antibiotics, AMPs are in the spotlight of biomedical research for a few decades. The naturally occurring antimicrobial peptides are more numerous rather than their synthetic analogs. These synthetic forms are even more effective than their native variety (Shwaiki et al. 2021). Consequently, intense efforts are currently underway and have aimed to preserve or improve their efficacy also by reducing their toxicity and maintaining lower resistance development along with their functional improvements.

The function of antimicrobial protein initially was brought into the community by Alexander Fleming for the first time in the year 1921, which was the lysozyme. In the year 1339, the isolation of gramicidin from the soil bacteria *Bacillus brevis*

revolutionized the study of AMPs from many sources (Berditsch et al. 2016). Until then, numerous AMPS are identified and isolated, but the future direction as therapeutics is still in compulsion, which should be addressed wisely and logically.

Antimicrobial peptides have many opportunities concerning therapeutics, animal husbandry, and food industries mainly. AMPs mainly interact with the bacterial membrane by electrostatic interaction to exert their antimicrobial action and hit many targets by numerous mechanisms of action (Zhang et al. 2021; Wu et al. 2018). But the main limiting factors of their modes of action are still intangible. The AMPs are usually safe, not prone to develop resistance, and not easily destroy normal compared to target bacteria, although their toxicity and incidence of resistance development are in focus in many pharmaceutical companies (El Shazely et al. 2020; Huan et al. 2020; Lee et al. 2018). A few AMPs are approved by the Food and Drug Administration (USA), and most of these AMPs are for topical utilities only, like mupirocin, neosporin, pexiganan, and bacitracin, whereas few AMP has systemic uses against pathogenic invasion (Mahlapuu et al. 2020). Most of the AMPs are not resistant to intestinal juices and can't penetrate mucosal linings therefore limiting their activities due to poor absorption. Peptide modification by inserting other compounds or pectin or whey protein-mediated encapsulation may plausibly restrict the peptide digestion by hydrolytic enzymes of body fluids (Knappe et al. 2010; Gomaa et al. 2017). It is noteworthy to mention that a little number of AMPs have obtained approval for intravenous treatment (Cubicin, Vancocin, Orbactiv, Dalvance, Vibativ, and Coly-Mycins), which can circumvent these aforementioned problems, and the injectable form of such antimicrobial peptides would be of great choice as it may perhaps gain mileage on prolonged retention time, extended half-life with appreciable pharmacokinetics (Moncla et al. 2011; Starr and Wimley 2017). All these features are important to be future drugs against various topical and systemic infections. Hence, from modest and slow beginnings as antimicrobial components from various sources, AMPs have been timely honored as a unique therapeutic agent and would be an important element in the pharmaceutical landscape in near future (Lau and Dunn 2018). It is also mentionable that continuous research has identified a different class of AMPs, namely, "selectively targeted AMPs" (STAMPs), with a heightened sensitivity toward specific pathogens without affecting normal microbial flora (Chung and Khanum 2017). These kinds of AMPs are in prime focus within many research groups over antibiotic materials and should be given the importance that would help to destroy a pathogen exclusively. Numerous means are implemented nowadays for improving the functionality of AMPs by exerting minimum toxicity whose futuristic vision is very relevant and unique, of which database curation, high definition screening, structure-guided designing followed by molecular dynamics and development of AMP, and synergistic uses of AMP with other drugs are significant (Chen and Lu 2020; Chen et al. 2019; Touti et al. 2019; Ballantine et al. 2018; Haney et al. 2018). The amidation and esterification could be used to penetrate the biological membrane, besides adding sugar moiety to increase the solubility of AMPs along with protease inhibitors which can be used to minimize the action of proteases as well (Greber and Dawgul 2017).

However, the obstacles aiming for antimicrobial drug development have been reviewed in past few years (Rima et al. 2021; Chen and Lu 2020; Silver 2011), where the identification, purification, purification cost, high probability of proteolytic digestions, less bioavailability, and cytotoxicity are major issues and may be reversed by nanomaterials or liposome-mediated encapsulation, chemical modifications, and structural adaptation. Today, nanoformulations of AMPs for delivery have only been evaluated in vitro and a few tested animals, although more work in this area is needed for product uplifting and their quality assessment to prove their clinical effectiveness. Moreover, sustained release of AMPs can be achieved either by amphiphilic hydrogel or by using a polyester-mediated AMP delivery system (Piotrowska et al. 2018; Yang et al. 2018). Both natural and engineered antimicrobial peptides are very prone to proteolytic cleavage (Gan et al. 2021). Moreover, numerous intricate methods like peptidomimetics and modifications of artificial amino acids can be adopted in designing AMPs to solve the proteolysis problem. Novel chemical strategy, peptide diversity, and peptide engineering along with new scientific innovations will substitute the uses of high-risk antibiotics certainly.

Apart from the direct killing of bacteria, new AMPs should be searched to neutralize the function of toxic proteins, or virulence factors should be targeted (Duperthuy 2020). Despite a huge repertoire of AMPs, the study on the animal of such peptides is very little (Pfalzgraff et al. 2018). More animal studies should be performed instead of testing the efficacy on cell lines under normal physiological conditions to find out the mode of action of these AMPs along with the study of their side effects, toxicity, and tolerance label in the experimental animal (Greco et al. 2020). Various drug combinations along with AMPs can be administered in case of MDR bacterial infection and their doses, and the mechanism of action should be addressed with priority to avoid side effects and resistance development mainly (Huan et al. 2020). Hence, the usage of AMP in therapeutic grade would be a grand fortune for human beings.

The naturally occurring antimicrobial peptides are numerous rather than their synthetic analogs as mentioned earlier. These synthetic forms are even more effective than their original form (Shwaiki et al. 2021). Although the identification of AMPs is less hard than their purification (Huan et al. 2020). Expertise should be developed more in this area for the attainment of more functional peptides having efficacy against pathogenic bacteria. It is very well known that the availability of these naturally occurring antimicrobial peptides is very less and usually gets upregulated upon microbial invasion. To address this issue mass production of AMPs with the help of recombinant DNA technology would be promising, which should be encouraged and has a great opportunity for the researcher to characterize AMPs in many ways along with their mass production.

The structure of AMP is very much essential for its antagonistic action against bacteria (Wang et al. 2021). Further contemporary research is needed on the reported AMPs to better understand the structure-function relationship (Ahmed and Hammami 2018). As a branch of peptide drugs, AMPs need to progress with the advancement of medical science against the background of the current low success rate of the clinical application of AMPs.

Antimicrobial peptides (AMPs) have long been projected as a hopeful measure of new or alternative antimicrobial means partly because they are less prone to bacterial resistance evolution and they are very prone to proteolytic digestion, and hence it can't be used systematically generally. The main limiting factor for the systemic use of AMPs is their sensitivity to proteolytic digestion by different body fluids (e.g., intestinal mucosa, gastrointestinal tract, and blood plasma), which directly affect their in vivo stability and pharmacokinetic profile simultaneously. Besides this, AMPs are extremely flexible molecules that put forward many avenues by chemical means for their modification intending for the development of new, effective, safe, and improved varieties of AMP with enhanced functional characteristics (Sierra and Vinas 2021). It is obvious to mention that a huge number of such agents are presently tested in various phases of functional improvement (Sierra and Vinas 2021; Greber and Dawgul 2017).

For the development of AMPs as anti-infective drugs, rational design should be taken into account in several parameters, including environmental factors (pH and ionic strength), peptide length, net charge, hydrophobicity, stereochemistry, and topology. All these parameters strongly influence the antimicrobial activity and cytotoxicity toward the mammalian cell, hemolytic activity, and immunomodulatory properties of AMPs.

The Antimicrobial Peptide Database (APD) has a repertory of 3283 antimicrobial peptides from life kingdoms having various performances. And only a few of them have been tested and gone under trial to develop as alternative therapeutics. It appears that AMPs may be utilized for clinical use, and we need to exercise with some caution. Not only it can be used clinically, but it can be applied in agriculture, the food industry, animal husbandry, and aquaculture also (Keymanesh et al. 2009).

19.8 Conclusion

It is impending to consider AMPs into a new class of bactericidal agents and is unquestionably promising. Their wide range of activity, synthesis simplicity, and means of activity put together them ideal aspirants for therapeutical purposes. We are approaching the post-antibiotic era as deadly MDR bacteria are arising rapidly; hence, antimicrobial peptides would be a hopeful measure to confront such deadly pathogens effectively. The progress of AMP is drawn backed by quite a few disputes which are huge manufacturing cost, cytotoxic effect on eukaryotic effect, lower specificity, enzymatic lysis, and short lifetime. Due to the broad spectrum of bustles and rare resistant development possibilities, many pharmaceutical companies are focusing on the clinical uses of antimicrobial peptides. To exploit AMPs more effectively, the structure-function relation along with physicochemical characterization is needed. Most of these AMPs internalize themselves by binding and distorting the lipid bilayer of bacteria and specifically inhibit macromolecules. Sometimes their pleiotropic action is also observed and can interact with multiple intracellular targets. Although genetic and protein engineering can transform antimicrobial therapeutic

approaches into the generation of novel and improved versions, AMPs remain in dilemma. The rational designing of AMPs with computational approaches and peptide mimetics will be very fruitful in better understanding the functionalities of AMPs with their improved version. FDA has already approved several antimicrobial peptides for potential clinical uses, and a significant number of AMPs are under clinical trials. The search for natural peptides and their delivery systems in the tested animal would be the new focus of the upcoming research. Also fetching the nonclinical aspirant AMPs into medical use is acknowledged. The expansion of AMPs as healing mediators is likely to speed up-gradation in coming years by illustrating their specific function, tidy medicinal preparation through improving chemical and stability in vivo conditions, and superior low-cost synthesis methods. We believe that despite having several limitations and difficulties, enormous progress has been achieved in this area in the last few decades, which indicates the effectiveness and possibilities of AMPs as alternative therapeutic weapons in near future. This book chapter sheds light on the contemporary classification of antimicrobial peptides, their biological sources, mechanism of action, molecular targets, and clinical aspects which broaden many avenues for further exploration and enlighten the knowledge on antimicrobial peptides in the current scenario as a next-generation medicine.

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Chapter 20
Development of Probiotics for *Helicobacter pylori* Infection Management

Vanita Mulay, Dhanashri Satav, Austin Fernandez, Priyanka Pisalwar, and Shadab Ahmed

Abstract The probiotics market is a fast-growing business which has already crossed approximately USD 60 billion in 2021 and further is most likely to cross USD 91 billion by the year 2026 as per the recent market surveys. What these stats tells us is that there is going to be a constant high demand for probiotics when it comes to consumption of supplementary foods. This is more evident now as globally people are more aware about their health benefits and are willing to consume such products even if it comes with a premium price tag. This is also evident in many infections as doctors are prescribing such supplements with strong antibiotics. There is more acceptance for consumption of fermented or similar products with known health benefits (probiotics). This chapter focuses on various facets of probiotics for *Helicobacter pylori* infections and also risks associated with clinical use and regulatory aspects.

Keywords Adhesins · Autolysins · Bacteriocin · Biocine · Blood antigen-binding protein A (BAPA) · Chronic gastritis · Combinatorial therapy · Complementation · Cytokines · Double-strain probiotics · Gastric cancer · Gastric epithelium · Gram-negative · *H. pylori* · Heat shock proteins (HSP) · Immune modulation · Inflammation · Labs · *Lactobacillus* · Lipopolysaccharide · Mucosal layer · Multi-strain probiotics · Neuraminidase · Pathogenesis · Peptic ulcer · Peptic ulcers · Probiotics · Reactive oxygen species (ROS) · Selection criteria · Supplementary food · Toll-like receptor (TLR) · Urease activity

Abbreviations

BQT	Bismuth quadruple therapy
GRAS	Generally regarded as safe

V. Mulay · D. Satav · A. Fernandez · P. Pisalwar · S. Ahmed (✉)
Institute of Bioinformatics and Biotechnology (Merged to Department of Biotechnology), Savitribai Phule Pune University, Pune, Maharashtra, India
e-mail: sahmed@unipune.ac.in

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LAB	Lactic acid bacteria
LPS	Lipopolysaccharide
MALT	Mucosa-associated lymphoid tissue
ROS	Reactive oxygen species
TLR	Toll-like receptor
WHO	World Health Organization

20.1 Introduction

Helicobacter pylori, a spiral shaped, flagellated, gram-negative bacteria, was first isolated and identified by Barry Marshall and Robin Warren in 1982 (Marshall et al. 1985; Marshall and Warren 1984). They proved that *H. pylori* is the cause of peptic ulcers and chronic gastritis for which they were awarded the Nobel Prize in Medicine in the year 2005. It was later discovered that *H. pylori* infections also cause cancer of the stomach (Peek Jr. 2002), and WHO has classified the organism as a type I carcinogen (Saxena et al. 2020).

An estimated 4.4 billion individuals are infected with *H. pylori*, of which only 20% show any symptoms of infection while the rest 80% are asymptomatic (Chen et al. 2018). Transmission of *H. pylori* is via the fecal-oral route and is prevalent in developing nations such as India, Bangladesh, Pakistan, etc. (Saxena et al. 2020). Infection of *H. pylori* is known to cause peptic ulcers, gastric MALT (mucosa-associated lymphatic tissue) lymphoma, chronic gastritis, and gastric cancer (Peek Jr. 2002; Marshall et al. 1985; Marshall and Warren 1984).

Current treatments for *H. pylori* include treatments via antibiotics and proton pump inhibitors (Harb et al. 2015; Kim et al. 2008; Malfertheiner et al. 2007). But the increase in antibiotic resistance and various other factors have led researchers look for alternative ways to treat *H. pylori* infections (Saxena et al. 2020; Pohl et al. 2019; McFarland et al. 2016). There are attempts to find inhibitors for major cell pathways which may be synthetic or natural (Zaidi et al. 2009; Coggins et al. 2003). Vaccine development for *H. pylori* infections is being done as well (Czinn and Blanchard 2011). A novel way to treat *H. pylori* infections is with the help of probiotics (Bhatia et al. 1989).

Probiotics, defined by WHO, are "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO 2002). In this chapter we shall cover how probiotics are screened for treatment against *H. pylori* based in their anti-*H. pylori* activity (Goderska et al. 2018). Development of single-strain and multi-strain probiotics and complementation of antibiotic treatment with probiotics have shown to help in the better treatment and recovery of the patient and their gut microbiota (Haghdoost et al. 2017; Hauser et al. 2015; Francavilla et al. 2014). Efficacy and safety of these treatments will also be discussed along with their limitations and future developments.

20.2 Mechanism of Infection by *Helicobacter pylori*

20.2.1 Entry into Mucosal Layer

Epidemiology studies have shown that the transfer of *H. pylori* is person to person and hence has to be via the oral route (Amieva and El-Omar 2008). The bacteria cannot survive in the acidic environment of the stomach but has adapted several mechanisms to survive.

The bacteria, via chemotaxis, can swim toward the mucosal layer of the stomach with the help of its polar flagella (McGee et al. 2005; Ottemann and Lowenthal 2002). The bacteria also survive the environment with the help of surface associated or cytosolic urease which creates a buffer like microenvironment for the bacteria (Saxena et al. 2020; Chen et al. 2018). The bacteria further borrow into the mucosal layer to reach the gastric epithelial cells where it adheres to the cells via adhesins.

20.2.2 Adhesion to Gastric Epithelial Cells

The bacteria adhere to the epithelial cells for the following reasons:

1. **To Aid in Cellular Damage and Inflammation**
   The release of various toxins by the bacteria into the cell is aided by the adhesion of the bacteria to the epithelial cells (Petersson et al. 2006; Guruge et al. 1998).
2. **To Avoid Clearance from the Stomach**
   To combat the constant movement of the stomach environment and clearing mechanisms employed by the gastric epithelia, the bacterial cells adhere to the host cells (Amieva et al. 2002; Martinez et al. 2000).
3. **To Colonize and Multiply**
   The adherence of the bacterial cells helps in the proliferation of the cells and formation of colonies (Amieva and El-Omar 2008).

   The adherence of the bacteria is carried out by outer membrane proteins which bind to modified carbohydrates present in host glycoproteins. These include blood antigen binding protein A (BapA) (Ilver et al. 1998), sialic acid binding adhesins (SabA) (Mahdavi et al. 2002), *H. pylori* outer membrane proteins (HopZ, HopH) (Peck et al. 1999), adherence-associated proteins (AlpA, AlpB) (Odenbreit et al. 1999), heat shock proteins (HSP60) (Yamaguchi et al. 1996), neutrophil-activating protein (NapA) (Teneberg et al. 1997), and many more.

20.2.3 Virulence and Cell Damage

The binding of the bacterial cells to the epithelial cells causes the release of reactive oxygen species (ROS) and cytokines by the host cells. At the same time, the bacterial cells release certain toxins which cause cell damage along with the cytokines and ROS. NapA and HSP60 causes the release of IL6 and IL8 by activating the NF-κB and MAP kinase pathway via the Toll-like receptors (TLRs) (Teneberg et al. 1997; Yamaguchi et al. 1996). This release of cytokines causes inflammation of the epithelial cells and thus causes the recruitment of $CD4^+$ and $CD8^+$ T cells leading to cellular degradation.

The bacteria release two major cytotoxins, cytotoxin-associated gene A protein (CagA) and vacuolating cytotoxin A (VacA). CagA translocated into the host cells via phosphatidylserine and after undergoing phosphorylation will cause damage to the cell cytoskeleton and adhesion. It also causes the release of IL8 and affects the proliferation of the cells (Argent et al. 2004; Higashi et al. 2002). CagA is also found in conjugation to gastric cancer formation in some cases. VacA causes the formation of large vacuoles in the host cells due to the release of anions into the host cell cytoplasm (Akazawa et al. 2013; Palframan et al. 2012). This causes the release of cytochrome C from the mitochondria resulting in apoptosis and ER stress.

20.2.4 Persistence and Immune Modulation

To remain within the epithelial tissue, *H. pylori* needs to modulate the immune response generated by the host against the specific toxins and proteins of the bacteria. The bacterial flagellar proteins have evolved such that they cannot be recognized by the TLRs, but proteins such as HSP60 and NapA can be recognized (Gewirtz et al. 2004; Yamaguchi et al. 1996; Teneberg et al. 1997). Also, the lipopolysaccharide (LPS) coat of the bacteria is highly non- pyrogenic and nontoxic as compared to other gram-negative bacteria (Muotiala et al. 1992). The LPS coat also has several modified sugar groups on its surface which mimic the human Lewis blood group antigen. This host mimicry causes the autoimmune response within the host cells and helps the bacteria evade the immune system (Moran et al. 2002; Appelmelk et al. 1996, 2000).

The immune response generated by the other proteins of the bacteria is modulated such that the bacteria are able to escape being degraded but at the same cause inflammation and cellular damage to the host. This ensures its persistence in the system and can lead to various types of clinical phenotypes of the infection (Amieva and El-Omar 2008; Amieva et al. 2002).

20.3 Selection and Screening of Suitable Probiotics

It was first demonstrated in the 1980s that lactic acid bacteria inhibit the growth of *H. pylori* in vitro (Midolo et al. 1995; Bhatia et al. 1989). Lactic acid alters gastric pH and inactivates urease for *H. pylori* viability. However, anti-*H. pylori* effects of *Lactobacillus* are variable among its different strains (Sgouras et al. 2004; Stingl et al. 2001; Aiba et al. 1998).

For probiotics to successfully inhibit the *H. pylori* growth, they must possess certain characteristics (Fig. 20.1). The criteria for the selection of probiotics include (Fig. 20.1):

1. Adhesion to intestinal epithelium and mucus (Gao et al. 2021; Shokryazdan et al. 2014; Chenoll et al. 2011; Servin and Coconnier 2003; Conway et al. 1987).
2. Proliferation under acidic condition (Gao et al. 2021; Khoder et al. 2016; Kimura 2004; Jacobsen et al. 1999).
3. Suppression of *H. pylori* by production of inhibitors such as antimicrobial compounds (Gao et al. 2021; Shokryazdan et al. 2014; Kaur et al. 2010; Fujimura et al. 2012; Felley and Michetti 2003).
4. Resistance to gastric acid, bile salts, and pancreatic enzymes (Gao et al. 2021; Shokryazdan et al. 2014; Chenoll et al. 2011; Kimura 2004).

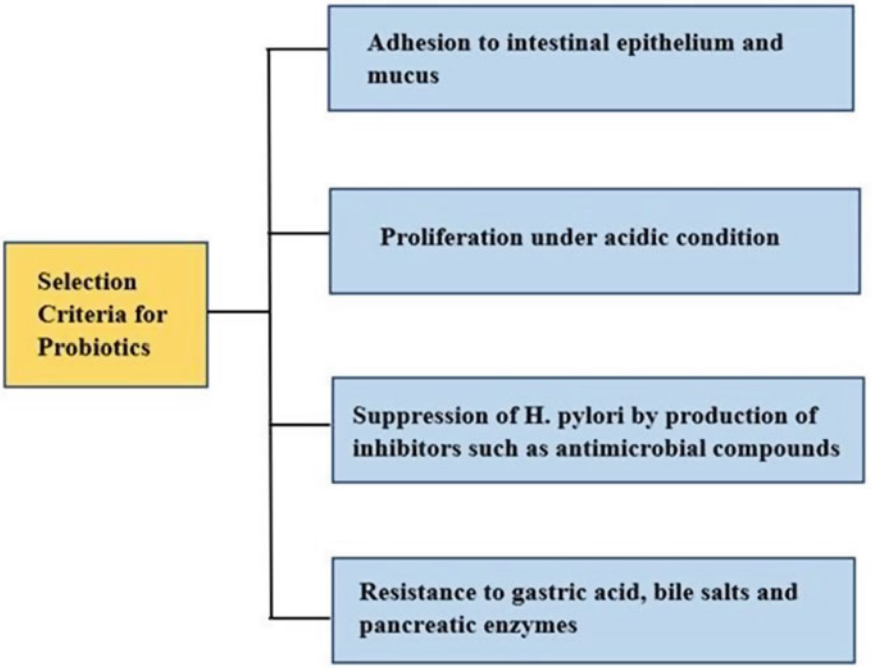

Fig. 20.1 Selection criteria for probiotics to be used clinically

The mechanisms by which *H. pylori* infection causes gastric mucosal damage includes the direct effects of virulence factors such as urease, cagA, or vacA; propagation and prolong inflammation; oxidative stress; and induction of apoptosis in infected gastric epithelial cells (Lee et al. 2008; Dhar et al. 2003; Stewart Goodwin 1997; Graham 1997). Probiotics are able to inhibit *H. pylori* by producing and secreting a variety of compounds such as short-chain fatty acids and bacteriocins including nisin A, leucocin K, reuterin, and pediocin PO_2 (Rolfe 2000; Fuller and Gibson 1998).

The mechanisms of anti-*H. pylori* activity of lactobacilli may be associated with decreases in urease activity and adhesive capability of *H. pylori*; secretion of lactic acid, bacteriocins, autolysins, and proteinaceous compounds; and suppression of *H. pylori*-associated IL-8 production (Lesbros-Pantoflickova et al. 2007; Servin 2004; Lorca et al. 2001). Another critical mechanism involving probiotics relates to changes in host immune responses to infection via reduced tumor necrosis factor alpha and interleukin-8 (IL-8) (Corr et al. 2007; Matsumoto et al. 2007). *Lactobacillus gasseri* and *L. casei* strains have been found to decrease *H. pylori* density in the gastric mucosa (Sakamoto et al. 2001; Sgouras et al. 2004), and later *L. casei* was identified as the best for *H. pylori* eradication rates (Feng et al. 2017). Lactobacilli is known to decrease both gastric inflammation and side effects of therapy for *H. pylori* eradication (Lesbros-Pantoflickova et al. 2007). When used as probiotics, LAB (lactic acid bacteria) help to decrease the occurrence of gastritis and reduce the risk of *H. pylori* infection without inducing side effects (Kim et al. 2014). A broad range of *Lactobacillus* probiotics increased the success of *H. pylori* treatment and reduced side effects (Shi et al. 2019). The probiotic strains along with their mechanism of inhibition are shown in Table 20.1.

20.3.1 Single/Double or Mixed Strain Probiotics Against Helicobacter pylori

Probiotics are available as single-strain products or in mixtures of two or more probiotic strains. Multi-strain probiotic compound has not beneficial effects in the treatment of *H. pylori* infection. It might be related to the low dosage of our probiotic regimen and/or high frequency of upper gastrointestinal adverse effects which in turn could decrease the eradication efficacy (Shavakhi et al. 2013). The neuraminidase activity of *Saccharomyces boulardii* selectively removes α (2–3)-linked sialic acid from surfaces of duodenal epithelial cells to prevent binding with *H. pylori* adhesin and thereby impedes bacterial adherence (Sakarya and Gunay 2014). In most of the meta-analysis, it was seen that single-strain probiotics are beneficial against the *H. pylori* (McFarland 2015). Only one probiotic strain (*S. boulardii CNCM I-745*) significantly prevented any adverse events, and two strains of probiotics (*S. boulardii or L. rhamnosus GG*) decreased the adverse events of eradication therapy (McFarland 2015). Double-strain probiotics are more effective

Table 20.1 Mechanisms of inhibition of *H. pylori* by probiotics strains

Sl. No	Probiotic strain	Mechanism of inhibition	References
1.	*Lactobacillus casei* subsp. *rhamnosus* *L. acidophilus*	Lactic acid	Midolo et al. (1995)
2.	*Lactobacillus salivarius* WB1040 *L. acidophilus* 4356 *L. casei* 393	Lactic acid	Aiba et al. (1998)
3.	*Lactobacillus acidophilus strain LB*	Heat-stable protein	Coconnier et al. (1998)
4.	*Lactobacillus johnsonii* La1	Heat-stable substance	Michetti et al. (1999)
5.	*Lactobacillus acidophilus* CRL 639	Autolysins	Lorca et al. (2001)
6.	*Bacillus subtilis* 3	Amicoumacin A	Pinchuk et al. (2001)
7.	*Weissella confusa strain* PL9001	Class II bacteriocin	Nam et al. (2002)
8.	*Limosilactobacillus reuteri* TM 105	Glycolipid binding protein	Mukai et al. (2002)
9.	*Lactobacillus casei* strain Shirota	Biocines	Cats et al. (2003)
10.	*Lactococcus lactis subsp. lactis* A164 *L. lactis subsp. lactis* BH5	Bacteriocins	Kim et al. (2003)
11.	*Lactobacillus casei* strain Shirota	Lactic acid	Sgouras et al. (2004)
12.	Bifidobacterium strains	Antimicrobial peptide	Collado et al. (2005)
13.	*Lactobacillus salivarius* (UCC118 and UCC119	Decreased the production of IL-8	Ryan et al. (2009)
14.	*Bifidobacterium bifidum* CECT 7366	Inhibit *H. pylori* growth	Chenoll et al. (2011)
15.	*Lactobacillus gasseri* OLL2716	Suppression of *H. pylori* adhesion	Fujimura et al. (2012)
16.	*Lactobacillus plantarum* ZDY 2013	Gastric microbiota alteration	Pan et al. (2016)
17.	*Lactobacillus delbrueckii* subsp. *bulgaricus*	Heat-stable bacteriocin	Boyanova et al. (2017)
18.	*Lactobacillus fermentum* UCO-979C	Inhibits the production of IL-8	García et al. (2017)
19.	*Lactobacillus rhamnosus* yoba 2012	Lactic acid	Westerik et al. (2018)
20.	*Lactobacillus* spp., GMNL-74 and GMNL-185	Suppresses *H. pylori* adhesion	Chen et al. (2019)
21.	*Lactobacillus acidophilus* *L. bulgaricus*	Inhibits the secretion of IL-8	Song et al. (2019)
22.	*Lactic acid bacteria*	Bacteriocin	Kouitcheu Mabeku et al. (2020)
23.	*Lactobacillus gasseri* ATCC 33323	Inhibits the secretion of IL-8	Yarmohammadi et al. (2021)
24.	*Lactobacillus casei T1*	Suppression of *H. pylori* adhesion	Wu et al. (2021)

than multi-strain probiotic in improving the rate of *H. pylori* infection eradication (Haghdoost et al. 2017; Wang et al. 2017).

20.4 Development of Probiotics for *Helicobacter pylori* Infection Management

20.4.1 Combinatorial Therapy: Probiotics with Antibiotics

20.4.1.1 Antibiotic Regimens Used for *Helicobacter pylori* Eradication

In the 1990s, the standard triple therapy was accepted as the most optimal treatment for the eradication of *H. pylori* infection. It uses two antibiotics, usually amoxicillin combined with clarithromycin (although many variations are used) and a proton pump inhibitor (PPI) (Goderska et al. 2018) (Fig. 20.2). However, the effectiveness of this standard therapy was observed to decrease over the years, due to prevalence of *H. pylori* strains resistant to antibiotics, particularly to the key antibiotic, clarithromycin (Malfertheiner et al. 2007).

Bismuth quadruple therapy (BQT) was later suggested and became the preferred first line of treatment in areas with greater prevalence of clarithromycin resistance (Fig. 20.2). It is also used as the second line of treatment when standard triple therapy fails (Papastergiou et al. 2014; Goderska et al. 2018). BQT includes a bismuth salt (e.g., bismuth subcitrate potassium) in addition to a PPI and the antibiotics metronidazole and tetracycline (Harb et al. 2015). Apart from standard

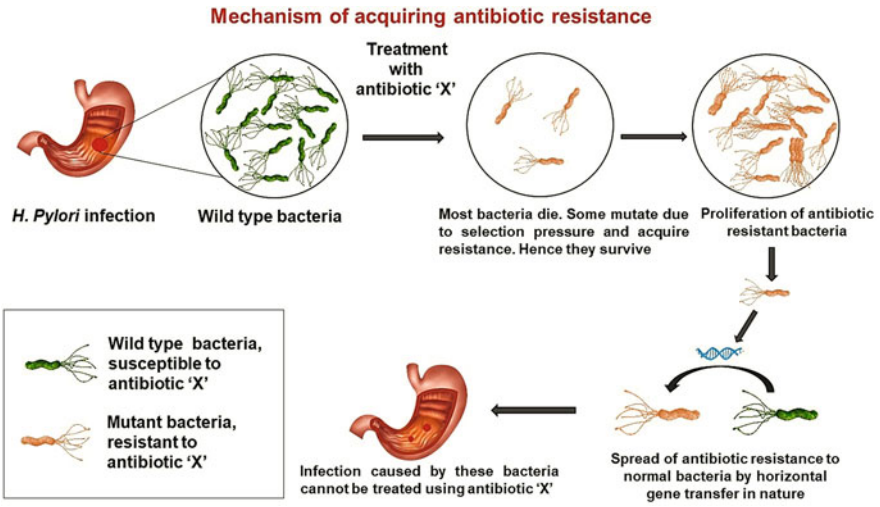

Fig. 20.2 Development of resistance due to widespread use of antibiotics

triple therapy and BQT, many other antibiotic regimens including a wide range of antibiotics are in use for the management of *H. pylori* infection (Goderska et al. 2018). Despite their initial success, over the years, antibiotic-based therapies have increasingly been reported to be associated with two major drawbacks:

1. Adverse effects

   Antibiotic therapy has been associated with many undesirable side effects, with diarrhea and vomiting/nausea being very common (Bell et al. 1992). These are one of the major reasons for poor patient compliance, which in turn may lead to treatment failure (McFarland et al. 2016).
2. Increase in prevalence of antibiotic-resistant strains

   Probiotics have been suggested to alleviate the problems associated with antibiotic-based therapies, due to their proven beneficial effects. Multiple studies over the years have shown that the administration of certain probiotics in conjunction with antibiotic therapy helps to ease its adverse symptoms. In many studies, the addition of probiotics also helped to increase the rate of eradication of the infection, that is, probiotics enhanced the efficacy of the antibiotic therapy (Fig. 20.1). The key features or inferences derived from these studies have been summarized in Table 20.2.

20.5 Antibiotic Resistance

Metronidazole, amoxicillin, and clarithromycin are the antibiotics that are primarily used in *H. pylori* eradication therapy. Several reports indicate that increasing prevalence of antibiotic resistance may be the major reason for failure of eradication therapy (Kim 2015). Clarithromycin resistance has been increasing in many countries over the past few years. Therapies based on this antibiotic therefore are not used in regions with a high incidence of clarithromycin-resistant *H. pylori* infection (Figs. 20.3 and 20.4). Other antibiotics like tetracycline and metronidazole are recommended instead (Eslami et al. 2019). However, resistance to fluoroquinolones like levofloxacin and metronidazole has also been reported in many countries (Eslami et al. 2019; Hu et al. 2017), with metronidazole resistance rates being higher in developing nations, as compared with those of developed nations (Eslami et al. 2019) (Fig. 20.3). Comparatively, the resistance to tetracycline and amoxicillin is lower (Eslami et al. 2019; Hu et al. 2017). The *H. pylori* isolates from different regions have shown a wide range of resistance to the different antibiotics tested over the years (Figs. 20.3 and 20.4). Consequently, there has been a variation in responses shown by them to the eradication therapies used; this has been extensively reviewed by many research groups (Eslami et al. 2019; Goderska et al. 2018; Hu et al. 2017; Kim 2015; Papastergiou et al. 2014). The choice, dosage, and duration of antibiotic eradication therapy therefore depend on the extent of susceptibility of the infection-causing strain in a particular region to the antibiotics under consideration.

Table 20.2 Summary of studies for combinatorial therapy using probiotics and antibiotics for *H. pylori* infection treatment

Sl. No.	Probiotic strain(s) used	Antibiotic regimen used[a]	Effect of addition of probiotic	References
1.	*Clostridium butyricum*	BQT (PAN, AMX, furazolidone, colloidal bismuth pectin)	Improved GI symptoms (defecatory function in particular), increased *Bacteroidetes to Firmicutes* (B:F) ratio	Chen et al. (2018)
2.	*Lactobacillus GG* *Saccharomyces boulardii* Multi-strain probiotic (containing *Lactobacillus* spp. and *Bifidobacteria*)	TT (CLM, tinidazole, rabeprazole)	Incidence of diarrhea and disturbance of taste reduced (for all probiotic strains used)	Cremonini et al. (2002)
3.	*Lactobacillus acidophilus* LA-5 and *Bifidobacterium lactis* BB-12	TT (CLM, AMX, OMP)	Duration of antibiotic associated diarrhea was reduced, and other GI symptoms like duration and intensity of pain also improved	De Vrese et al. (2011)
4.	*Lactobacillus acidophilus*	TT (CLM, AMX, OMP)	Increased eradication rate of infection	Du et al. (2012)
5.	*Enterococcus faecium* strain L-3 *Bacillus subtilis*	TT (CLM, AMX, OMP)	Eradication rates increased, and dysbiosis was significantly reduced in case of both probiotic groups, as evaluated using various parameters	Ermolenko et al. (2018)
6.	Two strains of *Lactobacillus reuteri* (DSM 17938 and ATCC PTA 6475)	TT (CLM, AMX, and a PPI)	Decrease in incidence of antibiotic associated side effects	Francavilla et al. (2014)
7.	Prodigest® (*Lactobacillus* and *Bifidobacterium*)	TT (CLM, AMX, PAN)	Significantly higher eradication; adverse effects of therapy (like epigastric pain, diarrhea) were reduced	Haghdoost et al. (2017)
8.	*Lactobacillus rhamnosus* GG (LGG®) and *Bifidobacterium* (BB-12®)	TT (varied combinations of antibiotics and PPIs were used)	Higher eradication rates and improved symptoms	Hauser et al. (2015)
9.	*Clostridium butyricum* (CBM588)	TT (CLM, AMX, LAN)	Significantly lower incidence of antibiotic induced diarrhea	Imase et al. (2008)
10.	*Lactobacillus acidophilus* HY2177, *Lactobacillus casei* HY2743, *Bifidobacterium longum*	TT (CLM, AMX, and a PPI)	Increased eradication rate, but *no reduction* in side effects of triple therapy	Kim et al. (2008)

(continued)

Table 20.2 (continued)

Sl. No.	Probiotic strain(s) used	Antibiotic regimen used[a]	Effect of addition of probiotic	References
	HY8001, and *Streptococcus thermophilus* B-1			
11.	(*Lactobacillus rhamnosus* GG, *L. rhamnosus* LC705, *Bifidobacterium breve* Bb99 and *Propionibacterium freudenreichii* ssp. *shermanii* JS)	TT (CLM, AMX, LAN)	Antibiotic treatment-related symptoms reduced	Myllyluoma et al. (2005)
12.	Medilac-S® (*Streptococcus faecium* and *Bacillus subtilis*)	TT (CLM, AMX, LAN)	Reduced incidence of antibiotic-resistant bacteria in the GIT	Oh et al. (2016)
13.	*Clostridium butyricum* MIYARI 588	TT (CLM, AMX, and a PPI)	Significantly higher eradication rate	Mukai et al. (2002)
14.	*Bifidobacterium lactis*	TT (CLM, AMX, LAN)	Significantly higher eradication rate and reduced symptoms like diarrhea, vomiting, and abdominal pain	Şirvan et al. (2017)
15.	*Bifidobacterium* DN-173010	TT (CLM, AMX, PAN)	Frequency of constipation and stomatitis decreased	Yaşar et al. (2010)

[a] *BQT* bismuth quadruple therapy, *TT* triple therapy, *AMX* amoxicillin, *CLM* clarithromycin, *OMP* omeprazole, *PPI* proton pump inhibitor, *PAN* pantoprazole, *LAN* lansoprazole

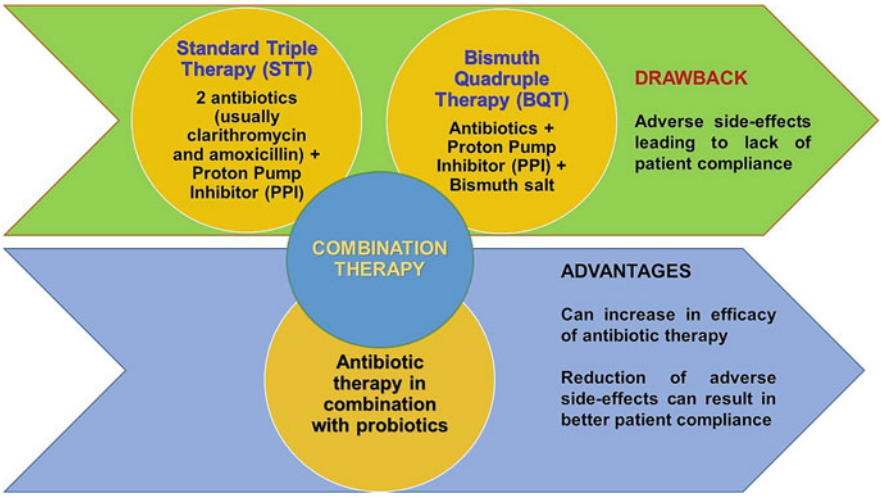

Fig. 20.3 Antibiotics and probiotics given together as part of combinatorial therapy against *H. pylori*

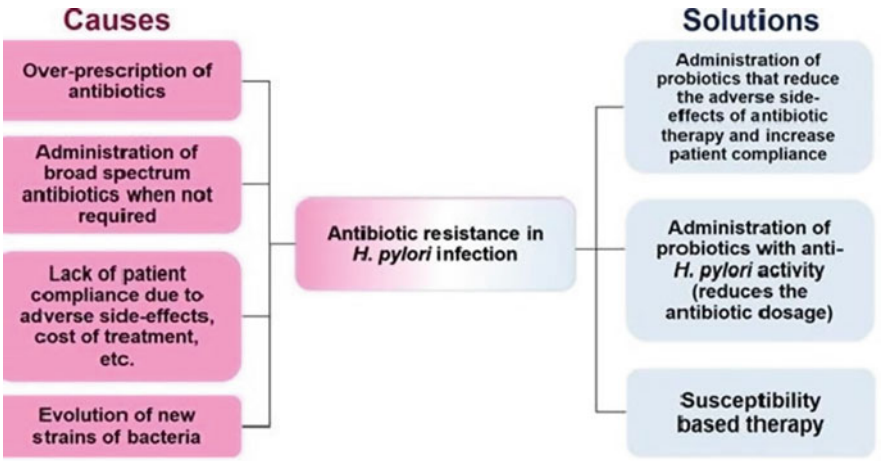

Fig. 20.4 Antibiotic resistance causes and newer strategies or solutions

Many broad-based antibiotic combination therapies expose the patients to at least one unnecessary antibiotic that in fact only serves to contribute to the global resistance without having any beneficial effect whatsoever (Figs. 20.3 and 20.4). Susceptibility-based therapy would avoid the administration of such unnecessary antibiotics, but the cost involved in this approach is high, and hence it is not always feasible (Hu et al. 2017; Papastergiou et al. 2014). Another major reason for increase in resistant strains of the bacterium is poor patient compliance, due to the adverse effects caused by antibiotic therapy, among other factors (Figs. 20.3 and 20.4). This leads them to stop the treatment before the regimen is complete (Hauser et al. 2015; Tong et al. 2018).

20.6 How Can Probiotics Be of Aid?

As demonstrated by many studies (Table 20.2), probiotics have been shown to reduce many of the adverse effects of antibiotic therapy. They increase patient compliance and also eliminate the need for administration of more antibiotics (that may be required due to failure of first line of treatment) (Fig. 20.4). This in turn reduces the chances of development of more resistant strains (Fig. 20.4) (Hauser et al. 2015).

Apart from this, addition of probiotics has proven to increase the efficacy of the antibiotic eradication therapies (Fig. 20.4). Pre-treatment with probiotics was shown to reduce the bacterial load and thus increase the efficacy of the eradication therapy, despite some antimicrobial resistance (Sheu et al. 2006) .Thus, supplementing antibiotic therapies with probiotics could cure the infection with a lesser dosage requirement of the antibiotic (Fig. 20.4). This reduction in antibiotic usage can go a

long way in curbing the rise of resistant species. Kim et al. noted that therapies containing probiotic are relatively free from the burden of resistance and drug complications are also reduced (Kim 2015).

As outlined in the previous sections, many single-strain and multi-strain probiotic compositions have been shown to have anti-*H. pylori* activity (without supplementation with antibiotics), both in vitro and in vivo. More research in this area can lead to the development of probiotic therapies without the need for antibiotics to cure the infection. In an era where antibiotic-resistant species are on the rise, this approach holds great promise for the future and is the need of the hour.

20.7 Efficacy and Safety of Probiotics for *H. pylori* Eradication

20.7.1 Efficacy

Many groups have reviewed the efficacy of probiotics for *H. pylori* treatment. Their results can be summarized as follows:

Probiotics improved the eradication rate of the antibiotic therapy when they were co-administered and decreased the adverse side effects of antibiotic therapy, like nausea, vomiting, diarrhea, pain, etc. (Shi et al. 2019; Lü et al. 2016; Lv et al. 2015). *Lactobacillus* spp., *Bifidobacterium* spp., *Saccharomyces boulardii, Enterococcus* spp., and *Clostridium butyricum* are some species that have shown favorable results across many trials (Shi et al. 2019; Zhang et al. 2020; Wang et al. 2013, 2017; Lü et al. 2016; Lau et al. 2016).

A few studies have shown contradictory results—for example, in one study the probiotic was revealed to be unsuccessful in the reduction of adverse effects (Kim et al. 2008), whereas in some others, probiotics did not increase eradication rate (Zojaji et al. 2013)—but these (and a few more) appear to be outliers. A majority of studies have reported results in favor of probiotics (Zhang et al. 2020; Shi et al. 2019; Wang et al. 2013, 2017; Lü et al. 2016; Lau et al. 2016). This being said, probiotic therapy has largely been shown to be beneficial only as a supplementary therapy to antibiotics. With the evidence we have so far, it is difficult to confidently advocate the use of probiotics as the primary treatment for *H. pylori* infection management. Although a few promising results have been obtained (as demonstrated in the previous sections), their efficacy as the sole treatment for eradication remains questionable and requires more study and experimentation.

Additionally, it should be noted that the studies on the basis of which the above conclusions have been drawn are very heterogeneous with respect to sample size, demographics, strains used, treatment regimen, etc. There is therefore a need to carry out uniform experimentation with standardized parameters.

20.7.2 Safety

So far, in all the studies and reviews analyzed, no significant adverse effects or reactions have been reported that could be attributed to the administration of probiotics (Zhang et al. 2020; Shi et al. 2019; Wang et al. 2013, 2017; Lü et al. 2016; Lau et al. 2016).

20.8 Probiotics for *H. pylori*: Future Scope and Directions for Research

20.8.1 Shortcomings of Current Infection Management

As seen in the previous sections, probiotics show great promise in treatment of *H. pylori* infections. However, there is scope for improvement. The major limitations are as follows:

1. So far, they have only shown results when used in combination with antibiotic therapy. Probiotics alone have not yet proven to be capable of eradicating infection.
2. Choice of strains and dosage required needs to be standardized based on the infection causing strain and load of infection.

20.8.2 Strategies to Overcome These Hurdles

As seen before, it is difficult to make a generalized statement about the use of probiotics for *H. pylori* infection management due to the large heterogeneity that exists in the studies carried out so far. Hence one major goal of future studies should therefore be standardization, so that results obtained can be reproduced with confidence. This would involve setting exclusion criteria for patients in the trials, evaluating the type of strains causing infection in a particular cohort and how this varies with factors like demographics, geographical location, etc. Upon identification of the strain(s), susceptibility tests to antibiotics being currently used should be carried out to get an idea of the extent of resistance.

Subsequently, the possible probiotic strains that can be used for eradication would need to be tested. Which antibiotics would the chosen probiotic strains work best in combination with will also have to be determined.

The second (and more important) goal for future research would be to develop probiotic strains that can manage the infection without the aid of antibiotics. Genetic engineering of the strains currently in use to secrete compounds that are toxic specifically to *H. pylori* is a possible approach. Multi-strain probiotics may also be developed for the same. Engineering strains such that they outgrow the *H. pylori* in

the stomach by competing for binding sites and other nutrients can also be engineered. This can be achieved in part, by synbiotics. It involves the co-administration of the probiotic organism along with a substrate that can be metabolized by it so that it can proliferate well in the host. With this view in mind, strains that can utilize novel substrates may be identified/engineered. Co-administration of these with their substrate would favor probiotic growth over *H. pylori* in the host.

A combination of the above approaches, instead of implementing each in isolation, may yield better results. Once the strain/combination of strains to be used has been identified, then parameters like dosage, duration of treatment, mode of administration, etc. can be standardized.

20.8.3 Immunity Against H. pylori *Infection*

20.8.3.1 Vaccines

Given the burden of *H. pylori* infection and the increasing prevalence of antibiotic-resistant strains worldwide, taking steps toward preventing infection is as vital as developing better treatment strategies. Consequently, there have been efforts over the years to develop a vaccine that will confer immunity against *H. pylori* infection. Common *H. pylori* antigens used in vaccine development studies are urease A and B, vacuolating cytotoxin A (VacA), and *H. pylori* adhesin A (HpA) among others (Banga Ndzouboukou et al. 2021). The results of some of the most recent studies have been reported below.

Zhong et al. (2020) studied the effect of an oral recombinant subunit vaccine using the *H. pylori* antigens with double mutant heat-labile toxin (dmLT) on mice. It was shown that the vaccine significantly reduced colonization by *H. pylori* when the mice were challenged post immunization. Cen et al. (2021) used the *Saccharomyces cerevisiae* display system to express the urease antigen. It was administered as an oral vaccine to mice, upon which they exhibited a significantly large mucosal and humoral immune response (Cen et al. 2021). Xie et al. (2021) designed a multivalent vaccine using several epitopes from *H. pylori* antigens. The vaccine was successful in reducing gastric colonization of *H. pylori* in mice. Jafari and Mahmoodi also designed a multi-epitope vaccine candidate containing three *H. pylori* antigens linked to the sequence of Melittin, which was used as an adjuvant. Melittin is the prime active protein in the venom of honey bees. The design was confirmed to be nonallergenic and antigenic, but it has not been tested in vivo (Jafari and Mahmoodi 2021). Cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) was also tested as an adjuvant for a parenterally administered vaccine, and it showed promising results in mice (Chen et al. 2020).

Computational tools have also been extensively used by many groups to design/identify novel antigens as vaccine candidates (Ghosh et al. 2021; Ma et al. 2021; Rahman et al. 2020).

20.8.3.2 Probiotics for Prevention *H. pylori* Infection

Previous sections have described the role of probiotics in infection management. However, if they are administered before infection as a prophylactic measure, probiotics can help in reducing the extent of colonization or even inhibit it altogether. This can be achieved using strategies similar to those described above (competing with *H. pylori* for colonization sites and nutrients, secretion of *H. pylori*-specific toxins, ensuring better growth of the probiotic organism by added a novel substrate, etc.)

Probiotics can be administered orally. This would make distribution of the formulation in the developing countries with poor healthcare facilities much easier. Another possibly major advantage is that a single dose of an ideally designed probiotic formulation would be enough. This is because probiotics being viable organisms can proliferate in the host and maintain their population.

This is an interesting avenue for further studies and research and should definitely be looked into.

20.9 Constraints or Risks Associated with Clinical Use of Probiotics

Apart from the many clinically documented benefits of probiotics, one should be very careful as there is also a risk associated with probiotic therapy in certain health conditions and patients (Boyle et al. 2006; Zielińska et al. 2018). Even the immune compromised patient and similar clinical scenarios required very careful and cautioned practice. In certain cases it has been seen that probiotics can interact with commensal bacteria and directly impact the host (Zielińska et al. 2018), and understanding these interface is one of the key challenges for future study. Other challenges are to know their mechanisms of action, to explain more specifically which probiotics strains can give which health benefits and intake levels required to achieve those effects (Pagliaro and Battino 2010) which leaves a gap in the selection of specific strain to treat *H. pylori* or any other specific pathogen. To date a number of clinical trials have been documented which are from different geological regions and populations of the world. There is lack in homogeneity in the research design which leads to inconsistent results.

Clinical and mechanistic studies are essentially needed to know the interface among the microbes, host cell, immune defense, and mucus and to create beneficial interventions (Doron and Snydman 2015; Boyle et al. 2006). Theoretically probiotics may responsible for five types of side effects in an individual with underlying medical conditions, deleterious metabolic activities, systematic infection, excessive immune stimulation in susceptible individuals, gene transfer, and minor gastrointestinal symptoms (Kothari et al. 2019; Doron and Snydman 2015).

20.10 Regulatory Guidelines Regarding Safety and Efficacy

Many developed countries like the United States, Japan, Norway, Canada, and the European countries have already come up with strict guidelines and criteria to ensure safety and efficacy of clinically administered probiotics which is in line with the various costumer protection laws (Zielińska et al. 2018; Gupta 2016). But many African and Asian countries still lack a concrete policy in this regard, and hence the spurious products and their sale are still at large. In India scientific bodies like Department of Biotechnology and Indian Council of Medical Research came up with DBT-ICMR guidelines for evaluation of probiotics in food (Indian Council of Medical Research-Department of Biotechnology 2011). The Food Safety and Standards Authority of India (FSSAI) has also come up with guidelines in 2015 (FSSAI 2015). So far, many species of bacteria and yeasts have been listed as safe by these regulatory bodies across globe, and further many are in the process of being declared safe. Such a practice is going to become vital if we are to ensure that no infections or deleterious effects after consumption as supplementary food (Gupta 2016; Hoffman et al. 2016; USFDA 2015; EFSA 2015).

20.11 Future Prospects

Probiotic either singly or in combination with antibiotics has shown great promise in risk management of *H. pylori* infection and also, in many cases, prevention. With further research and development and better understanding of the mechanism, probiotics have definitely shown their potential to be developed as primary treatment strategy against treatment for *H. pylori* infection even without the aid of antibiotics. Although it remains a challenge now, but the recent reports do indicate positive findings in support of probiotics use. Given the increasing prevalence of antibiotic-resistant strains, this approach is the need of the hour and the future.

Similarly, they can also be developed as prophylactics that can be administered to prevent onset of infection altogether and thus nip the problem in the bud. Many reports have indicated that probiotic monotherapy has higher eradication rate than placebo (Handa et al. 2020; Qureshi et al. 2019). That is why it is essential to further increase our understanding of mechanism of infection by *H. pylori* as this could lead to further revealing of better and more specific targets for molecular targeting via drugs or antibiotics and success of combinatorial therapy. This will not only help against the antibiotic resistance of the bacteria but will also help in the development of better probiotic and therapeutic formulations against the bacteria.

20.12 Summary and Conclusion

The future study of probiotics needs to continuously evolve for identification and selection of novel strains with GRAS or QPS (qualified presumption of safety, Europe) or suitable status along with the usual beneficial effects. A thorough investigation with respect to safety like analysis of possible infections and side effects caused by target probiotic should also be conducted before clinical use. Apart from that the analyses with respect to immunological impacts and aspects of each new probiotic with detailed understanding of the mechanism are also warranted. In essence safety of probiotics for clinical use must include animal studies to check for side effects, and also the methods used to assess the safety of probiotics also need to be standardized. There is also the issue of standard guidelines being implemented across the globe so as to minimize spurious products circulating in the markets. With advancement in genome-based techniques and other high-throughput processes and screening processes, it is now even more important to implement all the relevant guidelines and selection criteria globally, and WHO initiatives in underdeveloped and developing countries could play an important role in it.

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Chapter 21
Implications of Probiotics in Management of Bacterial Infections

Sandipan Mukherjee

Abstract The rise and spread of antibiotic-resistant pathogenic bacteria has led to intractable recurrent infections, strain on the healthcare system, and a sustained drain on the economy. Antibiotic resistance is acquired through plasmids, phenotypic changes, and genotypic mutations. Further, treatment with antibiotics has been linked to development of diseases, such as antibiotic-associated diarrhea (AAD). In this regard, penicillin and its derivative act by targeting the bacterial membrane. Bacterial membrane is an attractive target for developing antibacterials given it is a multivalent target and its essential role in maintaining cell shape and processes. Bacteriocins from probiotic lactic acid bacteria (LAB) are a class of antimicrobial peptides (AMPs) which act on gram-positive bacteria by insertion, pore formation, and lysis of the target cell. Further, bacteriocins act at nanomolar concentrations, are produced by generally recognized as safe organisms (GRAS), and have a selective target profile. Probiotic LABs are also being investigated for antibacterial applications. LABs are routinely consumed as indigenous fermented foods, are considered to be GRAS organisms, and are a key species of the food industry. Recently, probiotic LABs are being investigated for medical applications, such as in *Clostridioides difficile*-associated AAD and in keratinocyte models, owing to their ability to inhibit the adhesion of pathogens onto target sites, such as mucin and collagen. Probiotic LAB are known to produce organic acids, which can reduce pathogen viability. Additionally, bacteriocins are being explored as adjuvants with chemotherapeutic agents to target pathogenic bacteria. The present book chapter will explore the implications of probiotic LABs in mitigating bacterial infections.

Keywords Antibiotic resistance · Bacteriocins · *C. jejuni* · *E. coli* · *E. faecalis* · *L. casei* · *L. monocytogenes* · *L. plantarum* · *L. rhamnosus* · Lactic acid bacteria · Lactic acid · Pediocin · Organic acids · Plantaricin · Probiotic · *S. aureus* · Shiga toxin · Salmonella

S. Mukherjee (✉)
Department of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Mumbai, India
e-mail: m.sandipan@iitb.ac.in

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Abbreviations

ECM Extracellular matrix
FAO Food and Agricultural Organization
LAB Lactic acid bacteria
WHO World Health Organization

21.1 Introduction

The advent of drug-resistant pathogens is due to the acquisition of resistance biomarkers and excess use of modern chemotherapeutic molecules. The challenge of antibiotic resistance is enhanced due to dwindling armory of antibiotics and a slowing drug discovery pipeline. This has led to a breakdown in therapeutic modules, leading to poorer outcomes, recurrent infections, and higher mortality. Antibiotic-resistant pathogens have also been discovered in food, indicating that the gut may serve as a reservoir for dissemination of resistance genes among the microbial pool (Yousef and Abdelhamid 2019; Doyle 2015). The broad-spectrum activity of antibiotics has led to significant collateral damage to the gut, causing enhanced colonization by pathogenic bacteria. This colonization often leads to bloodstream infections, chronic diseases, and associated metabolic disorders.

Based on the challenging scenario associated with antibiotic resistance, a novel approach can be conceived. It is anticipated that such an approach will target a multivalent target with lesser chances of developing resistance. Probiotic lactic acid bacteria (LAB) can be utilized to serve this end, considering their use in food industry and various beneficial attributes. Food and Agricultural Organization (FAO) has defined probiotics as "live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host" (Hill et al. 2014; FAO and WHO 2001). Probiotic LAB has been used to immunomodulate the host in order to bring about a beneficial outcome (Wang et al. 2015b). Further, since probiotic LAB possess resistance to bile, low pH, and gastric proteases, adhering in high numbers to host cells, they can be used in the gastrointestinal niche (Begley et al. 2005, 2006; Liong and Shah 2005; Lee et al. 2000; Charteris and Morelli 1998). Probiotic bacteria are being considered for use in host treated with antibiotics, in order to serve as bulwark against opportunistic infections (Pamer 2016; Caballero and Pamer 2015; Buffie and Pamer 2013; Taur and Pamer 2014; Abt and Pamer 2014; Ubeda and Pamer 2012; Ubeda et al. 2010; Brandl et al. 2008). Further, probiotic bacteria are coming into scientific focus in order to decipher the mechanisms of their action and utilize them in food industry and clinical settings (Mikulski et al. 2012; Messaoudi et al. 2012b; Oelschlaeger 2010; Borchers et al. 2009; Ng et al. 2008; Zuccotti et al. 2008; Schlee et al. 2007; Wegener 2003). Further, probiotic bacteria are also known to protect from lethal listerial infections and bacterial toxins such as Shiga toxin and modulate tight junction function in animal model (Drolia et al. 2020;

Wu et al. 2016; Yang et al. 2015; Sears 2005; Asahara et al. 2004). The inactivation of Shiga toxin is particularly important given the prevalence of Shiga toxin-producing *E. coli* in food products (Douëllou et al. 2016, 2017; Bugarel et al. 2010, 2011; Espié et al. 2006).

Probiotic lactic acid bacteria (LAB) hold GRAS status (generally recognized as safe) (Jankovic et al. 2010; Brisson et al. 2010). These have been extensively used in food and fermentation industry and hence can serve as important candidates to be tested against drug-resistant pathogens (Burgain et al. 2014; de Carvalho et al. 2010; Ayala-Hernandez et al. 2008; Leroy and De Vuyst 2004). LAB from nonhuman sources are being explored for antibacterial as well as in industrial applications (García-Cano et al. 2019; Pidutti et al. 2018; Foligné et al. 2013; de Carvalho et al. 2010; Singh and Ramesh 2008, 2009). LAB functions by colonizing the gut, excluding the pathogens and via immunomodulation (Hurmalainen et al. 2007). Bacteriocins secreted by LAB act on the bacterial membrane and hold considerable potential given their specific spectrum of activity against gram-positive pathogenic bacteria. Therefore, bacteriocins are anticipated to lead to minimal collateral damage to human commensal microbiota and limit the threat posed by opportunistic gastrointestinal pathogenic bacteria. Bacteriocins are effective against *Listeria monocytogenes*, *Staphylococcus aureus*, *Enterococcus faecalis*, and *Clostridium difficile* (Walsh et al. 2014; Cotter et al. 2005).

The ways by which LAB display antibacterial against bacterial pathogens are discussed in detail in the following sections.

21.2 Production of Organic Acids and Other Inhibitory Substances

Probiotic LAB is known to produce copious amounts of organic acids and alcohol such as ethanol as by-product of metabolism (Agriopoulou et al. 2020). The production of organic acids lowers the pH of the media, lending a competitive edge to the LAB (Cotter and Hill 2003; Russell and Diez-Gonzalez 1997). The organic acids produced are as follows: acetic acid, citric acid, fumaric acid, lactic acid, and malic acid (Agriopoulou et al. 2020). Organic acids are naturally occurring molecules, utilized in the food industry to inhibit bacterial spoilage by pathogenic bacteria such as *Listeria monocytogenes* and *Escherichia coli* as well as by other microbial species (Carpenter and Broadbent 2009; Theron and Lues 2007; Doyle 2005; Samelis and Sofos 2003). For example, sodium salt of lactic acid is sprayed or added to meat products, as well as on sea-based food products to prevent spoilage by foodborne pathogens (Erickson 2019; Dussault et al. 2016; Dave and Abdel 2011; Apostolidis et al. 2008; Mejlholm and Dalgaard 2007; Manju et al. 2007; Kostrzynska and Bachand 2006). Bacteria tend to maintain their pH when the surrounding pH ranges from 7 $\pm$ 2 (Carpenter and Broadbent 2009). The mode of action of organic acids against bacteria stems from several factor, such as their protonated nature, ability to

cross the cytoplasmic membrane, and release of protons and anions inside the cell (Carpenter and Broadbent 2009). The excess concentration of anion and external pH drives the antibacterial action associated with organic acids. The process is conceived to be as follows: Since organic acids are membrane soluble, the acids can cross the membrane and enter the cytoplasm. Some pathogens, such as *L. monocytogenes*, have the cytoplasmic pH close to 8, when the surrounding pH ranges from 7 ± 2 (Carpenter and Broadbent 2009). This causes the dissociation of acid into protons and anions. If the buffering capacity of the cell is not able to compensate for the excess protons in the cytoplasm, the cellular functions are likely to be inhibited (Stancik et al. 2002; Booth 1985). For example, the inhibition of acid-sensitive enzymes associated with glycolysis can cause cessation in ATP production, along with other bystander effects (Diez-Gonzalez 2019; Carpenter and Broadbent 2009; Tuomanen et al. 2001). The increase in proton concentration in conjunction with an increase in K^+ leads to an increase in cellular osmolarity and turgor pressure (Roe et al. 1998; Kroll and Booth 1981). Simultaneously, accumulation of counterions, such as acetate, can cause inhibition of critical pathways, such as methionine biosynthesis pathways, leading to accumulation of toxic intermediates (Roe et al. 2002; Arnold et al. 2001). Lactic acid was found to have a higher anti-listerial effect as compared to propionic acid and acetic acid (Romick and Fleming 1998). The concentration of organic acid to be used to deter bacterial growth should be calculated based on the pKa of the test organic acid and the pH of the surrounding environments (Carpenter and Broadbent 2009; Romick and Fleming 1998). The extracellular concentration of organic acids leads to an increase in the concentration of the anions inside the bacterial cell, leading to cell growth arrest (Buchanan et al. 1994). In another study, lactic acid led to formation of alternative fermentative end products (Pieterse et al. 2005). It was surmised that presence of elevated concentration of lactic acid affected the differential expression of genes involved in bacterial growth (Pieterse et al. 2005). LAB and intestinal pathogens are known to display resistance to elevated concentration of organic acids (Azcarate-Peril et al. 2004; van de Guchte et al. 2002; Wilkins et al. 2002; Cotter et al. 2001). The resistance to elevated concentration of acid can be ascribed to enhanced expression of anion efflux pumps and acid resistance genes as well as decrease in the internal pH (Diez-Gonzalez 2019). In this context, expression of acid resistance is a cardinal feature of probiotic LAB and is considered important for durable adhesion in gastrointestinal tract (Mukherjee and Ramesh 2015, 2017; Mukherjee et al. 2013). In another study, the combination of lactic acid and the bacteriocin and pediocin led to a decrease in the viability of the gram-negative bacterial pathogen, *Aeromonas hydrophila* (Wang et al. 2020). Lactic acid and phenyllactic acid were found to inhibit the growth of *Salmonella* enteritidis, *E. coli*, and *L. monocytogenes* by acting on the bacterial membrane and genomic DNA (Ning et al. 2017; Wang et al. 2015a). These results suggest the role of organic acids produced by LAB in inhibiting bacterial growth and consequent spoilage. Coincubation of enterohemorrhagic *E. coli* O157:H7 with *L. rhamnosus* GG, *L. plantarum*, and *L. reuteri* led to downregulation of Shiga-toxin 2 gene expression in *E. coli* O157:H7 (Carey et al. 2008).

21.3 Bacteriocins

Bacteriocins are antimicrobial peptides secreted by gram-positive and gram-negative bacteria (Liao and Nyachoti 2017; Umu et al. 2016; Azevedo et al. 2015; Rea et al. 2010; Heng and Tagg 2006; Garneau et al. 2002). Bacteriocins are positively charged, amphipathic, and those secreted by LAB specifically target gram-positive bacterial pathogens (Rea et al. 2011; Walsh et al. 2014; Cotter et al. 2005; Liu et al. 2010; Dobson et al. 2012; Belguesmia et al. 2010). Bacteriocins from probiotic LAB are routinely utilized in food industry, to prevent food spoilage by bacterial pathogens and in animal husbandry, to treat infections, such as those caused by *S. aureus*, *L. monocytogenes*, and *Campylobacter jejuni* (Liao and Nyachoti 2017; Kadariya et al. 2014; Cizeikiene et al. 2013; Messaoudi et al. 2012a). Bacteriocins differ from antibiotics in the following ways: (a) Bacteriocins act at nanomolar range as opposed to antibiotics which require sub-micromolar to millimolar concentration to act on bacteria; (b) bacteriocins are largely considered to have lesser collateral damage as compared to antibiotics; (c) bacteriocins are primary metabolites, synthesized by ribosomes and produced at lag phase, while antibiotics are secondary metabolites and are produced after the completion of microbial growth; (d) bacteriocins are susceptible to proteolytic degradation and are thermally stable, whereas antibiotics are largely unaffected by proteases but become deactivated at high temperatures; and (e) bacteriocins work by forming pores and dissipating the membrane potential, while antibiotics target the membrane and have intracellular targets as well (Sharma et al. 2021; Agriopoulou et al. 2020; Umu et al. 2016; Garsa et al. 2014; Perez et al. 2014). Bacteriocins are also known to possess antiviral and antifungal activities (Juturu and Wu 2018; Bulgasem et al. 2016). Bacteriocins act on the bacterial membrane by forming pores, though there have been reports where bacteriocins can show inhibitory activity via non-membrane pathways (Daba et al. 2018; Chikindas et al. 2018; Jiang et al. 2016; O'Connor et al. 2015; Drider et al. 2006). Given the potent action of bacteriocins, various mutagenic or chemical approaches are utilized to obtain cell-free bacteriocins (Juturu and Wu 2018; Bédard et al. 2018; Healy et al. 2013). However, loss of bacteriocin activity is observed in both of these approaches. Disulfide bonds in bacteriocins are important in preserving its activity and are dynamic in nature (Bédard et al. 2018; Belguesmia et al. 2013). Interestingly, linear analogues of pediocin PA-1 displayed potent antibacterial activity against *L. monocytogenes* and *Clostridium perfringens* (Bédard et al. 2018). Class IIa bacteriocins are especially sought after given their potent activity against *L. monocytogenes* (Umu et al. 2016; Perez et al. 2014). Bacteriocins are being explored for use against multidrug-resistant bacterial pathogens since they are known to target bacterial membranes, an underexplored multivalent target (Mukherjee et al. 2013, 2019; Mukherjee and Ramesh 2015, 2017; Nes et al. 2013; Lohans and Vederas 2012; Hurdle et al. 2011; Heng and Tagg 2006; Rodríguez et al. 2002). Bacteriocins from LAB are also known to inactivate pathogens such as VRE, MRSA, and *Streptococcus mutans* as well as MRSA biofilms (Okuda et al. 2013; Galvin et al. 1999). Bacteriocins are also explored for their

adjuvant potential (Chi and Holo 2018). Nisin has been found to be active against planktonic and biofilm cells of *S. aureus* in combination with vancomycin and oxacillin (Angelopoulou et al. 2020; Fernanda et al. 2020). The enhanced activity stems from membrane damage and increase in oxidative stress (Fernanda et al. 2020).

Bacteriocins are divided into the following classes based on their structure: class I (lantibiotics), class IIa (pediocin-like), class IIb (two peptide systems), class IIc (circular), IId (leaderless bacteriocins), and class III (heat-sensitive large peptides) (Hegarty et al. 2016; Karpiński and Szkaradkiewicz 2016; O'Connor et al. 2015; Nes et al. 2013; Cotter et al. 2013; Dobson et al. 2012). Nisin is a class I bacteriocin and is the only authorized bacteriocin to be used in food packaging, in solutions, and in veterinary applications (Perez et al. 2014; Delves-Broughton 1990, 2012; Lee and Kim 2011). The sequence YGNGVXC is conserved in class IIa bacteriocins and is a distinctive feature (Wang et al. 2018; Cui et al. 2012; Ennahar et al. 2000; Bhunia et al. 1991). The sequence is present at the N-terminal end and is considered to be responsible for the enhanced anti-listerial activity (Jang et al. 2014; Liu et al. 2010; Loessner et al. 2003; Bennik et al. 1998). Of the classes of bacteriocin, class III bacteriocins act on the outer and inner membrane of both gram-positive and gram-negative bacteria (Sun et al. 2018; Nigutová et al. 2008).

Resistance to bacteriocins needs to be taken into account before pathogens such as *S. mutans* and *S. aureus* are known to develop resistance against bacteriocins and other antimicrobial peptides (Barbosa et al. 2021; Kawada-Matsuo et al. 2013a, b).

The development in resistance stems from change in bacterial membrane and enhanced production of enzymes such as glutamate decarboxylase (Begley et al. 2010). In another study, mutations in purine operon repressor and in an unidentified gene cluster in *S. aureus* led to 32-fold increase in nisin concentration required to inhibit cell growth (Blake et al. 2011). In *L. monocytogenes*, change in membrane potential mediated by a lowering in transmembrane pH and electric charges (Bonnet et al. 2006) is responsible for resistance to bacteriocins. In *C. difficile* and *Bacillus subtilis*, the resistance to bacteriocins is mediated by the ABC transporters (Clemens et al. 2018). Therefore, application of bacteriocin in clinical settings must be rationalized with efficacy and the development of resistance before use.

21.4 Competitive Exclusion

Inhibiting bacterial adhesion to surfaces and host cells can be an attractive way to impede antibiotic resistance, given that adhesion to proteins and cells is a critical step in initiating infection (Foster et al. 2014; Chagnot et al. 2012; Cusumano and Hultgren 2009; Pizarro-Cerdá and Cossart 2006; Shin et al. 2005; Zong et al. 2005; Jaradat and Bhunia 2003; Wilson 2002). Since inhibiting bacterial adhesion leads to minimal selection pressure, it can be anticipated that the chances of developing antibacterial resistance will also be less. Competitive exclusion can occur by inhibiting pathogen adhesion onto host cells, or onto protein factors such

as extracellular matrix (ECM) (Mukherjee and Ramesh 2015, 2017; Mukherjee et al. 2013; Chagnot et al. 2012; Ofek and Doyle 1994). This is anticipated to decrease pathogen numbers from intestinal mucosa, inhibit access to nutrients, and enable the host to remove the pathogens through digestive flow (Liao and Nyachoti 2017; Callaway et al. 2008). Probiotic LAB are known to autoaggregate, form biofilms, and release autoinducer-like factors (Tannock et al. 2005). Probiotic LAB are known to inhibit adhesion through competitive exclusion, by showing higher adhesion capacity to mucus, ECM, and production of organic acids (Lopes et al. 2017; Alemka et al. 2010; Ingrassia et al. 2005). The presence of S-layer proteins on the membrane of LAB has been found to be responsible for inhibiting adhesion of gastrointestinal pathogens such as *E. coli* O157:H7, *Clostridium sporogenes*, and *E. faecalis* to epithelial cells (Jensen et al. 2014; Zhang et al. 2013; Ramiah et al. 2008; Collado et al. 2007; Johnson-Henry et al. 2007). The S-layer proteins are being explored as antigen delivery vehicles given their excellent binding properties to host cells (Åvall-Jääskeläinen and Palva 2005). Various domains in LAB membrane such as SpaCBApilli, collagen-binding protein, fibronectin-binding protein, and mucin-binding protein are conceived to be responsible for the enhanced binding to ECM components and host cells (Ardita et al. 2014; Le et al. 2013; MacKenzie et al. 2010; von Ossowski et al. 2010; Tallon et al. 2007; Miyoshi et al. 2006). The trait of bacteriocin production has also been correlated to enhanced displacement of *L. monocytogenes* and *E. faecalis* from mouse gut model (Kommineni et al. 2015; Corr et al. 2007). Interestingly, adhesion determinants can be degraded to form antimicrobial peptides, aiding in colonization of probiotic LAB (Bøhle et al. 2010). The expression of adhesion determinants in conjunction with expression of bile salt hydrolase gene and production of bacteriocins can be used as biomarkers for assessment of probiotic LAB (Duary et al. 2012; Li et al. 2008). The combination of adhesion inhibition and bacteriocin activity has shown a decrease in pathogen numbers adherent on HT-29 cells (Mukherjee et al. 2019; Mukherjee and Ramesh 2017). Adhesion by probiotic LAB can also mediate other beneficial attributes such as expression of hormones and suppression of colonic dysfunction (Gareau et al. 2007). Probiotic LAB can be explored for application as anti-adhesion agents based on their exhaustive documentation of high adhesion capacity. Some examples are represented in Table 21.1.

21.5 Future Prospects

The rise of antibiotic-resistant pathogens has necessitated a paradigm shift in ways of developing alternative antibacterial approaches. The advent of antibiotic resistance, in conjunction with a drying arsenal of antibiotics and chemotherapeutic molecules, creates a dire situation for healthcare providers. In such a situation, alternative modes of combating antibiotic-resistant pathogens have to be explored. Probiotic LAB possesses several attractive properties such as resistance to bile salt, low pH, gastric proteases, ability to produce antibacterial molecules such as organic acids and

Table 21.1 Representative examples of adhesion inhibition by LAB

Target pathogen	LAB species used	Mechanism	Reference
Salmonella Typhimurium ATCC 29631	*Lactobacillus acidophilus* GK20, *L. paracasei* GK74, and *L. plantarum* GK81	Adhesion inhibition	Sung-Mee (2012)
Staphylococcus aureus *Bacillus subtilis* *Clostridioides difficile* *Escherichia coli*	*L. casei* g9		Guan et al. (2020)
			Darmastuti et al. (2021)
Escherichia coli	*Lactobacillus plantarum* Dad-13 and *L. plantarum* Mut-7		
Escherichia coli K88, *Salmonella enterica* serovar Typhimurium KCCM 40253	*Lactobacillus mucosae* LM1		Valeriano et al. (2014)

bacteriocins, and ability to adhere in high numbers to ECM and host cells. It is important to understand the implications of these properties. Organic acid produced by probiotic LAB inhibits pathogenic growth and in conjunction with high adhesion can aid in displacing prebound intestinal pathogens. Bacteriocins can function as scaffolds and adjuvants to enhance the clearance of antibiotic-resistant bacterial pathogens. Given these properties, probiotic LAB can be explored for antibacterial applications.

21.6 Conclusion

The rise and advent of antibiotic-resistant pathogens has led to renewed focus on alternative strategies. The accumulation of antibiotic resistance in pathogenic bacteria has led to chronic shortage of important drugs and caused severe illnesses in patients. Nosocomial infections such as antibiotic-associated diarrhea usually occur in immunocompromised or recently hospitalized patients. These factors indicate the need to investigate newer sources of drug therapies. One of the strategies can be the use of probiotic LAB. Probiotic LAB which can survive the gastric transit, produce organic acids in the gut niche, compete for adhesion sites and nutrients, and selectively target the pathogenic bacteria are candidates to be used against bacterial infections. Armed with bacteriocins, probiotic LAB can prove to be pivotal in selectively abrogating pathogenic bacteria, without the development of antibiotic resistance. However, more research is needed before probiotic LAB can be deployed for therapeutic applications.

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Chapter 22
Nanocarriers for the Molecular Targeting of Pathogenic Bacteria

Satendra Singh Gurjar and Poulomi Dey

Abstract The rise of antibiotic-resistant pathogenic bacteria is a worldwide health problem that necessitates the creation of a novel treatment strategy. This approaching crisis, along with the slow rate of innovative drug discovery, has heightened the need for the creation of strong bactericidal agents that can act on deep cellular targets while being antagonist to resistance development. In this regard, nanocarriers for the specific targeting of pathogenic bacteria hold special interest as the likelihood of developing resistance against them can be circumvented by targeting the underlying molecular mechanisms used by these pathogens to contravene the action of antibiotics and come up with alternate drugs (synthetic or natural compounds) and therapeutic interventions which play a key role in inhibition of bacterial pathogenesis. Of special interest are nano-drug delivery systems that can target key virulence attributes and pathogen physiology like temperature or pH-sensitive polymeric micelles, nanocarriers made of lipids and polymers, and antibiotic-replete synthetic amphiphilic micelles which facilitate passive as well as active targeting. These alternates can lead to enhanced uptake of antibiotics, stability of the drug, and heightened bioavailability by improving permeability of the pathogen membranes, along with prolonging the controlled release of drug from carriers leading to minimal adverse events and having modulable physiochemical attributes in order to maximize their bactericidal potency. Based on the aforementioned premise, the present investigation ascertains the potential of nanocarriers for the molecular targeting of pathogenic bacteria.

Keywords Alternate antibacterials · Antibiotic resistance · Amphiphiles · Antibiofilm · Antimicrobial peptides · Chitosan · Dendrimers · Liposomes ·

S. S. Gurjar
Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, Assam, India

P. Dey (✉)
Kusuma School of Biological Sciences, Indian Institute of Technology Delhi, New Delhi, India
e-mail: poulomidey@bioschool.iitd.ac.in

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Membrane target · Micelles · Nanocarriers · Nanocomposites · Pathogenic bacteria · Stimuli responsive

Abbreviations

AMP	Antimicrobial peptides
MRI	Magnetic resonance imaging
PA	Peptide amphiphile
PAMAM	Polyamidoamine
PAMAMOS	Polyamidoamine-organosilicon

22.1 Antibiotic-Resistant Pathogens

The advent of antibiotic-resistant microbial pathogens is a growing concern in modern medicine. Pathogenic bacteria have evolved a slew of modifications that have rendered them extremely resistant to commonly used therapeutic antibiotics. Based on the degree of resistance to therapy, CDC has characterized antibiotic-resistant pathogens into three tiers, namely, urgent threats, serious threats, and concerning threats as shown in Fig. 22.1.

Furthermore, the excessive utilization of antibiotics in the prescribed treatment for patients has resulted in the evolution of drug-resistant characteristics in pathogenic bacteria, exacerbating the situation (Peterson and Kaur 2018; Petchiappan and Chatterji 2017; Fair and Tor 2014). There are various mechanisms by which bacteria exhibit resistance to antibiotics including drug efflux, creating a diffusion barrier, modifying the drug target, adopting a metabolic bypass, or secreting antibiotic altering/degrading enzymes as shown in Table 22.1.

Biofilms are bacterial populations that are encased in a matrix and exhibit greater antibiotic resistance as well as the ability to elude the immune system. They can produce persistent infections that are resistant to antibiotic treatment (Forier et al.

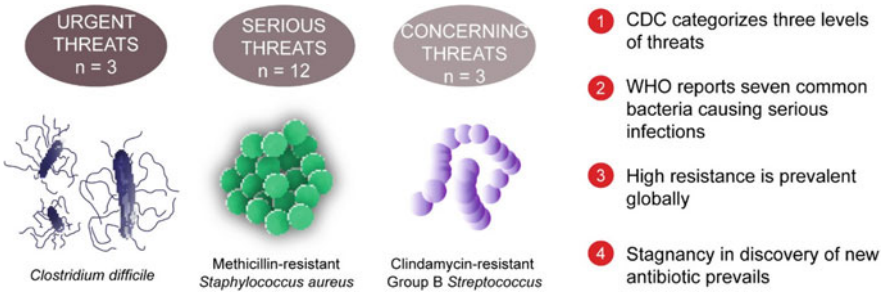

Fig. 22.1 Threat levels of antibiotic-resistant pathogenic bacteria based on the Lahsoune et al. (2007)

Table 22.1 Examples of antibiotic-resistant machinery in bacterial pathogens

Mechanism	Effect
Lipopolysaccharide (LPS) barrier	In gram-negative bacteria
Wall teichoic acid and peptidoglycan barrier	In gram-positive bacteria
Porin protein modification	Inhibition of antibiotic entry
Drug efflux	Expulsion of antibiotic from the cell
Antibiotic hydrolyzing and modifying enzymes	Inactivation of β-lactams, chloramphenicol, aminoglycosides, and macrolides
Modification of the target	β-lactam resistance and resistance against rifamycin, macrolides, quinolones, and cationic antibiotics

2014). Antibiotic resistance is promoted by the complex properties of bacterial biofilms, resulting in the rise of resistant device-related illnesses, which pose additional obstacles in their management. Indwelling medical devices are vulnerable to microbial colonization by biofilm-producing bacteria and if ignored become a source of persistent infection, unless removed from the patient. A few examples include catheter-related bloodstream infections, catheter-associated urinary tract infections, and ventilator-associated pneumonia (Singhai et al. 2012). Antimicrobial drugs have been used empirically to treat such infections, but the increased complexity of bacteria in biofilms colonizing the indwelling medical devices has led to the creation of resistant device-related illnesses.

Therefore, the rational in target selection and drug design against antibiotic-resistant pathogens should be such that vital cellular targets, which are germane to the survival, and virulence of the target strain (Johnston et al. 2016) is selected that lack structural homologs in the mammalian host. Candidate antibacterials having optimum pharmacodynamic and pharmacokinetic attribute (Onufrak et al. 2016; Silver 2011; Mueller et al. 2004) need to be developed which are nontoxic to host cells even at concentrations higher than their MIC (Wispelwey 2005) and are less likely to induce resistance development.

22.2 Advantages of Nanosystems Used for Antibiotic Delivery Vis-à-vis Conventional Antibiotic Therapy

Intracellular drug delivery using nanoparticles individually and or using them for encapsulation of carrier antibacterial particles is an attractive mode of therapy. The important determinants of such a model include efficient loading capacity of the drug of interest and an effectual sustained release profile. Nanocarrier systems have the capability to encapsulate antimicrobial drugs thereby enhancing their bioavailability on one hand and suppressing drug toxicity and antimicrobial resistance on the other hand (Devrim and Bozkır 2017). Such vectors can shield antibiotics from

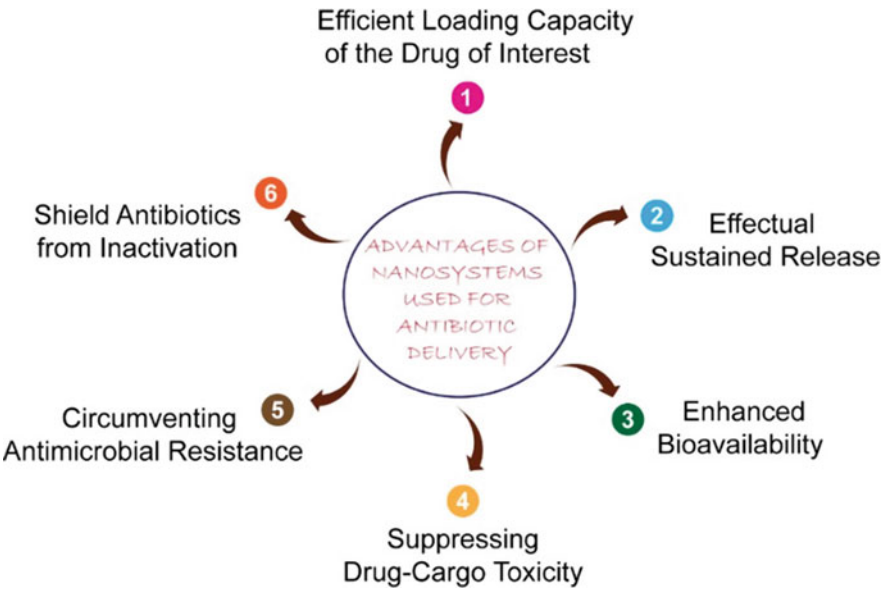

Fig. 22.2 Advantages of nanosystems over conventional antibiotic therapy

inactivation and aid to overcome physiological obstacles, allowing for faster transit and gradual release at the target region. As a result, medication accessibility advances, while adverse effects are reduced (Colzi et al. 2015). The physicochemical properties exhibited by nanocarriers are exceptional. They are ultrasmall having controllable size, a hefty surface area-to-mass ratio, enhanced reactivity, and structures which can be easily functionalized. Few of the advantages of using nanosystems for antibiotic delivery are listed in Fig. 22.2.

22.3 Lipid Nanocarriers

Lipid nanocarriers can be of a variety of types. Vesicles are universal transporters for both hydrophilic and hydrophobic compounds and are further subclassified as large unilamellar vesicles, multilamellar vesicles, and small unilamellar vesicles. The drugs which have a charge on them are also known to have charge base interaction with the liposome surfaces (Drulis-Kawa and Dorotkiewicz-Jach 2010). There are significant limitations of antibiotics due to the poor affinity for bacterial membranes; hence antimicrobials encapsulated in nanocarriers, such as liposomes, are regarded a promising approach (Colzi et al. 2015). There can be varying types of encapsulation of drugs depending upon the lipophilicity or charge as depicted in Fig. 22.3. Liposomes can engage with gram-negative bacteria's outer membrane (OM) and lipopolysaccharides, allowing therapeutics to be internalized at reasonably high

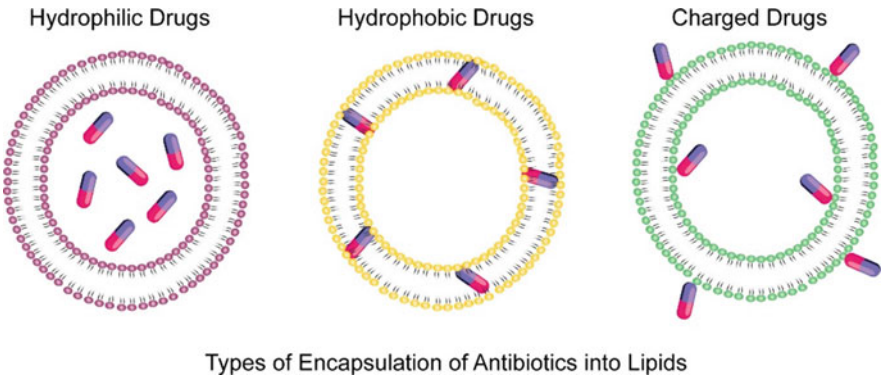

Fig. 22.3 Mode of loading of drugs into liposome nanocarriers

concentrations able to fully circumvent transmembrane pumps that accelerate enhanced efflux of active substance outside the bacterium (Allen and Cullis 2013; Basnet and Škalko-Basnet 2013). Colzi et al. has described the use of the membranes of prokaryotes to form unconventional liposomes for use as vectors for antibiotic delivery.

Modification of diverse physicochemical parameters of liposomes allows for the creation of specific carriers with the necessary pharmacokinetic and pharmacological qualities. A few of the known liposomal antibiotics include quinolones, aminoglycosides, beta-lactams, and polypeptide antibiotics (Drulis-Kawa and Dorotkiewicz-Jach 2010). Liposomal ampicillin, gentamicin, and ciprofloxacin have been used for annihilation of intracellular bacteria. Liposomal aminoglycosides and quinolones like amikacin, tobramycin, and ofloxacin have been known to be applied for eradication of extracellular bacteria. Metronidazole, polymyxin B, and vancomycin are few chemically formulated liposomal antibiotics used for extracellular bacteria eradication. A promising technique for reducing biofilm resistance is drug administration which is also using lipid or polymer nanoparticles which have the ability to increase antibiotic transport to bacterial cells, hence boosting treatment efficacy (Forier et al. 2014).

Anchored ligands help direct vesicles to specific bacteria or bacterial aggregates including biofilm. Schiffelers et al. provided evidence of better effectiveness of antibiotics encased in STEALTH liposomes (modified surfaces especially with PEG) relative to the unbound antibiotic (Schiffelers et al. 2001). The contact interaction of liposomes with the cytoplasmic membrane of bacteria has shown great promise in the eradication of drug-resistant *P. aeruginosa* strains by contravening the low permeability of the outer membrane and the efflux mechanisms (Beaulac et al. 1996). However, the limited shelf life of lipid vesicles affects its pharmacological stability and is a drawback of liposomal antibiotic carriers.

22.4 Stimuli-Triggered Drug Delivery Systems

A stimuli-responsive system can truly be considered a smart drug delivery nanocarrier. Stimuli-responsive materials provide several benefits for building mechanically dynamic devices at the micro and nano level. An ideal system should be able to preserve the efficacy of the drugs without disturbing the physicochemical properties of the entity like maintaining bioavailability, pH range, water solubility, and biocompatibility. One particular problem is to build astute or adaptive micro- and nano-sized capsules that safeguard their contents from extraneous forces and release them just in response to specific environmental scenarios at the intended location. There have been records of stimuli-responsive drug carriers which respond to various impetuses like pH, temperature, light, enzyme, magnetism, metal ions, microwave, and ultrasound as listed in Table 22.2. Various studies have reported multimodal stimuli-sensitive polymeric capsules as well as nanoshells made using the layer-by-layer (LbL) approach that are responsive to a wide range of stimuli including physical, chemical, and biological ones (Delcea et al. 2011). A representative image of chemical stimuli-driven drug delivery system is shown in Fig. 22.4.

The interaction of laser light with nanoparticles for biomedical applications resulting in triggered release of drugs is an interesting approach. The concept involves fine-tuning the wavelength of light used, so that there is preferential absorption by the nanoparticle as compared to the surrounding tissues thereby allowing selective targeting albeit being less toxic. Near-infrared light has been found to be the most suitable candidate for this endeavor (Timko et al. 2010; Roggan et al. 1999). Light can also have photothermal effects. It can be used to heat nanocarriers, especially noble metal ones, like gold nanoparticles, leading to their thermal disaggregation, thereby resulting in the release of the loaded drug cargo (Huang et al. 2009; Angelatos et al. 2005).

Another intriguing concept is magnetic field targeting using magnetic nanoparticles. One of the primary roles is magnetic field-driven release which can improve therapeutic effectiveness by modulating the permeability of the carrier

Table 22.2 Types of stimuli-responsive nanocarriers for drug delivery

Type of stimulus	Category
Physical	Light
	Electric
	Magnetic
	Ultrasound
	Mechanical
	Temperature
Chemical	pH
	Ionic strength
	Solvent
	Electrochemical
Biological	Enzymes
	Receptors

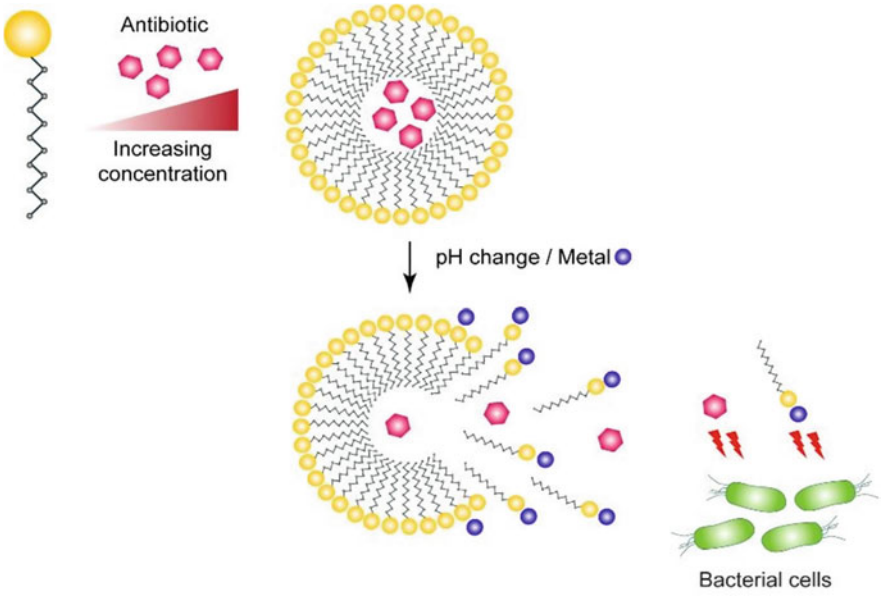

Fig. 22.4 Schematic representing chemical stimuli-driven drug delivery systems

under the effect of alternating electromagnetic field (Hu et al. 2008; Lu et al. 2005). Furthermore, magnetically triggered expulsion from magneto-responsive smart micro-/nanospheres made of magnetic nanoparticles, lipid bilayers, as well as polyelectrolytes has been explored (Katagiri et al. 2010; Wang et al. 2009).

The reversible protonation/deprotonation forms the basis of pH-responsive nanocarriers and allows regulation of the charge of the subunit moieties thereby modulating the acidity of the surrounding environment and controlling the interaction of the nanocarrier (Sui and Schlenoff 2004). The buildup of extra protons induces greater repulsion, which in turn induces the nano-capsules carrying the drug to swell, resulting in enhanced permeability (Shiratori and Rubner 2000). Deprotonation, on the contrary, reduces polymer interaction, promotes shrinkage, and hence reduces permeability (Burke and Barrett 2003).

Ionic strength also plays a major role in the electrostatic interaction between the oppositely charged polyelectrolyte carriers and salt ions which can lead to the disaggregation of the nanocarrier (Büscher et al. 2002). The interaction of metals with the surfactants forming the nanocarrier modulates their self-assembly behavior and antibacterial activity. Complexation can cause an amphiphile's electrostatic potential or structural conformation to shift, resulting in a noticeable transformation in aggregate shape (Dey et al. 2020).

There are also a few smart carriers which respond to biological stimuli for targeted release of the cargo. They may be switched on and off by enzymes or may get activated in the presence of specific receptors (Delcea et al. 2011). One such appealing model is using polyelectrolyte multilayer capsules which can be

biodegraded using enzymes. Such a system can be applied for the channeled delivery of drugs which can target intracellular components like nucleic acids and proteins (Shu et al. 2010; Zelikin et al. 2006). Glucose-responsive smart drug carriers are also in trend (De Geest et al. 2006). Researchers have also described a nano drug carrier with dual triggerable release features based on keratin-graft-PEG copolymers wherein trypsin can trigger the release of the loaded drug in the nanoparticles (Li et al. 2012). The most significant benefit of having an enzyme-degradable micro/nano delivery system is that they do not necessitate the involvement of any exogenous stimulus to disintegrate and therefore have a great capacity toward biomimetic uses and in vivo applications for intracellular administration of antibiotics (Delcea et al. 2011). Microcapsules and nanoshells functionalized with antibodies can be used as a smart system because they allow for selective targeting (Cortez et al. 2007). Antibody-conjugated nanoparticles are one of the most basic types of receptors. The increased cellular absorption and intracellular longevity may be two of the most significant features of employing antibody linked nanoparticles for biomedical applications (Arruebo et al. 2009). The effectiveness of the delivery is determined by the capacity of each antibody to penetrate its target in sufficient proportions, along with the small quantity of nanoparticles retained by the cell (Arruebo et al. 2009).

22.5 Biocompatible Micelles

Micelles have advanced and have now been investigated especially as therapeutic nano delivery systems to aid in the solvation of hydrophobic medicines, co-delivery of medications or antimicrobial substances, and as precision nanomedicine for site-specific delivery (Yao et al. 2017; Bartolami et al. 2016). Antibacterial effect of micellar systems obtained from self-assembly of strategically engineered oligomeric and polymeric amphiphilic frameworks has also been reported (Drouet et al. 2015; Fukushima et al. 2012).

Membrane-targeting amphiphiles having self-assembling property can be a promising outline to integrate several warheads and generate a potent therapeutic material against resistant bacteria. Amphiphiles can be engineered to self-assemble as micellar carriers for antibiotic cargo leveraging their antibacterial potential (Dey et al. 2018). A schematic representing the mechanism of action of membrane-directed amphiphilic micelles is shown in Fig. 22.5. Researchers helped create cationic peptide amphiphiles (PAs) capable of forming nanofibers, micelles, and twisted ribbons in order to effectively observe antimicrobial activity at the supramolecular level, and these micelles have been observed to have significant antibacterial activity against a variety of gram-positive and gram-negative pathogenic organisms (Rodrigues De Almeida et al. 2019).

Recently there has been a surge in attention toward polymeric micelles as a possible controlled delivery vehicle as they are less prone to disintegration at small concentrations as compared to surfactant micelles and can preserve the

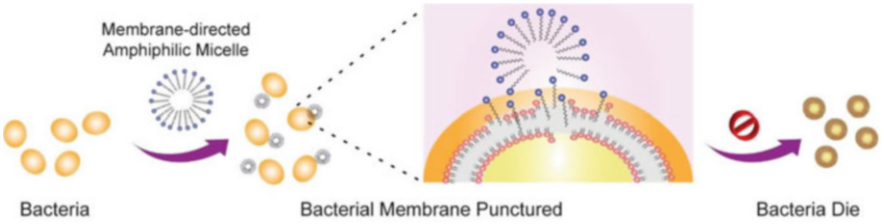

Fig. 22.5 Schematic representing mechanism of action of membrane-directed amphiphilic micelles

micellar framework that enables sustained circulation in the bloodstream (Rijcken et al. 2007). A hydrophobic core surrounded by a hydrophilic outer shell is their general morphology. Yuan et al. explored the use of stearic acid-g-chitosan polymeric micelles for the purpose of oral drug delivery (Yuan et al. 2010). Temperature, chemical or enzymatic hydrolysis of side chains, pH, oxidation, reduction as well as light can often be used as a trigger to destabilize drug-loaded polymeric micelles and vesicles for targeted therapy (Rijcken et al. 2007).The creation of biodegradable antimicrobial polymers like polycarbonate expands the arsenal of appealing antibacterial agents targeting antibiotic-resistant microorganisms (Qiao et al. 2012).

Mondol et al. have described the use of temperature-dependent FRET as a stimulus for the release of rifampicin from an anionic micellar carrier made from sodium dodecyl sulfate (Mondol et al. 2011). There are also records of pH-sensitive polymeric micelles for extracellular and intracellular drug smart release (Liu et al. 2013). Antimicrobials packaged in micellar nanocarriers can be released using not just pH but also bacterial enzymes (Liu et al. 2019). Fluorescently labeled micelles derived from amphiphilic star-block copolymers have the potential to be exploited in a wide range of applications, including cell labeling, imaging, and medicinal administration (Du et al. 2008).

22.6 Antimicrobial Peptide Loaded Nano-Composites

Antimicrobial peptides (AMP) in clinical use have the potential for broad-spectrum efficacy, quick bactericidal activity, and a low proclivity for resistance development, but potential drawbacks include their exorbitant pricing, restricted stability (particularly when constituted of L-amino acids), and uncertain toxicity and pharmacokinetics (Marr et al. 2006). The mechanism of action of AMPs is mentioned in Fig. 22.6. Loading them onto nanocarriers will not only enhance their efficacy but can also help reduce toxicity.

Yu et al. describe the creation of chitosan-polyethylene oxide nanofiber membranes incorporating varying amounts of an antimicrobial peptide NP10 exhibiting strong therapeutic potential against gram-positive as well as gram-negative bacteria along with having excellent biocompatibility in animal wound healing (Yu et al. 2021). The interaction of citrate-stabilized gold nanoparticles with AMP nisin has

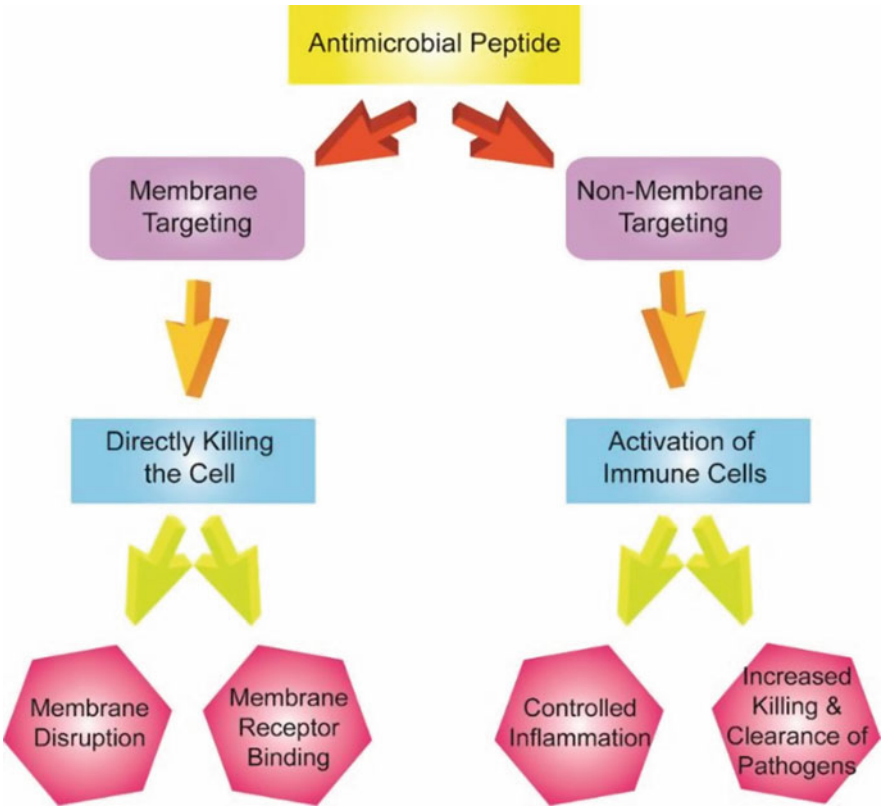

Fig. 22.6 Schematic representing mechanism of action of antimicrobial peptides

been described to result in a potent antibacterial agent. The researchers created a nisin–gold nanoparticle combination that can outmaneuver the problem of nisin's limited solubility and loss of activity at high pH, thereby retaining the unique nisin activity even at high pH (Adhikari et al. 2012). Selective activity and low proclivity to cause collateral damage to beneficial gut microbes present the AMP bacteriocins produced by lactic acid bacteria as effective therapeutic arsenals for targeting gastrointestinal pathogens and a promising alternative to antibiotics. Mukherjee et al. and colleagues established a biocompatible milk protein fraction-based nanocarrier for encapsulation of the bacteriocin pediocin which protects the AMP against proteolysis during delivery in the gastrointestinal environment (Mukherjee et al. 2019). Piras et al. investigated the antibacterial efficacy of AMP temporin B encapsulated in chitosan nanoparticles. The nanocarrier demonstrated persistent antibacterial activity against several *Staphylococcus* strains while exhibiting little cytotoxicity against mammalian cells (Piras et al. 2015).

22.7 Chitosan-/Alginate-Based Nanomaterials as Antimicrobial Carrier

In the recent years, biopolymers have gained great attention for the targeting of microbes. Mostly, these biopolymers are polysaccharides in nature which provide a lot of advantage like biocompatibility, minimal toxicity, and biodegradable nature in the host body. Polysaccharide biopolymers attach to the cells non-covalently due to the presence of carboxyl, hydroxyl, and amino groups, which enhance the half-life of the material into the body (Swierczewska et al. 2016). Polysaccharide biopolymers which are in use these days are chitosan, dextran, cellulose, hyaluronan, agar, and alginate (Das and Pal 2015).

Chitosan is a polysaccharide biopolymer developed from chitin that has a large number of C2 amino groups that can be protonated in slightly acidic environments, giving it antibacterial characteristics. Chitosan can bind to bacterial cell membranes including the cytoplasmic membranes, which are negatively charged causing osmotic instability, membrane breakdown, and intracellular element leakage. Chitosan can penetrate the bacterial/fungal nucleus and suppress mRNA and protein synthesis by attaching it to microbial DNA. Chitosan as a nano formulation has also received merit as an antimicrobial wound dressing agent. They have also been modified to have encapsulated antibiotics, antibacterial extracts, or metallic antimicrobial agents inside them before forming the wound dressing (Kravanja et al. 2019). A schematic representing the properties of chitosan-based nanocarriers is shown in Fig. 22.7.

Alginate is another polysaccharide biopolymer which is anionic in nature and formed by copolymerization of D-mannuronic acid and L-guluronic acid. Due to its biocompatibility and biodegradable nature, alginate has been widely used in wound dressing and development of novel drug delivery systems. Uyen et al. have developed curcumin-loaded alginate microspheres, which exhibit potent antibacterial activity against *S. aureus* and *E. coli* (Thanh Uyen et al. 2020). Moreover, alginate-derived surfactant and its complex with different divalent metals like copper, zinc, and cobalt also have significant antimicrobial activity against gram-negative and gram-positive bacteria (Tawfik and Hefni 2016).

Nanoscale formulation of chitosan, alginate, and metal ions is being developed to load antibiotics, antibacterial extracts, or metallic antimicrobial agents inside them before forming the wound dressing (Kravanja et al. 2019). Friedman et al. have discussed the use of chitosan-alginate nanoparticles for cell membrane-targeted therapy against cutaneous pathogens like *P. acnes.* These chitosan-alginate NPs also inhibit the inflammatory cytokines secreted by monocytes and keratinocytes at the site of infection. Moreover, benzoyl peroxide (a known anti-acne agent) encapsulated into the NPs showed a higher anti-acne effect than benzoyl peroxide alone (Friedman et al. 2013). ε-Polylysine-loaded chitosan-sodium alginate nanoparticles have been investigated by Liu et al. for their antibacterial activity against a number of resistant pathogens, including *S. aureus* and *M. luteus* (Liu et al. 2018). Susilowati et al. developed silver-chitosan-alginate (Ag-Chit-Alg) nanocomposite film,

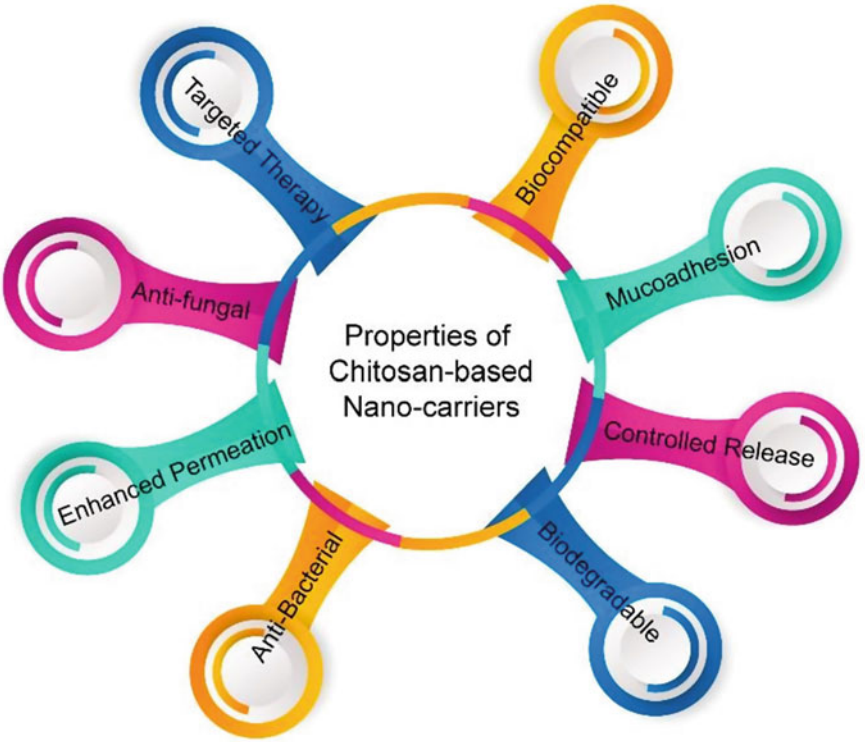

Fig. 22.7 Schematic representing the properties exhibited by chitosan-based nanocarriers

showing good antibacterial activity against *S. aureus* and *E. coli* in the disk diffusion method (Susilowati et al. 2020).

Camptothecin-loaded calcium alginate nanocomposites (Ca-Alg2/CPT) show high antimicrobial potency against *S. aureus, E. coli, and Klebsiella pneumonia.* However, camptothecin-loaded calcium alginate-chitosan nanocomposites (Ca-Alg2-CH/CPT) were active only against E. *coli and Klebsiella pneumonia* (Al-Gethami and Al-Qasmi 2021).

22.8 Quantum Dots

Quantum dots (QDs) are the specialized nanoparticles that are formed by assembly of crystalline clusters of semiconducting materials like cadmium-tellurium (CdTe), modified graphene, zinc oxide (ZnO), etc. QDs show higher photoluminescence and photostability than conventional fluorescent dyes, making them efficient tools in advanced biosensors, cell imaging, and in vivo animal tracking. QDs also play a crucial role in identifying bacteria and understanding complex microbial

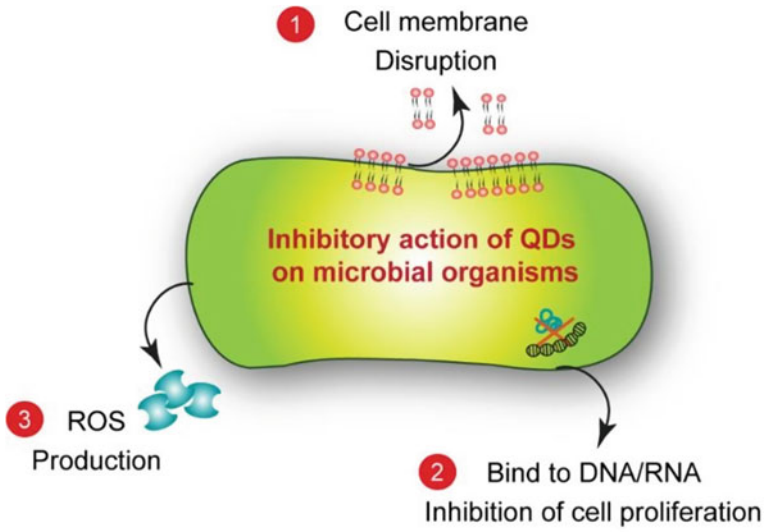

Fig. 22.8 Modes of action of quantum dots

arrangements in a colony. Single bacterium imaging is one of the most important applications of QDs in microbial research, which can be achieved by making probe-conjugated QDs (Hahn et al. 2005). The various modes of action of quantum dots are depicted in Fig. 22.8.

Besides diagnostics and sensing, QDs also have excellent antimicrobial properties, which can be further enhanced by functionalizing with polymers. There are three main modes of action: production of ROS, the disruption of cell walls/membranes, and the halting of expression of genes (Rajendiran et al. 2019). The electrostatic interaction of the positively charged ions of quantum dots with the negative charge of cellular constituents causes membrane distress. The introduction of metals/ions of the quantum dots into the cell enhances toxicity, culminating in cell destruction. The interplay of quantum dots and nucleus components inhibits cell respiration, division, and adenosine triphosphate (ATP) generation. Cellular apoptosis is the ultimate outcome of nucleus injury. Dong et al. and colleagues have discussed light-activated microbicidal carbon dots (Dong et al. 2020).

22.8.1 Cadmium Tellurium Quantum Dots (CdTe QDs)

CdTe QDs interact with the phospholipid bilayer anchored by the lipophilic tail disrupting the outer peptidoglycan layer of the cell wall. Further, they release the Cd^{2+} ion into the cytoplasm, which accumulates in the bacterial vesicle bodies, leading to heavy metal cytotoxicity. Alternately, CdTe also decreases the expression of superoxide dismutase (Mn-SOD) and endonuclease IV, which governs the

antioxidant activity in the bacterial cells (Kumari et al. 2017). Functionalized CdTe QDs show enhanced antibacterial activity compared to the conventional one. CdTe–rocephin QD complex exhibits enhanced cell wall damage leading to high Cd^{2+} toxicity and low antioxidase activity in *E.coli* (Luo et al. 2011). Furthermore, in rutin- conjugated TGA–CdTe QDs, rutin produces OH^-species, which interacts with the bacterial cell membrane creating positive holes leading to the enhanced entry of QDs into the cell. Cd^{2+} released by QDs and -OH (hydroxyl-free radical) generate higher ROS inside the cell and exhibit more toxicity in *E. coli* (Ananth et al. 2015). Coating CdTe QDs with a biocompatible polymer like poly-L-lysine (PLL) helps in protein-specific targeting in a bacterial system. PLL-CdTe QDs show reduced mammalian cell toxicity, while the PLL bilayer provides increased toxicity in *E. coli* (Li et al. 2013).

22.8.2 Graphene Quantum Dots (GQDs)

Graphene QDs (GQDs) are carbon-based zero-dimensional materials molded into spherical dots of quantum size that have greater photostability and edge effect (Thangamuthu et al. 2019). GQDs bind to the bacterial cell wall via electron transfer and induce oxidative stress in the membrane producing ROS and damaging it, leading to cell death. Functionalized and polymer-conjugated GQDs show higher antimicrobial activity. PEGylated GQDs have higher binding potential to the bacterial cell membrane showing greater antimicrobial activity against *S. aureus* and *P. aeruginosa*. Silver-containing PEGylated silver GQDs show even greater bactericidal activity due to synergistic activity provided by Ag^+ ion, which binds to the thiol group (-SH) containing proteins in the cell membrane and inactivates them, causing cell disruption (Habiba et al. 2015). Moreover, coating of graphene oxide onto the PVDF (GOQD-coated PVDF) produces an antibacterial membrane that can effectively inactivate *E. coli* and *S. aureus* cells and exhibits excellent anti-biofouling activity (Zeng et al. 2016).

22.8.3 Zinc Oxide Quantum Dots (ZnO QDs)

ZnO QDs act by producing electron-hole pairs leading to ROS and perhydroxyl ion formation, damaging the cell wall. Alternately, it directly interacts with the phospholipid bilayer and disrupts it. Accumulation of ZnO QDs into the cell can cause DNA damage and apoptosis (Premanathan et al. 2011). Functionalization further increases the antibacterial activity. Acetate ZnO QDs show excellent antibacterial activity against multidrug-resistant *E. coli* (Mir et al. 2018). PEG-capped ZnO NPs kill both gram-positive (*S. aureus*) and gram-negative (*E. coli*) bacteria (Meshram et al. 2018).

22.9 Hydrogen Dendrimers

Dendrimers are formed in two processes. The divergent dendrimer forms from its core and then extends outward, while the convergent dendrimer forms from the dendrimer's outer and moves inward. Polyamidoamine (PAMAM), poly(propylene imine) (PPI), liquid crystalline, core–shell, chiral, peptide, glycodendrimers, and PAMAM-organosilicon (PAMAMOS) dendrimers are the different types of dendrimers based on their functionalization moieties (Noriega-Luna et al. 2014). Of special interest are PAMAM-based dendrimers (Kannan et al. 2019). PAMAMs are the most researched dendrimer with potential in oral drug administration because they are water soluble and can penetrate past epithelial tissue, enhancing their transmission through the paracellular pathway. The selectivity of action is determined by the unique molecular structure of these entities. The macromolecules are distinguished by their high molecular weight, highly branching spherical 3D structure, and capacity to form monodisperse media (Aurelia Chis et al. 2020). Simple encapsulation, electrostatic contact, and covalent conjugation are methods for loading drugs into dendrimers (Hoffman 2013). The various surface moieties present on the PAMAM dendrimers have specific interactions with the bacteria membrane (Castonguay et al. 2012) as depicted in Fig. 22.9. In recent years, several self-assembled cationic amphiphilic dendrimers with various supramolecular structures have been shown to have significant antibacterial action with enhanced specificity. Furthermore, the comprehensive mechanistic analysis demonstrates that proper adjustment of the hydrophobic nature of amphiphilic dendrimers is critical in bacterial membrane rupture (Kannan et al. 2019).

22.10 Current Limitations and Future Prospects

Nanoparticles have shown synergy when combined with appropriate antibiotics which potentially help to limit the global challenge of escalating bacterial resistance especially by the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species) pathogens. Nanostructured materials are increasingly being employed as a bactericidal alternative for antibiotics as well as additives in a number of composites. The existing studies have impediments in that most antibacterial methodologies of nanocarriers remain currently unknown. One shortcoming of present investigations on the antibacterial effects of nanoparticles is the absence of coherent standards especially with regard to the duration of treatment for effective activity.

There is minimal knowledge regarding nanocarrier metabolism, clearance, and toxicity, as well as the nature of appropriate targets for specific pathogens and also the optimum dosage for therapeutic efficacy at pathogen target locations. As a result, no single method can address all of the conditions for obtaining data concerning the

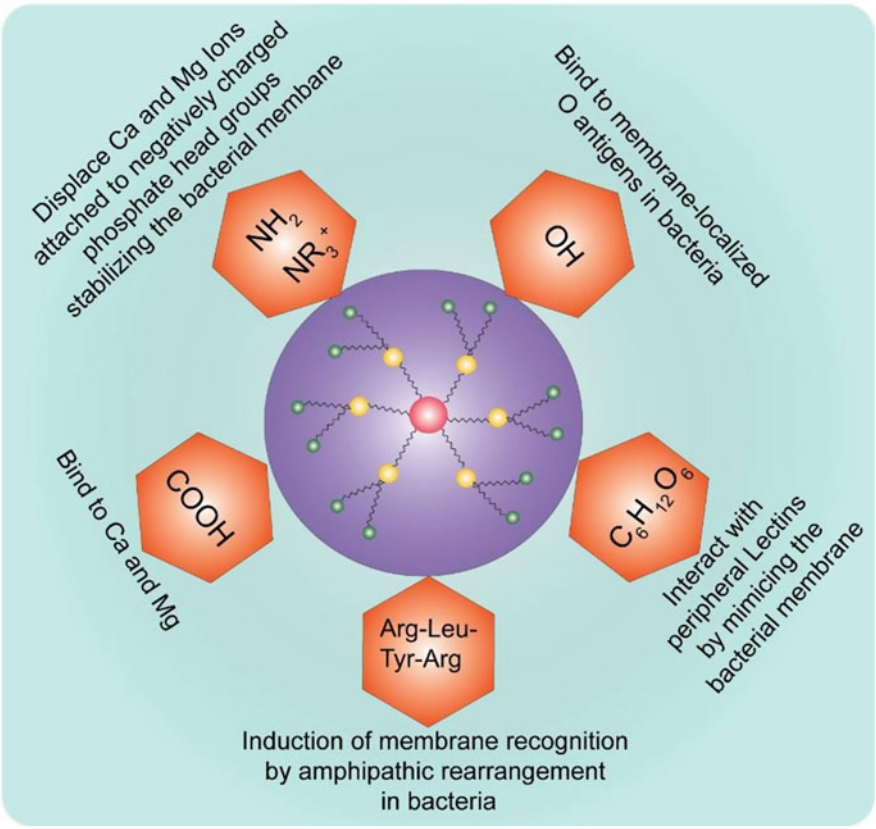

Fig. 22.9 Mechanisms of interaction between surface groups of PAMAM dendrimers and the bacteria

antibacterial processes of nanocarriers because each type of nanocarrier exhibits diverse bactericidal effects.

Moreover, because of their size, practically all nanocarriers have limited transport; however, porins which generally allow the passage of molecules <600 Da can mediate the passage of nanocarriers with diameters ranging from 1 to 9 nm through the bacterial cell membrane. The oxidative stress caused by nanoparticles needs investigation, and research into intracellular inhibitory pathways is very restricted, and few researches have looked at how nanocarriers affect gene expression, protein synthesis, and bacterial cell metabolism.

Early diagnosis of the bacterial infection can help us eradicate the complex bacterial infection and avoid the misuse of broad-spectrum antibiotics. Bacterial infection biomarkers like C-reactive protein (CRP), procalcitonin (PCT), soluble triggering receptor expressed on myeloid cells 1 (sTREM-1), hemoglobin scavenger receptor (CD163), and high mobility group box 1 (HMGB1), etc. can be exploited for the targeting of bacteria. The development of quantum dot- based

nanocomposites conjugated with biomarkers like PCT/CRP can help in early diagnosis and site-directed targeting of acute infection. These PCT/CRP-conjugated QD nanocomposites can be used for imaging and tracking the degree of infection. In addition, it can also impart antibacterial and anti-inflammatory effects in the in vivo models.

Conjugated as well as conducting polymers can be exploited as promising materials for delivery of drug and detection of localization of infection via fluorescence visualization. Another noninvasive method includes the use of magnetic resonance imaging (MRI) contrast reagents like gadolinium ions (Gd^{3+}) or iron oxide nanocomposites to construct drug carriers which can then be tracked via imaging.

The prospect of using nanocarriers which can inhibit the synthesis of bacterial proteins and DNA is very interesting, for example, nanocarriers influencing bacterial denitrification can lead to many downstream effects on genes regulating the expression of proteins involved in nitrogen metabolism, electron transfer, and substance transport leading to combatting resistance mechanisms.

Nanocarriers, which can cause the membrane potential to collapse, as well as limiting ATPase activities to lower the ATP level, can render a bacteria unable to exhibit resistance, so loading a drug into it will result in increased killing of pathogenic bacteria.

Nanocarriers that are capable of generating ROS through oxidative stress and stimulating the electron transport chain to create superoxide can enhance the death of remaining bacteria by catabolizing the carbon source and producing nicotinamide adenine dinucleotide.

Creation of nanocarriers which can regulate the expression of metabolic genes imperative for bacterial subsistence like glucose metabolism genes can be used to regulate bacterial cell pathogenicity and will act in synergy with the drug cargo for facilitated killing.

Overall, the future of antibacterial nanocarriers for drug delivery against pathogens leading to enhanced killing is open to imagination. There are a number of interesting branches which can be investigated to curb the present problem of antibacterial resistance.

22.11 Conclusion

Multidrug resistance and persistent microbial infections are a serious healthcare concern worldwide leading to the generation of superbugs. Efforts are in place for the discovery of new antibiotics; however, repurposing existing therapeutics seems to be a more practical approach. One of the major areas of focus in nanomedicine research in recent years has been the merging of therapy with diagnosis (theranostics). Nanocarriers are imperative in this endeavor. Synthetic polymer particles, liposome formulations, micellar nanoparticles, protein nanoparticles, nanocrystals, and many more nanotechnology-based products have skyrocketed,

typically in combination with pharmaceuticals or biologics. Not only do they help curb the resistance barrier by providing protection to the cargo but also provide additive, adjuvant, or synergistic effect thereby enhancing the outcome of the therapy. Targeted delivery also enhances the bioavailability, thereby reducing the dosage and in turn preventing any sort of toxicity to the adjacent human tissues. Nanocarriers are indeed a feasible alternative to antibiotics, and they promise great potential for addressing the problem of microbial multidrug resistance. Therefore, using nanocarrier-mediated antibiotic delivery is a smart approach which kills two birds with one stone.

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Conflict of Interest The authors declare no conflict of interest.

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