Nanotechnology in the Life Sciences

Vinay Kumar
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Nano-Strategies for Addressing Antimicrobial Resistance

Nano-Diagnostics, Nano-Carriers, and Nano-Antimicrobials

Springer

Nanotechnology in the Life Sciences

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Nano and biotechnology are two of the 21st century's most promising technologies. Nanotechnology is demarcated as the design, development, and application of materials and devices whose least functional make up is on a nanometer scale (1 to 100 nm). Meanwhile, biotechnology deals with metabolic and other physiological developments of biological subjects including microorganisms. These microbial processes have opened up new opportunities to explore novel applications, for example, the biosynthesis of metal nanomaterials, with the implication that these two technologies (i.e., thus nanobiotechnology) can play a vital role in developing and executing many valuable tools in the study of life. Nanotechnology is very diverse, ranging from extensions of conventional device physics to completely new approaches based upon molecular self-assembly, from developing new materials with dimensions on the nanoscale, to investigating whether we can directly control matters on/in the atomic scale level. This idea entails its application to diverse fields of science such as plant biology, organic chemistry, agriculture, the food industry, and more.

Nanobiotechnology offers a wide range of uses in medicine, agriculture, and the environment. Many diseases that do not have cures today may be cured by nanotechnology in the future. Use of nanotechnology in medical therapeutics needs adequate evaluation of its risk and safety factors. Scientists who are against the use of nanotechnology also agree that advancement in nanotechnology should continue because this field promises great benefits, but testing should be carried out to ensure its safety in people. It is possible that nanomedicine in the future will play a crucial role in the treatment of human and plant diseases, and also in the enhancement of normal human physiology and plant systems, respectively. If everything proceeds as expected, nanobiotechnology will, one day, become an inevitable part of our everyday life and will help save many lives.

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Preface

Antimicrobial drugs, especially the life-saving antibiotics, once the backbone of modern clinical medicine, are facing serious challenges from emerging antimicrobial resistance (AMR), a condition where microbes develop resistance against the most common antibiotics used against them. Initially considered a nosocomial or hospital-related issue, the AMR unfortunately is spreading at a fast pace in bacterial species of community origins. AMR has exploded in recent years and is posing a serious threat to human health and survival. The drying pipeline of novel and effective antibiotics effective against these drug-resistant "superbugs" has further aggravated the situation, and we are staring at a potential "post-antibiotic era." AMR has been declared a global risk by intergovernmental agencies. This necessitates novel and effective ways for diagnosis, drug delivery, and treatment of AMR infections. Nanostrategies including nano-diagnostics, nano-carriers, and nano-antimicrobials are gaining momentum and are hailed as a potential solution in containing AMR.

The main objective of this book is to promote research themed on the potential use of nano-approaches for diagnosis, detection, drug delivery, and as antimicrobial agents against drug-resistant pathogenic microbes, by providing an integrated blend of basic and advanced information for students, scholars, scientists, and practitioners interested or already engaged in research involving these areas and themes.

This book presents the current understanding and updates on nano-strategies for combating AMR. The chapters in this book cover aspects ranging from advances in nanomaterials synthesis, characterization, functionalization, and improvisation to their applications in the diagnosis of AMR and their therapeutic and drug-delivery potentials against AMR phenotypes. Chapters are written by highly acclaimed experts of international repute working on different aspects of AMR and targeting it with nano-based approaches.

We express our sincerest thanks and appreciation to our eminent authors for their first-rate and timely contributions. We gratefully acknowledge the reviewers for their valuable comments that helped in the improvement of the scientific content and quality of the chapters.

We also thank the Springer publishing team comprising the publisher, editorial project manager, and the entire Springer production team for their consistent hard work in the publication of this book.

Pune, India — Vinay Kumar
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Vinay Kumar is working as an associate professor in Department of Biotechnology, Modern College (Savitribai Phule Pune University), Ganeshkhind, Pune, India. He obtained his PhD in biotechnology from Savitribai Phule Pune University in 2009. He has published more than 65 peer-reviewed research/review articles with over 4000 citations, 27 book chapters, besides editing 6 books. He is a recipient of Young Scientist Award from SERB, Government of India. His research interests include antimicrobial resistance (AMR) in community settings, elucidating mechanisms underlying AMR, and combating it with phytochemicals and nanomaterials. He is on the editorial and reviewer boards of reputed journals.

Varsha Shriram has completed her PhD in natural products chemistry and biotechnology at CSIR-National Chemical Laboratory, Pune, and Savitribai Phule Pune University, Pune, India. She is currently working as an associate professor, Botany Department, Prof. Ramkrishna More College, Akurdi (Savitribai Phule Pune University), Pune, India. She has published more than 35 peer-reviewed research/review articles and is on the reviewer board for reputed journals. Her areas of research interest include medicinal plants, phytochemicals, and bioactivities including anticancer and antimicrobial resistance reversal. She has completed extramural research projects in these and allied areas.

Ravi Shukla is currently Associate Professor of Bioscience and Food Technology, and co-leader at Sir Ian Potter Biosensing Facility at RMIT University, Australia, and routinely engages and contributes to day-to-day university academics. He has over 20 years of research experience in the interdisciplinary research areas spanning biosciences, food science, and materials science. Dr. Shukla has supervised 15 PhD, 5 master's, and more than 50 undergraduate researchers through to completion. His peers regard him as a meticulous cell and molecular biologist and cross-disciplinary researcher working towards understanding of cellular responses to materials. He is a co-editor-in-chief of *Current Research in Nutrition & Food Science* and serves on editorial board of several journals including special sections on nanobiotechnology in *Frontiers* open access journals. He has authored more than 100 research publications with over 6500 citations and a Hirsch index of 37, and has contributed to over 20 patents and invention disclosures for his inventions.

Suresh Gosavi is currently working as Senior Professor of Physics, director of the School of Physical Sciences, head of Environmental Science Department, and director of the School of Basic Medical Science at Savitribai Phule Pune University, Pune, India. He has also worked as visiting professor at the University of Franche Comté, Basancon, France; King Saud University, Saudi Arabia; and the University of Melbourne, Australia, and is presently working at PIRC, Tokyo University of Science, Tokyo, Japan. He has more than 30 years of teaching and research experience. His research interests mainly include nanomaterials and nanotechnology/nano-bio systems and applications, besides nano-capsules and targeted DNA vaccines for immunotherapy of cancer; micro and nanofludics; and solar thermal technology for rural electrification. He has supervised 26 doctoral and 16 MPhil students, besides mentoring 14 postdoctoral fellows. He is a fellow of Maharashtra Academy of Science. Dr. Gosavi is on the editorial and reviewer boards of several journals of repute. He has published 227 journal articles so far and has 8 patents to his credit.

Chapter 1
The History of Antibiotics Illumes the Future of Antimicrobial Peptides Administered Through Nanosystems

Nazim Nassar, Stefan Kasapis, Suneela Pyreddy, and Taghrid Istivan

Abstract The discovery of antibiotics in the early nineteenth century and their introduction in the 1940s into clinical practice has revolutionised the global healthcare system. Infectious diseases were controlled, various medical procedures became attainable and the pharmaceutical industry greatly benefited. However, the indiscriminate overuse of these antibiotics has contributed to an emerging bacterial resistance. Hence, it encouraged the search for new antimicrobial peptides (AMPs) and nano-drug delivery systems, which can leverage these bioactive therapeutics with a tuneable controlled delivery system for clinical use against microbial infections. This review presents a brief history on the treatment of infectious diseases during the pre-antibiotic era, the golden era of conventional antibiotics, the emergence of the superbugs, and the surge in the AMP research and pharmaceutical exploration to offer a new class of pharmacotherapy through a wide range of different nano-vehicles as alternative options for antibiotics to treat various infectious diseases.

Keywords Antibiotics · Antibiotic resistance · Infectious diseases · Antimicrobial peptides · Nanobiotechnology · Nano-drug delivery systems

1.1 Introduction

Humans, alongside animal species, have shared the planet since the very beginning. Thus, it is fair to assume that human ancestors have adopted similar survival and reproducibility strategies that animal species successfully employed in their behavioural defence mechanisms and self-medicating habits against pathogens and parasites (Hart 2011, Shurkin 2014). For thousands of years, flora and herbal remedies played a crucial role in traditional medicine in eradicating a long list of historical

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pandemics, which includes the Plague of Athens in 430 BC, the Plague of Cyprian between 250 AD and 270 AD, the Plague of Justinian in 540 AD, the Black Death of China and then Russia in the fourteenth century, the Great Plague of London in 1665, the Cholera in Paris in 1832 and the Malaria in 1897, amongst many others (Swenson 1988; Hassel et al. 2002; Aminov 2010; Holmes 2011; Wray 2012; Spinney 2019). Nevertheless, inquisitive scientists' and medical historians' revelations often argue the distinctive abilities and the active adaptation of human beings for survival throughout history. Case in point, numerous research projects have shown how moulds were commonly used by different civilisations, including the Greek (during the sixteenth-century BC) and the Chinese (3000 years ago) to treat wounded soldiers with infected skin wounds (Wainwright 1989a; Bentley 2005). Moreover, several archaeological studies showed traces of tetracycline in human bones that stretch back to the Nubia and Egyptian civilisations that inhabited Southeast Africa more than 1400 years ago (Bassett et al. 1980; Cook et al. 1989; Nelson et al. 2010). These findings argue that different civilisations had different views and experiences in survival and fighting of the unknown microbial diseases long before biomedical sciences took control of disease diagnoses and treatments during the early decades of the twentieth century.

In 1928, Professor Alexander Fleming, a physician-scientist, experimented with the mutual biological interaction between *Staphylococcus aureus* (an aerobic bacterium) and airborne mould spores. His attempt was based on previous observations of *Staphylococcus aureus* colony lysis in a contaminated petri dish with fungal infection (Fleming 1929). Subsequently, Professor Fleming has stated that "It was found that broth in which the mould had been grown at room temperature for 1 or 2 weeks had acquired marked inhibitory, bactericidal and bacteriolytic properties to many of the more common pathogenic bacteria" which, thereupon, have changed the modern medical practice beyond recognition.

Thus, the antibiotic era started almost 90 years ago based on the "magic bullet" concept that Paul Ehrlich introduced in 1910 and the discovery of penicillin by Alexander Fleming in 1928 (Strebhardt and Ullrich 2008; Aminov 2010). Paul Ehrlich has connected chemistry to biology by introducing the "magic bullet" concept, which became the cornerstone of the pharmaceutical industry, and many medicines have been produced, including antimicrobials based on this concept (Strebhardt and Ullrich 2008). The "magic bullet" concept refers to the specificity of drug activity through its affinity to a specifically targeted receptor exerting its effect on the target cell or pathogen without affecting the rest of the biological system (Elliott 2010).

The golden age of antimicrobial pharmacotherapy, which was initiated by the discovery of penicillin in 1928, started gaining momentum in the early 1930s of the twentieth century when the German scientist Gerhard Domagk discovered the sulpha drugs in 1932 (Oesper 1954). Soon after, this group of drugs was developed to sulphonamides, one of the most effective antibiotics against a wide range of infections, including pneumonia and gonorrhoea (Stanwell-Smith 2007). However, penicillin stayed in the lead, and during the Second World War, it continued to do wonders in treating various infections between skin injuries and systemic

infections. Bacteriologists continued their research and have listed different penicillins, streptomycin, chloramphenicol and tetracyclines in their list of discoveries during the 1940s and the 1950s (Rebstock et al. 1949; Daniel 2005; Stanwell-Smith 2007; Liu and Myers 2016). Benjamin Duggar discovered aureomycin (chlortetracycline) in 1948, which was approved in 1950 for human use, followed with natural tetracycline in the early 1950s, such as terramycin, which Pfizer produced in 1950 (Finlay et al. 1950; Liu and Myers 2016). In 1952, Selman Abraham Waksman was awarded the Nobel Prize in medicine for his discovery of streptomycin (Daniel 2005). Streptomycin was tested in vivo against tuberculosis and proved successful in January 1945 at the Mayo Clinic after it was administered to Patricia T, who suffered from that pulmonary infectious disease. Moreover, in 1948, the Italian pharmacologist Giuseppe Brotzu efficaciously isolated *Cephalosporium acremonium* from sewage water in South Italy which resulted in the development of different classes of broad-spectrum cephalosporins (Bo 2000; Chizhik 2014).

However, the confidence in antibiotics and their effectiveness swiftly became sceptical. Since the mid-1970s and early 1980s, bacterial resistance to different antibiotics started breaking the surface leading to the regression of the nineteenth century in terms of infectious disease infestation, which became untrammelled (Stanwell-Smith 2007; Gould 2016). Over the second half of the previous millennium, a random, inconsiderable, unquestionable and unnecessary prescribing and administering of antibiotics to humans, animals, animal food and plants were practised for treatment and prophylactic purposes (Stanwell-Smith 2007; Landers et al. 2012; Stockwell and Duffy 2012). This irrational behaviour of healthcare specialists, pharmaceutical industry workforce and within agriculture has led to profound genetic mutations resulting in virulent strains to survive and thrive. Such risks require immediate and fast interventions to prevent imminent global health crisis.

Hence, in addition to the strict policies concerning antibiotic prescription and use that have been adopted by the World Health Organisation (WHO) and other health authorities around the world, such as controlled supply chains and drug cycling (Stallmann et al. 2006), scientists have started looking for an alternative to the conventional antibiotics. One way to do so is to go back to the early days of humanity and ask the question: what was the critical factor that helped humans and other prokaryotic life forms in their battle against nasty pathogens during their early primitive days? No doubt, it is our innate defence system. So AMPs are inherited natural antibiotics born with us and are available as part of our immune system to fight microbial infections with minimal chance of bacterial resistance (Boman 2003; Stallmann et al. 2006). Such atypical therapeutic molecules endure unique pharmacodynamic behaviours accompanied with fewer side effects (Leader et al. 2008). They are one of the leading categories of nonconventional molecules that exert a remarkable remedial potential against infectious diseases. They demonstrate their pharmacological effects via specific cellular pathways resembling natural molecular signalling.

Nevertheless, these peptides are immensely susceptible to physicochemical and biological degradations preventing them from reaching the microbial pathogen at the vital bioavailability. Poor luminal permeability, high cytosolic metabolism,

gastric degradation and the first hepatic clearance of these molecules have executed most of therapeutic peptides including antimicrobial from being translated into medicines (Gupta et al. 2013; Muheem et al. 2016). Moreover, all of the currently used antimicrobial peptides and proteins are formulated in a parenteral form that requires high-cost cold chains for storage and transport.

This chapter aims to shed some light on the history and the process of developing antibiotics and the emerging bacterial resistance. However, our focus will be on antimicrobial peptides as the inherent and intrinsic self-defence mechanism behind our powerful immune system and the latest developments in nanobiotechnology as an adopted strategy to develop systemic and topical targeted delivery vehicles.

1.2 Antibiotics: Key Events from Prehistory to the Golden Age

The awareness of the invisible pathogens, their effect on the human body and how to eradicate them was evident since the early days of our ancient history. Substantial historical evidence has shown that ancient civilisations, including Ancient Egypt and Southeast Africa, Middle East, China, Greece and the Romans, used different antimicrobial remedies including honey, herbs, clay and mouldy bread to treat systemic and skin infections (Wainwright 1989a; Martin and Ernst 2003; Newman and Cragg 2007; Falkinham III et al. 2009; Eteraf-Oskouei and Najafi 2013; Gould 2016; Kirchhelle 2018; Qiao et al. 2018). Surprisingly, even tetracycline was detected in human bones exhumed from Sudanese and Egyptian cemeteries going back to the Nubia and Roman occupation era (Nelson et al. 2010; Gould 2016).

Archaeologists, anthropologists and biologists frequently and silently travel for their adventures and share their knowledge to uncover the discreet. In the early 1980s of the last century, different archaeological scientific projects, carried out in Southeast Africa, uncovered human bone remains that exhibit traces of a 1400-year-old tetracycline (Bassett et al. 1980; Cook et al. 1989; Nelson et al. 2010). Fluorescence microscopy tests showed an identical fluorescence imaging between the antibacterial agent of tetracycline and the contamination from the bone recovered from the X-group cemetery (Bassett et al. 1980). Historically, the X-group cemetery belongs to one of the Sudanese Nubia tribes who lived on the west bank of the Nile River across from Wadi Halfa in Sudan (Bassett et al. 1980; Nelson et al. 2010). The counteracting claim argued that the fluorescence microscopy results from the bone discoloration are merely due to fossilisation or the so-called taphonomic process and the decomposition of the dead bone tissue performed by fungi (e.g. *Stachybotrys* and *Cladosporium*). This fossilisation mechanism produces an intermediate substrate with a leaching effect (Piepenbrink 1986; Schaller et al. 2015) rather than calcium complex formation between the tetracycline, as a chelating agent, and the calcium that exists in the bone tissue (Menon 2014; Shasmitha 2014). However, Bassett et al. have dismissed this possibility since the fluorescence

was patterned in the same manner as the modern tetracycline-labelled and not diffused as it appears in the post-mortem mould infestation.

Moreover, the bones were found undamaged, reducing the chances of mould infestation (Bassett et al. 1980). Furthermore, a histological ageing study performed by Cook et al. on femoral mid-shafts goes back to the Roman period in Dakhleh Oasis, Egypt, has shown a distinct fluorochrome labelling (Cook et al. 1989). These fluorochromes were also traced within the enamel matrix of some individuals of that population. Moreover, the fluorescent patterns obtained from these fluorochromes correspond to those obtained from patients treated regularly with tetracycline. These findings argue that the fluorochrome is most likely tetracycline that was administered in vivo over a prolonged time. In addition, tracing the fluorochrome in the enamel supports Bassett's theory, which argues that the fluorochrome found in the remains of the Nubia tribes was an ingested tetracycline rather than a fungi contamination based on the fact that enamel is an avascular body compartment and, unlike the bone, is more resistant to contaminations introduced by taphonomic processes (Cook et al. 1989). Finally, according to Cook's observations, including periosteal examinations, the effect of the tetracycline on these populations was for antibacterial and prophylactic treatment.

Based on the above, it is easy to agree that the usage of some antibiotics was available a long time before the discovery of penicillin. However, the argument is that the revolutionised treatment of infectious diseases, which started less than 100 years ago, was validated after introducing novel antibiotics into a scientifically regulated medical field (owing to Paul Ehrlich and Alexander Fleming). Statistical data from the USA has shown a significant annual decline of 8.2% in the infectious disease mortality rate between 1938 and 1952 (Armstrong et al. 1999). The early bright of this era started when Rudolf Emmerich has revealed the first antibacterial substance in modern history (Gould 2016). The pyocyanase, a proteolytic substance extracted from *Bacillus pyocyaneus*, exerts autolytic activity, against its source, and the lytic activity against *Staphylococcus* and *Streptococcus* species (Waksman 1948; Gould 2016).

Further advancements in the golden era of antibiotics are owed to the German chemist, Paul Ehrlich, and his team in 1909, after their famous discovery of the arsenic derivative, Salvarsan or dioxydiamidoarsenobenzol, for the treatment of syphilis (Scovil 1912). The yellowish powder produced in an injectable form has been selected from 606 other compounds despite its high level of toxicity, which pushed towards the new formulation (neosalvarsan) in 1912. The neosalvarsan was an optimised derivative of the original Salvarsan after improving its solubility. Also, the acidity of the new product was reduced, which eliminated the need to use soda as an alkaline base additive before the injection (McIntosh et al. 1912).

During the 1920s, Joseph Klarer, Fritz Mietzsch and Gerhard Domagk discovered the antibacterial sulpha agents or sulphonamides. This group consisted of three main categories of sulphonamide series of antibacterial drugs that have been given the generic name of Prontosil by the Bayer Products Ltd (Nature 1938). At that time, Dr. Doris Brown from Royal Maternity Hospital, Belfast, reported that Prontosil drugs were significantly effective against septicaemia caused by

haemolytic *Streptococci*. Three years of comparison trials have found that Prontosil has reduced the death rate caused by general septicaemia from 87.5% to 28.57% and the mortality rate caused by *Streptococcus* spp. from 23.5% to 6.6%. There were further encouraging results reported on the remarkable effectiveness of Prontosil usage against urinary tract infections caused by *E. coli* (Nature 1937).

During the same period and precisely on the third day of September 1928, Alexander Fleming witnessed the birth of penicillin. He observed accidental mould contamination in a petri dish-contained bacteria for influenza research purposes, inhibiting the growth of the nearby *Staphylococcus* colonies. This mould was *Penicillium notatum* (initially identified as *Penicillium rubrum*) which produces a substance against bacterial growth, which was named penicillin (Marshall 1946; Wainwright 1989a; Wainwright 2002). Indeed, the discovery of penicillin by Alexander Fleming was one of the most remarkable scientific achievements in the modern history of the medical field. However, Professor Fleming himself underestimated this attainment at the start. His evaluation of the initial product was described as good as a topical antiseptic substance, and after few years from his initial observations, he started consulting chemists for purification techniques and chemical stability studies of the product (Aminov 2010; Lobanovska and Pilla 2017).

Nevertheless, Fleming's publication in 1929 about the penicillin discovery has reached the hands of two gurus, Howard Florey and Ernst Chain, from Oxford's Sir William Dunn School of pathology. They followed Fleming's discoveries, including his 1922 work on antibacterial lysosome, trying to look for a product invention (Swann 1983). The Oxford team started the purification process of penicillin, and in 1940, they conducted their first trial on infected animals with haemolytic *Streptococci* where they found that the treated group survived as much as threefold of the untreated control (Swann 1983). Soon after, Florey and Chain discovered that penicillin is effective against different *Staphylococcus* spp., *Streptococcus* spp. and *Gonococcus* strains. These observations have advantaged penicillin over sulphonamides, which was limited to *Staphylococci* strains (Swann 1983). Consequently, in 1941, penicillin was commercialised and started saving the lives of millions of civilians and soldiers on the battlefields around the world during the Second World War (Aminov 2017).

Further advancement in the penicillin world occurred in 1959 when Batchelor, Doyle, Nayler and Rolinson from Beecham Research Laboratories, Ltd, reported the amine 6-amino-penicillanic acid (6-APA) in penicillin fermentation (Batchelor et al. 1959; Batchelor et al. 1961). Acetylation of 6-APA results in synthesising various semisynthetic penicillins, including penicillins, against Gram-negative bacteria such as *Escherichia coli*, *Haemophilus*, *Listeria*, *Neisseria*, *Proteus mirabilis*, *Shigella and Salmonella*. Other examples include ampicillin, amoxicillin, bacampicillin and penicillins against *Enterobacteriaceae* and *Pseudomonas aeruginosa* such as carbenicillin and ticarcillin penicillinase-resistant penicillins including methicillin, oxacillin and nafcillin (Wright 1999; Aminov 2017).

Moreover, in 1947, John Ehrlich and his team discovered chloramphenicol, the first member of the amphenicol group to be discovered (Ehrlich et al. 1948). They found that *Streptomyces venezuelae*, which was extracted from a soil sample near

Caracas in Venezuela, is the source of chloramphenicol (Ehrlich et al. 1948). In 1954, Gottlieb et al. have described the natural production process of the chloramphenicol by *S. venezuelae*, which is encouraged by adding P-nitrophenylserinol to a synthetic glycerol lactate medium, and soon after, it became the first antibiotic to be chemically synthesised (Gottlieb et al. 1954; Gottlieb et al. 1956; Dinos et al. 2016). The physiochemical characteristics of chloramphenicol support its permeability through the blood-brain barrier, and it was found to have a bactericidal effect against meningitis-related bacterial species including *Haemophilus influenzae*, *Streptococcus pneumoniae and Neisseria meningitidis* (Dinos et al. 2016). However, neurotoxicity and haematological disorders such as bone marrow depression and aplastic anaemia, amongst others, limited its usage (Aminov 2017).

This notable scientific success in antibiotic discovery in the golden era and the tremendous impact of the clinical translation on the medical field has inspired entrepreneurial scientists and commercial pharmaceutical companies worldwide for more innovations and blooming in the antimicrobial arena. Waksman's argument in the early 1940s that "a considerable proportion of all actinomycetes that can be isolated from soils or other natural substrates have the capacity of inhibiting the growth of, and even of destroying, bacteria and other microorganisms" has captivated the minds of many scientists as well as the attention of the pharmaceutical industry with the antibiotic rush (Waksman et al. 1946; Nelson and Levy 2011). In the early 1940s, a retired professor and a world specialist named Benjamin Minge Duggar has joint the Cyanamid as the head of the soil department looking for actinomycetes in a wide range of soil samples sent to him from all around the world with a vision to discover a novel antibiotic (Nelson and Levy 2011).

In 1945, Duggar and his team had witnessed an unusual antimicrobial activity of a yellow coloured sample that wiped off all the Gram-positive and Gram-negative bacteria in the tested group. Duggar named this broad-spectrum substance *aureo*, *meaning* gold (yellow) in Italian (Duggar 1948; Yan and Song 2014). Aureomycin, or chlortetracycline, was the first of the tetracycline class of antibiotics to be discovered and 3 years later was experimented successfully on a five-year-old Tobey Hockett who had a life-threatening post-operative wound infection at the John Hopkins Children Hospital in Washington, DC (Nelson and Levy 2011). Nowadays, tetracyclines belong to a large family of antimicrobials and are the second widely used antibiotic in humans, animals and agriculture (Zhang et al. 2011; Yan and Song 2014).

After discovering streptomycin, scientists have intensified the screening of actinomycetes resulting in further discoveries (Benedict 1953). In 1949, a new antibacterial agent was exposed in Iloilo City on the Philippine Island. Dr. Abelardo Aguilar collected a soil sample analysed at the Eli Lilly Research Laboratories, where they isolated the antibiotic erythromycin-A (Robertsen and Musiol-Kroll 2019). Erythromycin-A is a polyketide antibacterial substance produced by *Saccharopolyspora erythraea*, a Gram-positive soil bacterium (Jiang et al. 2013). Erythromycin-A has shown a great deal of similarity with penicillin. It is effective against a wide range of bacteria including Gram-positives and acid-fast rods, in addition to some of the following Gram-negative genera, *Brucella*, *Haemophilus*

and Neisseria, thereby providing an alternative treatment for patients with penicillin allergy (Benedict 1953).

Furthermore, in 1947, cyclic peptide with a hydrophobic backbone chain was named colistin, or polymyxin-E, after its discovery in Japan (Storm et al. 1977; Aminov 2017). Colistin is a member of the polymyxins family of antibacterial peptides produced by *Paenibacillus polymyxa*, a Gram-positive bacterium found in soil, plants and marine precipitants (Ainsworth et al. 1947; Stansly and Schlosser 1947). Colistin was found to be a remarkable substance as an active antibacterial agent against Gram-negative bacteria (Stansly and Schlosser 1947). Colistin's cationic cyclohepta peptide ring targets the anionic lipopolysaccharide (LPS) molecules in the outer cell membrane of the Gram-negative bacteria. Attacking the LPS by the cationic moiety of the colistin leading to a non-enzymatic disturbance of the magnesium and calcium ions' positioning (two metals that stabilise the membrane phospholipids by bridging the negatively charged phosphor-sugar molecule) destabilises the negatively charged LPS molecules, leading to cell membrane damage. Moreover, destabilisation of the cytoplasmic membrane allows the antimicrobial peptides to interact with the cytoplasmic organelles (Zasloff 2002; Falagas and Kasiakou 2005; Zhang and Gallo 2016). This cationic intervention results in local destruction of the outer membrane integrity, promoting membrane permeability and leakage of the cell content and cell lysis of the targeted bacteria (Newton 1956; Laporte et al. 1977). Based on the above results, it was approved in 1949 for clinical use, and it was utilised against pathogens resistant to standard treatments such as β-lactams, aminoglycosides and fluoroquinolones (Evans et al. 1999; Falagas et al. 2008). Moreover, colistin was the last resort antibiotic against life-threatening infections caused by multidrug-resistant bacterial pathogens including *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Endo et al. 1987; Falagas et al. 2008). However, due to systemic severe side effects such as nephrotoxicity, neuromuscular blockade and acute airway obstruction, colistin's usage was banned except for patients with cystic fibrosis (Evans et al. 1999; Falagas and Kasiakou 2006).

One of the significant breakthroughs during that golden era was the discovery of vancomycin in 1953. Vancomycin was purified from a soil sample sent from Borneo, in Southeast Asia, to Dr. Edmund C. Kornfeld, a chemist at Eli Lilly (Levine 2006), which represented, at that point, a new class of antibiotics produced by *Streptomyces orientalism.* Vancomycin is constructed from peptides with a carbohydrate moiety attached to the amino acid residues forming glycopeptides. These peptides possess high selective toxicity against different bacterial pathogens including the Gram-positive penicillin-resistant *Staphylococcus* spp., and the anaerobic *Clostridium* spp., in addition to the Gram-negative bacterium *Neisseria gonorrhoeae* (Reynolds 1989; Levine 2006; Chen et al. 2011; Aminov 2017). Vancomycin, like other glycopeptides, is a large and rigid molecule that configurationally fits into the particular structure of the bacterial cell wall precursor, sequenced as L-Ala-D-Ala-D-Ala, forming a stable hydrogen bonding which results in blocking of the transglycosylation process of bacterial cell wall peptidoglycan biosynthesis (Allen and Nicas 2003). Therefore, in 1956, the FDA has approved vancomycin as an

antibacterial drug; however, its usage was limited until the emergence of the methicillin-resistant *Staphylococci aureus* (MRSA) in the mid-1980s (Chen et al. 2011).

In 1959, a new class of antibiotics against one of the most indelible and debilitating pulmonary infectious diseases had been discovered. Sensi et al. from Lepetit laboratories in Milan, Italy, have managed to isolate an antibacterial substance which they named rifamycin based on its initial nickname (Rififi), via a process of broth fermentation performed by *Amycolatopsis mediterranei* – a member of *Actinomycetaceae* group (Lester 1972; Sensi 1983; Murray et al. 2015). Further studies have identified rifamycin as a constituent of five different substances, including rifampicins A, B, C, D and E (Lester 1972). Rifamycin-B portion composed less than 10% of the whole mixture, and it was the only substance that had been purified in a crystalline form where the rest were unstable to be isolated or characterised (Sensi 1975; Sensi 1983). Moreover, the purified rifampicin-B showed a low level of activity in its initial chemical form; nevertheless, it demonstrated an insignificant degree of toxicity and a notable therapeutic effect against pathogenic infections in animals which, in turn, inspired further pharmacotherapeutic-related chemical modifications of the molecule (Lester 1972; Sensi 1983). Hence, 1963 rifamycin SV was developed based on rifampicin-B's spontaneous activation chemical process (Sensi 1975, Sensi 1983). Rifamycin-B has demonstrated a unique ability to undergo a spontaneous and reversible transformation into a rifamycin-O in an oxygenated aqueous medium. In turn, rifamycin-O goes through a hydrolysis process which forms the so-called rifamycin-S by losing a glycolic acid, thereafter a mild reduction of the S form results in rifamycin SV. Rifamycin SV exerts a significant bactericidal effect against Gram-positive pathogens, including the acid-fast *Mycobacterium tuberculosis*, in addition to moderate antibacterial effect against Gram-negative bacteria (Sensi 1983).

The great activity of rifamycin SV against *M. tuberculosis* has inspired scientists to develop an oral form of the medicine which has seen the light of day in 1966. Maggi et al. have innovated a modifiable, semisynthetic and orally active substance (N-methyl-piperazine derivative of rifamycin SV) at the Lepetit Research Laboratories, which was named rifampin or rifampicin. Rifampicin has demonstrated a defined pharmacokinetic profile, including significant levels of solubility and stability throughout the gastrointestinal tract system, as well as an elevated transepithelial absorption. Such acceptable stability and solubility are attributed to unique physicochemical properties such as high lipophilicity of the molecule (Maggi et al. 1968; Lester 1972; Di Stefano et al. 2011; Grobbelaar et al. 2019).

The mechanism of action of rifampicin culminates in inhibiting the bacterial RNA polymerase synthesis, which is a crucial enzyme in bacterial RNA transcription. The bacterial RNA polymerase catalytic core consists of five different subunits including 2α, β, β', β and σ as an initiation unit. Rifampicin binds to the β-subunit (adjacent to DNA/RNA channel), leading to a physical blockade and, consequently, inhibition of the DNA-dependent RNA elongation process (Sensi 1983; Pang et al. 2013; Nusrath Unissa and Hanna 2017).

Nevertheless, another pivotal event occurred during the late stages of the golden era of antibiotics: the accidental discovery of metronidazole as a treatment for gingivitis. In 1962, the D. L. S. Shinn from King's College Hospital Dental School, Denmark Hill, London, reported that a treatment with metronidazole 200 mg, three times daily, for trichomonal vaginitis cured, also, acute marginal gingivitis, which was diagnosed at the same time (Shinn 1962). Before that, in 1959, the Rhone-Poulenc Labs in France produced a substance called nitroimidazole as a secondary agent of the extracted antibiotic azomycin from *Streptomyces* species. Then, the nitroimidazole was synthetically derived from metronidazole (a b-hydroxyethyl-2-methyl-5-nitroimidazole) for chronic trichomonas infections (Petrin et al. 1998; Samuelson 1999).

In addition to the activity of metronidazole against anaerobic Gram-positive bacteria *Clostridium* spp. and anaerobic Gram-negative bacteria like *Bacteroides* spp. by disrupting the bacterial DNA synthesis, it is effective against parasitic infections (Samuelson 1999). Metronidazole has demonstrated significant efficacy against the anaerobic protozoan parasite *Entamoeba histolytica*, which causes dysentery and liver abscess (Ravdin 1995). Moreover, it is highly efficacious against the protozoan luminal parasite *Giardia lamblia*, which stands behind severe malabsorption and epigastric pain conditions in developing countries (Zaat et al. 1997) (Fig. 1.1).

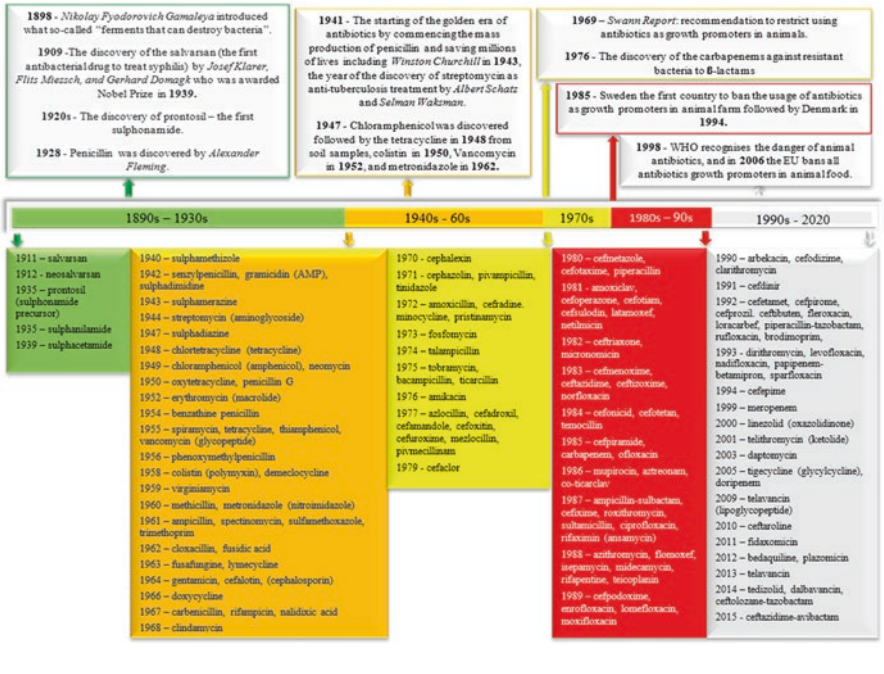

Fig. 1.1 Timeline of the key events in the history of the discovery of the natural antimicrobials and the introduction of the semi-synthetic and synthetic conventional antibiotics

1.3 Antibiotic Resistance: Health Crisis and Solutions

The habitation of Earth for more than 200,000 years by its inhabitants utilising its resources, coexisting amongst a remarkable diversity of a wide range of different species includes prokaryotes and eukaryotes and, concurrently, preserving the homoeostatic cycle of the life forms is owing to the symbiotic fashion of interaction between the various species. Nevertheless, predatism is the alternative way of practice when competing over limited essential resources for survival (Gauze 1934). Along with that, the victorious species are the elite which will continue to prevail. This instinctive principle, the essence of Darwin's theory of evolution, has been empirically applied to quantify the interaction between pathogens and their hosts, including the epidemiological outcomes of the struggling course for existence (Gauze 1934).

Gauze et al. argued that the relationship between the prey and the predator (i.e. the host and the pathogen) results in a cyclical oscillation of their numbers. Moreover, the species will never be able to eliminate each other (Gauze 1934). Hence, the declaration that was made by the clinician William H. Stewart in the early 1960s, "it is time to close the book on infectious diseases and declare the war against pestilence won" (Spellberg et al. 2008), was an invalid scientific statement. Furthermore, needless to say that the unsupported overconfidence of the medical practitioners in prescribing and the uncontrollable usage of antibiotics in livestock as well as in the agricultural industry has contributed, overwhelmingly, to the emergence of mutated and stubborn-resistant predators (Stockwell and Duffy 2012; Venter et al. 2017). Such microbial resistance counteracts the so-called magic bullet impact, or the therapeutic effect of the antibiotic, and aborts the host's combat during the downfalls of these oscillatory cycles (Sengupta et al. 2013).

Admittedly, acquiring resistance genes to antibiotics is a defensive biological mechanism adopted by single-cell microorganisms more than 30,000 years ago (Vanessa et al. 2011). Bacteria develop antibiotic substances to compete against other bacterial species over common living resources (Venter et al. 2017). Nevertheless, our argument concerns the augmented prevalence of bacterial resistance amongst communities, which correlates with the highest records of antibiotic consumption (Sun et al. 2012). Goossens et al. have found that antibiotic resistance incidences are significantly higher in southern and eastern European countries versus the northern ones where the prescribing rate in primary healthcare was 32.2 per 1000 residents compared to 10 per 1000 residents, respectively (Goossens et al. 2005).

Antibiotic-resistant infections have been declared by WHO and other global health organisations as a health "crisis" which demonstrates an imminent potential of a "catastrophic consequence" on the human race (WHO 2014; Sivalanka et al. 2016; Alam et al. 2019; Sprigg and Pietrangeli 2019). Hospital-acquired infections are one the most prominent infections that attack immunocompromised patients and are caused by different pathogens, including antibiotic-resistant bacteria such as the methicillin-resistant *Staphylococcus aureus* (MRSA), which is responsible for more

than 11,000 deaths annually in the USA alone (Gross 2013; Venter et al. 2017; Podolsky 2018). MRSA was initially encountered in 1962 (Sprigg and Pietrangeli 2019). Nowadays, MRSA has spread worldwide, and it is one of the most frequent and severe infections arising out of antibiotic-resistant bacteria (Ventola 2015). However, there are reports acknowledged by some European health authorities from the United Kingdom and the Netherlands stating that hospital-acquired MRSA incidences were declined by almost 31% between 2005 and 2011 due to the adoption of strict hygienic measures, while the trend in the community-acquired MRSA is still increasing (Ventola 2015).

MRSA is sensitive to glycopeptide treatment such as vancomycin (Rai et al. 2005), which prevents the biosynthesis of the bacterial cell wall by blocking the peptidoglycan production throughout a complex formation with the D-Ala-D-Ala residues of the building blocks, which, in turn, inhibits the trans-glycosylation process (Allen and Nicas 2003). Such a mechanism of action was considered highly immunised against bacterial resistance. In 1979, Sengupta et al. showed the development of resistance to vancomycin in coagulase-negative *Staphylococci* (Sengupta et al. 2013). Currently, vancomycin-resistant *Enterococci* (VER) pose a significant concern, predominantly, because of the wide range of hospital-acquired infections caused by these pathogens, such as post-operative and urinary tract infections, besides the limited options of specific treatments against them (Handwerger and Skoble 1995; Ventola 2015). Furthermore, in 1997, Hiramatsu et al. demonstrated the reduction of vancomycin susceptibility in a methicillin-resistant *S. aureus* (VISA) strain isolated from an infected surgical wound (Hiramatsu et al. 1997). This reduction in susceptibility to vancomycin by MRSA has been, also, extended to different advanced cephalosporins (e.g. ceftaroline) and vancomycin analogues (e.g. telavancin, oritavancin and dalbavancin) that have been used as alternative treatments (Venter et al. 2017).

Cross-transfer of antibiotic-resistant coding genes may happen between different species throughout plasmids or transposons (Handwerger and Skoble 1995; Podolsky 2018). A new vancomycin-resistant *S. aureus* (VRSA) strain has emerged due to conjugative transfer of a plasmid transposon from a vancomycin-resistant *Enterococcus faecalis* strain to the MRSA bacterium. In turn, these transconjugants depict the contributor's role regarding vancomycin resistance after their incorporation into the recipient's chromosome, via changing the bacterial cell wall precursor from D-Ala-D-Ala to D-Ala-D-Lac (Handwerger and Skoble 1995; Venter et al. 2017).

To sum up, during the golden era of antibiotic history between the early 1940s and the early 1960s, medical practitioners and the pharmaceutical industry took initial control over pathogenic infections but ignored critical scientific clues and the extraordinary and evolutionarily skills of the biological cell. The euphoria of victory ended prematurely and was buried under the new imminent threat of the superbugs with potentially catastrophic consequences. Between the early 1960s and the 1980s, scientists have comprehended the magnitude of the antibiotic resistance crisis; they have witnessed the transmission of resistant genes horizontally, such as the emergence of the VRSA throughout the plasmid transformation between two different

species they have cried out in desperation for help. Consequently, in 1985, Sweden was the first to ban the usage of antibiotics as promoters in animal farming (Wierup 2001), followed by Denmark in 1994 in an attempt to eliminate the resistance reservoir and to reduce the chances of transforming antibiotic-resistant genes into human pathogens (Hayes and Jensen 2003). Thereafter, in the 1990s, the awareness of drug resistance became a global concern, and in 2006, the EU imposed a total ban on the usage of antibiotics in animal farming. Moreover, in 2014, WHO has recognised the antibiotic resistance condition as a global crisis (Alam et al. 2019).

For the time being, hospital-acquired infectious diseases remain a serious risk. Increased antibiotic resistance reduces the treatment options and incurs painful financial costs worth billions of dollars of expenses on management. Moreover, the excessive use of antiseptics in hospitals and communities can lead to resistance increment against these sanitizers and increase the chances for cross-resistance to antibiotics. Hence, comprehensive management and control programmes are in desperate need of a comprehensive solution in the clinical and veterinary fields and for antimicrobials used in agriculture. Finally, reducing the frequent usage of conventional antibiotics requires a new alternative of less resistible pharmacotherapeutics such as antimicrobial peptides (AMPs).

1.4 Antimicrobial Peptides (AMPs): Conception and History

Humanity has branched out into different societies experiencing distinctive plagues along the way reshaping our history. Nevertheless, our long-term coexistence with microorganisms proved to be in a continual dynamic ecological equilibrium until a retaliation had taken place, as it happened when the microbial resistance to antibiotics emerged following the excessive use of antibiotics in the 1960s and 1970s (Anderson 2004). Hence, dealing with infectious diseases from the ecological and evolutionary point of view will be more advantageous than handling them from a solo and narrow clinical angle (Burnet 1941). As Dr. Joshua Lederberg has stated: “Perhaps one of the most important changes we can make is to supersede the twentieth-century metaphor of ware for describing the relationship between people and infectious agents. A more ecological informed metaphor, which includes the germ’s-eye view of infection, might be more fruitful… yet they are equally part of the superorganism genome with which we engaged the rest of the biosphere” (Lederberg 2000).

The relationships and interactions between microorganisms and microorganisms/host involve a complex of ecological aspects, including cellular signalling amongst others, as an integral feature of an instructive and controlling co-evolutionarily system that supports a peaceful adaptation (Braga et al. 2016). This part of the present review focuses on the AMPs and their roles in bacterial control’s intercellular/intracellular signalling mechanism.

AMPs are a diverse class of natural and multifunctional small molecules ranging between 10 and 150 amino acids that exert their biological function either

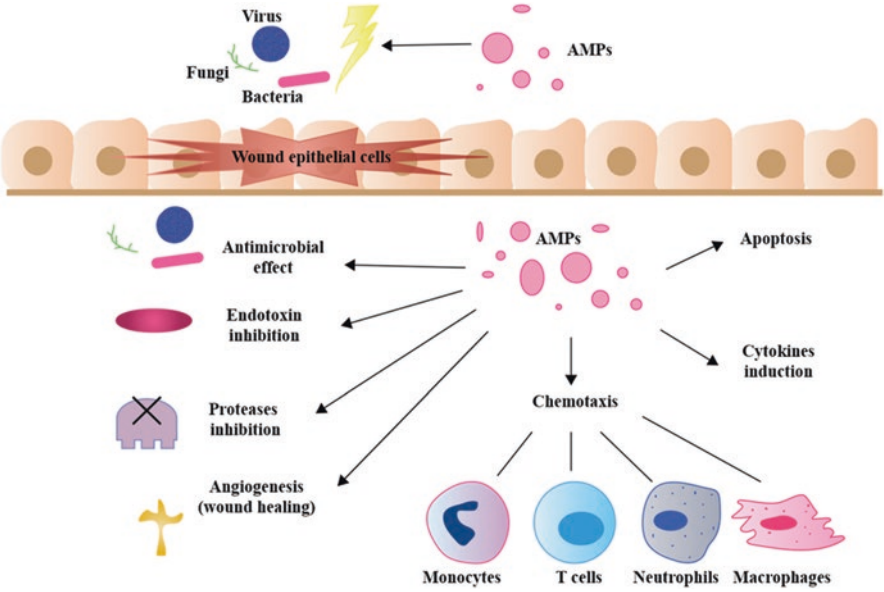

Fig. 1.2 Summary illustrating the general and various functions of the AMPs

constitutively or through an external factor inducement (Lei et al. 2019; Lazzaro et al. 2020). AMPs are evolutionarily preserved indigenous molecules that originated and are employed by all different life forms of prokaryotes and eukaryotes of the human and animal kingdom for different cellular functions, including antimicrobial protection (Epand 2016; Zhang and Gallo 2016; Wu et al. 2018). Hence, they serve as the first line of defence against different microbes, including viruses, bacteria, fungi and protozoa. Moreover, in higher life forms, AMPs are ubiquitously dispersed in epithelial cells, and some of them can be expressed as immunomodulatory agents (Boman 2003; Gordon et al. 2005; Lai and Gallo 2009; Lee et al. 2015; Scorciapino et al. 2017; Pfalzgraff et al. 2018; Xia et al. 2018) (Fig. 1.2).

The initial detection of AMPs was in 1921 when Alexander Fleming observed a lysis process in a bacterial culture obtained from nasal mucosa of a patient with the common acute cold (Fleming 1932; Tan and Tatsumura 2015). Dr. Fleming named the responsible effector for the bacterial lysis, lysosome, which is known, nowadays, as a generic name of various proteolytic organelles that possess more than 50 different intracellular digestive enzymes (Bainton 1981). Soon after this discovery, Alexander Fleming stated that this bacteriolytic element is dispersed throughout the human body, confirming Metchnikoff's synopsis: "Nature, to protect the skin and mucous membranes does not use antiseptics. The fluids which bathe the surface of the mouth and other mucous membranes are not bactericidal or only very imperfectly so. Nature removes from the mucous membranes and the skin quantities of microbes, eliminating them by epithelial desquamations and expelling them with the secretions and liquid excretions. Nature has chosen this mechanical procedure

just as the surgeons who replace the antisepsis of the mouth, the intestine and other organs by lavage with physiological saline" (Fleming 1932).

The first defined AMP was found in 1939 in a culture of an aerobic sporulating bacilli species. Dubos and his group isolated an alcohol-soluble substance named tyrothricin, which was bactericidal against a large number of Gram-negative and Gram-positive bacteria (Dubos 1939). After that, two distinct crystalline substances have been purified from the tyrothricin, including tyrocidine, which showed bactericidal activity against both Gram-positive and Gram-negative pathogens, and gramicidin which showed apparent effectiveness against Gram-positive species when it was applied topically (Dubos and Hotchkiss 1941; Mootz and Marahiel 1997).

The euphoria of the golden era of antibiotics between the 1940s and the 1960s has overshadowed the discovery of AMPs. However, the redemption of the AMPs to be considered a novel remedy for infectious diseases started gaining momentum in the 1960s through the exploration course for a new ecological resolution to counteract the emerging bacterial resistance for conventional antibiotics (Davies and Davies 2010; Aminov 2017).

A sequence of exclusive studies conducted by Hans G. Boman and his group in the 1970s and the 1980s has led to the discovery of insect-based antimicrobial peptides with an overwhelming stimulant effect on the immunity response in Drosophila (Faye and Lindberg 2016). Boman's great deal of interest in the functionality of the human immune system has diverted his focus towards the preliminary defensive processes in the vertebrates' immune system that take place before the maturity of the required antibodies during the early stages of infections. Thus, he employed insect species, which lack lymphocytes and immunoglobulins, as a fundamental model for his manipulative studies of the immune system via selected stimulus (Hultmark et al. 1982). In 1981, Boman and his group identified and characterised a new group of AMPs named cecropins that deliver a significant antibacterial effect against Gram-negative bacteria (Steiner et al. 1981).

Moreover, in 1987, a new family of AMPs which consists of two similar small peptides (23 amino acids) has been discovered by Zasloff et al., for which he named magainins (Zasloff 1987). They have isolated the broad-spectrum amphiphilic peptides with antimicrobial activity against a wide range of bacteria, fungi and protozoa, from the skin of the *Xenopus laevis*, an African frog. Furthermore, in 1994, scientists have isolated the first mammalian skin of AMPs, which belongs to a larger group of small cationic antimicrobials named cathelicidins, which was found in humans and other animals, birds and marine species (e.g. cattle, sheep, pigs, chicken and some fish) and exert a broad-spectrum antimicrobial activity against bacteria, viruses and fungi (Kościuczuk et al. 2012).

Undoubtfully, such fascinating research, alongside other studies that identified and characterised a wide range of AMPs for the last 60 years, has instigated a widespread campaign behind the comprehensive appreciation of the role of the AMPs in our innate immune system (Hultmark et al. 1982; Tracey et al. 1995; Lung et al. 2001; Faye and Lindberg 2016; Wu et al. 2018).

1.5 Diversification, Structural Characterisation and Mechanisms of Action of AMPs

AMPs are circulated and diversified in nature across and within the different species of vertebrates, bacteria, fungi and plants (Lei et al. 2019). The AMP database is updated regularly, and it includes natural AMPs with a defined sequence and biological activity (Wang et al. 2016). The latest version of the AMP database shows the sum of 2169 antibacterial peptides in addition to 277 antivirals, including anti-HIV, 959 antifungals, 80 antiparasitics and 185 bioactive peptides listed for different anticancer activities (Wang et al. 2016). In humans, the defensin family is the more significant ubiquitous type of AMPs followed by the cathelicidins, the first family to be identified (Dhople et al. 2006; Mojsoska and Jenssen 2015). Moreover, cathelicidins were discovered ubiquitously in monkeys, rats, mice, rabbits, pigs, cattle, goats, sheep and horses (Gudmundsson et al. 1995; Zanetti 2004; Lei et al. 2019).

The characterisation of AMPs is very complex (Zasloff 2002) due to their diversified origin and the crossover of functionalities against different microorganisms and target cells (e.g. cancer cells). However, there is a simple classification approach based on the geometrical (structural) features of the AMPs upon their contact with the microbial membrane (Zasloff 2002). This approach broadly categorises the AMPs into four main types based on their secondary structure, including the alpha-helix, the beta-sheet, the loop (mixed) and the extended conformation (Steinberg et al. 1997; Mojsoska and Jenssen 2015). The formation of these different structural characteristics of the AMPs depends on the blend of the different amino acids throughout the peptide, which supports the folding of the AMP that leads to the formation of its three-dimensional conformation, shaping the physicochemical characteristics, and consequently influences the biological activity based on the structure-activity relationship (SAR) concept (Zasloff 2002; Bahar and Ren 2013; Phoenix et al. 2013; Lei et al. 2019).

For the different AMPs' structures to exert their biological activities, they need to achieve the following:

(i) Matchmaking between the length of the AMP residues and the thickness of bacterial membrane dimensions (Tossi et al. 2000). Given that the reaction is not based on receptor-ligand mediation, complete configurational matchmaking (lock-key theory) is not required; however, for the alpha-helical structure, it is optimal to have a complete penetration through the bacterial membrane (see discussion under *AMPs formed in alpha-helix type*));
(ii) An ideal amphiphilic balance between the hydrophilicity and the lipophilicity ratios, as well as an appropriate cationic level of charge to govern the efficacy of the antibacterial activity (Lei et al. 2019).

As we have seen thus far in the present review, AMPs exert their antibacterial effect in various ways, including disruption of protein synthesis and cell wall formation, inhibition of genetic material expression and hindering different enzymatic

activities. However, the primary mechanism of action is based on the disintegration of the bacterial membrane (Strömstedt et al. 2010). Moreover, the above particulars argue that the physicochemical characteristics of AMPs are the primary key behind their biological activity against the integrity of the bacterial membrane (Lei et al. 2019). The AMPs are predominantly short cationic (net charge between +2 and +9) peptides due to amino acids such as lysine and arginine with an amphiphilic composition (Bahar and Ren 2013; Pushpanathan et al. 2013; Zhang and Gallo 2016; Mirski et al. 2018). The amphiphilic nature governs the peptides' pharmacokinetic profile of peptides through the balancing between the required optimal solubility in an aqueous physiological medium supported by the hydrophilic chemical groups versus the membrane permeability and the therapeutic bioavailability of the AMPs owing to the hydrophobic moiety. However, pharmacodynamically, this amphiphilic property of the AMPs, also, allows a direct interaction between the peptide and the microbial membrane rather than a receptor-mediated interaction which plays a major role in the bacterial resistance development (discussed below) (Tossi et al. 2000). On the other hand, the cationic property of the AMPs owing to lysin and arginine residues (Strömstedt et al. 2010) controls the pharmacodynamic effect of the AMP through its interaction with the negatively charged bacterial membrane (Zasloff 2002).

To exhaust our discussion within the scope of this review, we present a selected debate for each structural type with an example of a peptide and a related mechanism of action based on the expected physicochemical characteristics.

1.5.1 Alpha-Helical AMPs

Arguably, the effectiveness of the AMPs depends on the partitioning of the peptide molecule (distribution at physiological pH) into the phospholipid membrane of the microorganism. Thus, the higher the partitioning into the lipid phase, the more influential the peptide. Moreover, this partitioning is proportional to the molecule's hydrophobicity, or it is inversely related to the availability of hydrogen bonding. This hypothesis has been tested by quantifying the free energy cost (using Gibbs free energy module) for partitioning the native bee venom peptide melittin, which is in alpha-helical form of 12 residues versus the unfolded form D_4,L-melittin. The results showed a reduction of 0.4 Kcal $mole^{-1}$ in the free energy cost per each alpha-helical residue distribution into the lipid phase (Ladokhin and White 1999). Hence, these results confirm that the secondary alpha-helical structure reduces the availability of hydrogen bonding and exposes the hydrophobic groups of the peptide to the lipophilic interfaces of the membrane's phospholipids, helping in tunnelling through the membrane (Almeida et al. 2012). Nevertheless, the alpha-helical structural characteristic of the peptide plays only a partial role in its biological activity, which is determined by several other effectors, including the flexibility and self-assembly of the peptide, the amphiphilic balance of the peptide, the cationic charge of the peptide and the ionicity of the bacterial membrane (Juretić and Simunić 2019).

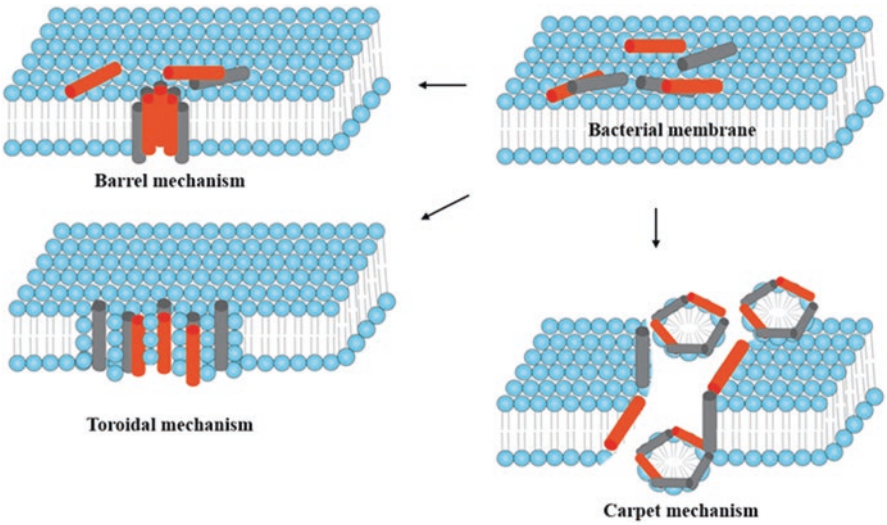

Fig. 1.3 Summary illustrating the multistep of the mechanism of action of the AMPs

The alpha-helical segment results from the peptide sequence enrichment with specific amino acids such as arginine, alanine, phenylalanine, isoleucine, leucine and lysine (Dathe and Wieprecht 1999; Phoenix et al. 2013). These AMPs, generally, are more frequent and more prolonged peptides that can channel the bacterial membrane (Juretić and Simunić 2019). The disintegration of the bacterial membrane results from different ways of AMP activity, including the barrel-stave mechanism, the toroidal mechanism and the surfactant-induced lysis (Fig. 1.3) (Shai 2002; Strömstedt et al. 2010).

Usually, the alpha-helical type of AMPs with more significant residues (over 22 amino acids) adopts the barrel-stave mechanism, causing few transmembrane pores, dissociating the membrane integrity and depleting the transmembrane potential (Shai 2002). The peptide must present enough hydrophobic moiety which faces the hydrophobic bilayer of the bacterial membrane after the electrostatic attraction between the cationic sides of the peptide, and the anionic charge of the bacterial membrane occurs. Magainin, the clawed frog of *Xenopus laevis*, is one of the prototypes of alpha-helical AMPs that inhibits bacterial and fungal growth and mediates protozoa lysis (Zasloff 1987).

Another mechanism of action adopted by different structures of AMPs includes the alpha-helical type, called the carpet-like mechanism, which can progress to either the toroidal or the surfactant-induced mechanisms depending on the AMP. The carpet-like mechanism is an alternative module to the barrel-stave mechanism but with fewer requirements for activation in terms of peptide's specifications such as transmembrane insertion and porosity forming or a minimal peptide length or the number of amino acids (Shai 2002). Therefore, it is available for a broader range of AMPs. In this module, the peptide accumulates parallelly on the surface of the

pathogen's membrane and causes a lysis-type reaction due to surfactant effect and micellisation (Pokorny and Almeida 2004; Strömstedt et al. 2010; Bertelsen et al. 2012). However, some other peptides augment their concentration until they reach a critical level on the membrane surface which results in an adsorbent reaction of the peptide on the polar surface of the membrane due to the development of chemical imbalance in the ionic surface of the membrane (Zasloff 2002). Following the peptide's adsorption on the membrane surface, a translocation process of the peptide takes place across the membrane throughout the inner components, which leads to toroidal pore formation (Fig. 1.3) (Strömstedt et al. 2010).

Furthermore, the incorporation of the peptide into the outer layer of the membrane expands the surface of the membrane laterally, which, in turn, thins the membrane to the cleaving point (Mecke et al. 2005). The human cathelicidin (LL-37) is another example of the alpha-helical structure. However, it adopts the carpet-like mechanism of action against bacterial membrane (Pokorny and Almeida 2004). LL-37 is produced by phagocytes and skin keratinocytes and released into phagocytic vacuoles and skin wound fluids (Tossi et al. 2000).

1.5.2 Beta-Sheet AMPs

Beta-sheet family of AMPs adopts the anti-parallel beta-sheet structure upon their contact with aqueous medium due to disulphide bond formation between the cysteine residues positioned on the adjacent beta-strands (Kier et al. 2015; Moravej et al. 2018). Folding adjoining beta-sheets with no less than two disulphide covalent bonds creates a rigid platform that harbours the peptide's essential functional moieties, including the cationic and the hydrophobic groups (Zasloff 2002). Generally, beta-sheet AMPs such as human defensins, bactenecin, cateslytin, protegrin and tachyplesin are less abundant than alpha-helix peptides. They are ubiquitous across the different levels of life forms (Phoenix et al. 2013; Moravej et al. 2018).

Although the beta-sheet family of antimicrobials employs the same barrel-stave and carpet-like mechanisms in their attack on bacterial membrane, different studies have presented various arguments concerning the structure-activity relationship. Generally, the highly rigid constructed beta-strands are occupied by polar and non-polar domains (cationic and hydrophobic groups). The cationic component initiates the electrostatic interaction between the peptide and the bacterial membrane, settling the hydrophobic residues on the membrane surface ready for partitioning and disrupting the membrane integrity via the formation of transmembrane channels (Yeaman and Yount 2003).

Amongst the most comprehensively studied peptides that fit as a model of SAR for this debate are the tachyplesins, which exist in three main types, including tachystatins A, B and C, consisting of 44, 42 and 41 amino acids, respectively (Osaki et al. 1999). Tachyplesins have been isolated from Japanese horseshoe crap *T. tridentatus* (Powers and Hancock 2003). The structural motif of tachystatin C is distinctively different from A and B. It can form an amphiphilic beta-sheet on

terminal C which drives the partitioning of the peptide into the microbial membrane and enables the peptide to function as a haemolytic and cell lysis effector against *Pichia pastoris.* Moreover, tachystatin C shows an unusual antimicrobial activity against Gram-positive, Gram-negative and fungi throughout specific recognition and interaction with the lipoteichoic acids, the lipopolysaccharide and the chitin, respectively (Osaki et al. 1999).

1.5.3 Loop AMPS

Cyclic or loop peptides, in general, are linear peptides that adopt a loop-shaped segment due to either a single disulphide bridging or other types of bonds such as isopeptide, ester or amide, which lead to the so-called heterodetic cyclic peptide (Fehlbaum et al. 1996; Powers and Hancock 2003; Davies 2003). Restriction and freedom reduction of the amino acids' residues by cyclisation leads to a higher degree of configurational rigidity. Subsequently, a higher affinity between the ligand and the target site results in a notable improvement in the biological activity of the peptide (Davies 2003).

Mika et al. have shown in a comparative study between the biological activities of a linear AMP named BPC194 and its analogue, a de novo designed cyclic decapeptide, against the plant pathogens *Erwinia amylovora* and *Xanthomonas vesicatoria* (Mika et al. 2011). The cyclic peptide demonstrated adoption of a beta-sheet structure which supported a higher affinity and more significant partitioning into the bacterial membrane whereas the linear analogue resided on the surface of the membrane (Mika et al. 2011).

Moreover, Hirakura et al. examined the relationship between the structural diversity of AMPs and specific antimicrobial activities (Hirakura et al. 2002). They tested the activity of the cyclic tachyplesin versus the activity of the alpha-helical magainin. The cyclic beta-stranded tachyplesin demonstrated higher affinity (by 280-folds) towards the lipopolysaccharide component of the cell membrane compared to its affinity to the acidic phospholipids, whereas the linear alpha-helical magainin acted equally towards both membrane components (Hirakura et al. 2002).

1.5.4 Extended AMPs

The extended type of AMPs lacks a steady as well as specific structural shape. However, they are rich in certain amino acids such as arginine, glycine, histidine, proline and tryptophan (Powers and Hancock 2003; Mishra et al. 2018). Moreover, their active configuration is an outcome of their electrostatic interaction, such as Van der Waals forces, with the microbial membrane rather than their intrinsic chemical/physical bonding amongst the amino acid residues (Powers and Hancock 2003). Thereupon, the extended conformational structure of certain AMPs has minimal impact on the microbial membrane integrity (Mika et al. 2011).

Indolicidin is an example of an extended cationic AMP isolated from cow neutrophils that exert its antimicrobial effect through its interaction with the bacterial membrane due to the amphiphilic cloud of the side chains around peptide (Mishra et al. 2018). It consisted of 5 tryptophan residues out of 13 and had demonstrated antibacterial activity against *E. coli*. Nevertheless, its affinity to lipopolysaccharides was notably low compared to the beta-sheet type of AMPs (Powers and Hancock 2003). Thus, the [AMPs] mode of action is derived from the amino acid composition rather than on a presumed secondary structure. Tryptophan is one of the most common amino acids that has been identified in the extended AMPs (Powers and Hancock 2003). Tryptophan residue is a critical interactive element in the interfacial vicinity of the lipid bilayers (Chan et al. 2006). It can form hydrogen bonding with the microbial membrane promptly. Likewise, arginine residues enhance the peptide interaction with the microbial cell membrane by providing the cationic characteristic and hydrogen bonding capabilities to attract the anionic bacterial membrane as an initiation for the peptide partitioning across the microbial membrane (Chan et al. 2006). On the other hand, proline disrupts the protein synthesis throughout its interaction with the 70S ribosomal subunit, which inhibits specific molecular signals and the production of certain constructive microbial proteins (Gennaro et al. 2002; Mishra et al. 2018).

Bellamy et al. demonstrated the chemical characteristic-activity relationship via an experimental measurement of lactoferrin B (47 amino acids) as an AMP against different bacteria, including *Pseudomonas fluorescens* and *Enterococcus faecalis* and *Bifidobacterium bifidum* (Bellamy et al. 1992). Lactoferrin B is one of the two active forms of lactoferrin (Bellamy et al. 1992), which is an extended broad-spectrum AMP found in most exocrine secretions in mammalians (Wakabayashi et al. 2014). Bellamy's group reported two main findings (Bellamy et al. 1992): (i) the activity of the lactoferrin B was pH-dependent; hence, the peptide was more active at pH 7.5 compared to pH 5.5, and (ii) the antibacterial activity against the tested panel was reduced with the addition of Na^{+}, K^{+}, Mg^{++} or Ca^{++} ions. Bellamy et al. argued that the reduction in the peptide's susceptibility was related to the changes in the ionicity of the membrane after the introduction of the cationic minerals. This argument was supported by their report about the higher effectiveness of the lactoferrin B activity in an alkaline environment compared to the acidic conditions (Bellamy et al. 1992), where the sensitivity of the peptide was reduced in the protonated medium presumably because of the changes in the ionicity of the bacterial membrane.

1.6 Immunomodulatory Signalling of AMPs

AMPs' functional roles are widely versatile between the innate and the acquired immune systems of the complex life form. The ubiquitous presence of the indigenous AMPs in circulating and epithelial barrier cells across the different compartments of the living physiological system capacitates them as immunomodulators throughout various signalling pathways of the immunological and inflammatory

processes (Hancock et al. 2016). That is to say, AMPs can serve as antibiotics and controllers of inflammatory mechanisms via immunomodulation and up−/down-regulation of different cytokines (Phoenix et al. 2013).

In 1989, Mary C. Territo was the first to relate the AMPs and the immunomodulatory concept. Mary's team argued that monocytes' recruitment at inflammatory sites by neutrophils ought to be due to the AMP defensin mediation (Territo et al. 1989). Their argument was based on the observation that showed an unusual monocyte chemotactic activity that resulted after releasing HNP-1 (a human defensin) from neutrophil granules (Territo et al. 1989). Thereafter, further studies evidenced that alpha-defensins isolated from circulating human neutrophils stimulate T cells which, in turn, express CD4/CD45RA and CD8 antigens (Zasloff 2002). Nevertheless, the involvement of certain AMPs, such as the human cathelicidins in the signalling pathways of the immune system, can be even more convoluted. For instance, the human cathelicidin LL-37 interacts with more than 16 different proteins resulting in over 1000 successive interactive signalling molecules due to an expression of more than 900 different genes (Lau et al. 2005; Hancock et al. 2016).

Based on the mentioned above, AMPs play vital roles in mediating an array of cellular regulatory signalling during microbial infection and the subsequent inflammation (Scott et al. 2007). The roles of these cellular signals range over the preparation of the acquired immune response, such as the attraction of monocytes as well as the formation of antibodies against the invading pathogens, and the up-/downregulation of the pro-inflammatory cytokines such as interleukins (IL) (6, 8 and 18) and tumour necrosis factor-alpha (TNF-alpha), in addition to the anti-inflammatory cytokine interleukin-10 (Chaudhry et al. 2013; Li et al. 2017; Muñoz-Carrillo et al. 2018).

Inflammation is a complex process; nevertheless, it is a crucial segment of the immune system's response to an infection. It is a vital signal for the immune system to prepare the body for protection and a successive healing process (Muñoz-Carrillo et al. 2018). However, inflammation can be too dangerous and more harmful, in some cases, than the actual bacterial infection. The role of bacterial infection in the pathophysiology of inflammation is related to the bacterial cell wall and membrane-associated LPS endotoxin (Ginsburg 2002). The release of the LPS into the bloodstream enhances the production and release of pro-inflammatory cytokines (e.g. TNF-alpha and interleukin-6 and interleukin-8) from monocytic and phagocytic cells (Sun and Shang 2015). Overreaction of the immune system to the titre level of LPS in the blood system can lead to overexpression of cytokines which, in turn, ought to lead to sepsis followed by multiple visceral organ injuries or what so-called flesh-eating syndrome (over 700,000 cases in the USA alone, with mortality rate up to 50%) (Ginsburg 2002).

Cathelicidins family of AMPs, particularly LL-37, were found to reduce inflammation by neutralising the endotoxin LPS via a direct interaction between the cationic moiety of the AMP and the anionic groups of the glycolipid (Nagaoka et al. 2001). Moreover, the antiseptic activity of the LL-37 is resulted from the expression of anti-inflammatory cytokines such as interleukin-8 (Scott et al. 2002). Scott et al. took their study further by performing gene expression experiments aiming to reveal the effect of LL-37 on the modulation of macrophages (Scott et al. 2002). The

results revealed that LL-37 directly affects the downregulation of 20 genes; nevertheless, it causes upregulation of 29 genes, some of which coding for chemokines and their receptors, in addition to the anti-inflammatory interleukin-8 (Scott et al. 2002).

Besides the antimicrobial activity and the mediation/regulation of the inflammatory process of the AMPs, LL-37, the human beta-defensin (hBDs) group plays a significant role in the wound pathophysiology by controlling the healing process (Diamond et al. 2009). The skin produces and enhances the activation of LL-37 and hBDs to prevent and eliminate microbial infections and, also, to support the healing of cutaneous injuries (Lehrer and Ganz 1999). It promotes the production of cytokines/chemokines, attracting keratinocyte migration, angiogenesis and cell proliferation (Heilborn et al. 2003). Niyonsaba et al. have found that three out of four different hBD peptides that are produced in the skin mediate the production of IL-6, IL-10, interferon-gamma-inducible protein (IP-10), macrophage inflammatory protein-3-alpha (MIP-3alpha) chemokine, monocyte chemoattractant protein-1 and RANTES, by stimulating the epidermal keratinocyte cells which, in turn, increase their gene expression (Niyonsaba et al. 2007). Moreover, hBDs induce the phosphorylation process of the epidermal growth factor receptor (EGFR), which promotes skin wound healing via the induction of the epidermal and the rejuvenation of the dermal cells (Niyonsaba et al. 2007; Bodnar 2013).

Along with the significant role of the hBDs and the LL-37, which are expressed in leukocytes and epithelial cells (Koczulla et al. 2003), they are immensely filtrated into wound bed during the resorptive phase of the injury before it starts declining towards the end of the regeneration phase and the closure of the wound (Heilborn et al. 2003). Heilborn's team argued that LL-37 has a great deal of influence in wound healing and closer mediation. They validated their hypothesis by showing the induction of LL-37 during the re-epithelialisation of the skin wound. Furthermore, this re-epithelialisation was halted by antagonising the LL-37 with specific antibodies (Heilborn et al. 2003).

The human cathelicidin LL-37 has been further presented as a critical intrinsic factor in cutaneous wound healing. Koczulla et al. demonstrated that LL-37 directly manipulates the angiogenesis and arteriogenesis processes via the activation of the formyl peptide receptor-like-1 on the endothelial cells (Koczulla et al. 2003). They showed that administration of LL-37 into the chorioallantoic membrane assay resulted in neovascularisation. Furthermore, they demonstrated a reduction in wound bed vascularisation in mice with cathelin-related antimicrobial peptide (CRAMP) (LL-37 murine homologue) deficiency (Koczulla et al. 2003).

To sum up, the AMPs' portfolio of pleiotropic bioactivities involves a wide range of vital roles and mechanisms. They can exert direct action against microbes based on the incapacitating potential of the physicochemical characteristics of the AMP, adopting chemotactic mediation to assist the migration of different leukocytes to the site of the infection and stimulating the production of cytokines and chemokine to promote keratinocyte migration and prefoliation in wound beds. Concurrently, AMPs mediate a homoeostatic equilibrium in which all the biological processes are subject to perpendicular regulatory feedback.

1.7 Bacterial Resistance to AMPs

The focus on AMPs as a feasible solution for antibiotic resistance has been intensified in recent years. AMPs have been in the scientists' spotlight since the early days of the emerging of bacterial resistance to conventional antibiotics. The initial assumptions stated that the probability of bacterial resistance development against AMPs is negligible. This hypothesis was based on the preliminary perception of AMPs, as they are simple and lack specificity in their mechanisms of action against microbes (Lazzaro et al. 2020). Furthermore, the early descriptive module of AMPs' antimicrobial activity was presented as a platform involving various effectors that target different microbial biological aspects (Zasloff 2002). However, recent comprehensive research findings have revealed the inaccuracy of that early approach concerning the AMPs' pharmacodynamic ligand binding modelling. The new studies argue that AMPs execute their biological activities based on high specificity and affinity to their target sites, which occur synergistically with other AMPs (Lazzaro et al. 2020). Pharmacodynamically, these findings provide more evidence that the development of microbial resistance against AMPs is possible; nevertheless, it can be counteracted by employing various types of peptides to achieve a broader spectrum and mode of activities for a specific target (Diamond et al. 2009).

Different species of microorganisms adopt various techniques to evade the antagonism of antimicrobial peptides. In general, bacterial resistance to AMPs is categorised into two main types based on their broadness of spectrum. These types can be specific resistance against a particular type of AMP or broad resistance against multiple AMPs sharing the same motif (Nawrocki et al. 2014). For the scope of the present chapter, we reviewed the last 20 years' research data related to, predominantly, bacterial mechanisms of resistance that can be adopted by Gram-positive and Gram-negative bacteria against AMPs to provide further insight into the tangled relationship between AMPs and microorganisms.

The AMPs' efficacy, as well as the hindrance to them, is a complex of complicated mechanisms based on physiochemical characteristics of the peptides and the microbial wall/membrane, the vicinity of the environment including stressful parameters and the transport kinetics of the AMPs as well as the synergy between them (Groisman et al. 1992; Devine et al. 1999; Hancock 2001; Perron et al. 2006; Lazzaro et al. 2020). The AMP's mode of action predominantly depends upon the molecule's physicochemical characteristics and the microbe's cell surface, where both, in turn, determine the magnitude of their mutual attraction to each other. Thus, the primary mechanisms of antagonism between the AMP biological activity and the microbial cell ought to be initiated through cell surface alteration or the so-called extracellular mechanisms of resistance which can be exerted via shielding of the binding site from the AMP and the enzymatic degradation of the peptide (Nawrocki et al. 2014).

1.7.1 Extracellular Mechanisms of Resistance

It is the predominant category of adopted bacterial mechanisms against AMPs' bioactivities. Several studies have investigated the susceptibility of different bacterial species to the human cathelicidin LL-37. Schmidtchen et al. showed that human pathogens act on epithelial surfaces, including wound beds, such as *Enterococcus faecalis*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Streptococcus pyogenes*, capable of producing proteinases against the cathelicidin LL-37 (Schmidtchen et al. 2002). A mass spectroscopy analysis demonstrated the cleavage of LL-37 at Arg-Ile and Asp-Phe, which took place in the presence of the elastase, an enzymatic product of *P. aeruginosa* in coetaneous wounds (Schmidtchen et al. 2001). Similarly, Sieprawska-Lupa et al. have examined the degradation of LL-37 by *S. aureus*-derived proteinases (Sieprawska-Lupa et al. 2005). Magdalena and her group have tested the susceptibility of LL-37 to metalloproteinase (aureolysin), which is generated by *S. aureus*. The results of the mass spectroscopy test demonstrated a cleaving enzymatic activity of aureolysin between Arg19-Ile20, Arg23-Ile24 and Leu31-Val32 peptide bonds of the LL-37, which inhibited the antibacterial activity of LL-37 in time- and concentration-dependent manner (Sieprawska-Lupa et al. 2005).

Exploring the extracellular mechanism of resistance against other AMPs, Schmidtchen and his group tested the susceptibility of alpha-defensin AMP against a mixed panel of bacterial species. They reported that pathogens that habituate connective tissues in wound beds, such as *E. faecalis*, *P. aeruginosa and S. pyogenes*, can evade the antimicrobial activity of the AMP alpha-defensin (Schmidtchen et al. 2001). Ultimately, these bacteria degrade the existing proteoglycans, such as decorin, biglycan and versican, in the host's connective tissues via extracellular microbial proteases such as *S. pyogenes* cysteine proteinase, which, subsequently, leads to the generation of dermatan sulphate that binds and neutralizes the AMP alpha-defensin (Schmidtchen et al. 2001).

Another example of bacterial resistance to antimicrobial peptides concerning extracellular proteases is depicted in the products of *Porphyromonas gingivalis* and *Prevotella* species. It relates to oral anaerobic and highly proteolytic pathogens known to neutralize the antibacterial activity of some AMPs, including the wasp venom mastoparan and magainin II (Devine et al. 1999). The inhibition of the mastoparan and the magainin II was due to a specific structural cleavage at the Arg residues. Nevertheless, this type of inhibition was ceased after administering protease inhibitors, proving that the resistance's nature is protease-based (Devine et al. 1999). It is worth noting that cecropin B's activity was not affected by these proteases, which was most likely due to the higher rate of the cecropin B activity than the inhibition rate of the protease, which explains the superior efficacy of these AMPs (Devine et al. 1999).

Gelatinase is a notable representative of the extracellular proteases that contribute to bacterial resistance to AMPs in different ways. Gelatinase is produced by *E. faecalis* which is a prime nosocomial pathogen that causes various types of acquired infectious diseases such as urinary tract infections, post-surgical infections

and endocarditis (Engelbert et al. 2004; Thurlow et al. 2010). Thurlow et al. showed that gelatinase is the primary factor behind the virulence of *E. faecalis* as the cause of endocarditis. Gelatinase was found to be an enhancer for bacterial resistance against the LL-37 peptide by cleaving it. Moreover, it was found to break down the anaphylatoxin complement C5a, reducing neutrophil migration and incrementing pathogen virulency. This action is in addition to its degradation bioactivity of the extracellular protein matrix in the connective tissue such as collagen and clotting factors, including fibrinogen and fibrin (Thurlow et al. 2010).

Furthermore, gelatinase is involved in biofilm formation as a valuable mechanism to evade the host's defence mechanism (Hancock and Perego 2004). Hancock et al. showed that *E. faecalis* biofilms, which increase the resistance to the innate immune system and, hence, the bacterial virulence, are controlled through the production of gelatinase (Hancock and Perego 2004). The biofilm formation in *E. faecalis* was shown to follow the quorum sensing principle, a cell density controlling mechanism (Nakayama et al. 2001). Quorum sensing system regulates different characteristics of the *E. faecalis*, including biofilm development throughout the up-/downregulation of the extracellular gelatinase genes following the accumulation of the required threshold of a cyclic lactone peptide on the bacterial surface, which, typically, happens when the bacterial population is ready for an aggressive phenotype (Nakayama et al. 2001).

In addition to the proteases' employment as a mechanism against AMPs' hostile biological activity, isolation of the AMP can be another helpful mode of resistance. It has been found that some Gram-positive bacteria can produce surface-linked peptides that bind to the AMP and block its bioactivity against the bacterial membrane (Nawrocki et al. 2014). *S. pyogenes*, a Gram-positive bacterium that can lead to different infectious diseases in humans, is a typical example of human pathogens that adopt the sequestration methodology against mammal AMPs (Akesson et al. 1996). In addition, *S. pyogenes* was found to produce a streptococcal inhibitor of complement-mediated protein (SIC). This 31 KD extracellular protein lacks the typical structural characteristic (COOH-terminal) of the Gram-positive's cell wall protein anchor, suggesting atypical biological functionality of the SIC (Akesson et al. 1996). After that, SIC was found to block different constituents of the innate immune system in the mucosal epithelial cells, including secretory leucocyte proteinase inhibitors (SLPI), cathelicidin LL-37, human alpha-defensin I and human beta-defensins I, II and III, throughout a complex formation with the target via ionic bonding, for example, interaction with the NH_2-terminal of the SLPI group (Frick et al. 2003; Fernie-King et al. 2004; Pence et al. 2010).

Extracellular polysaccharides are polymeric carbohydrates produced by Gram-positive bacteria, which are attached to the microbial cell wall via covalent bonding forming capsular polysaccharides (CPS) (Fig. 1.4) (Nwodo et al. 2012), as an additional extracellular resistance mechanism, along with proteases and sequestration, to resist AMPs' actions (Nawrocki et al. 2014). Campos et al. reported that *Klebsiella pneumonia*, a common nosocomial pathogen, upregulates the transcription of CPS when it is exposed to AMPs such as lactoferrin and polymyxin B aiming for limited interaction between the AMP and the bacterial cell surface (Campos et al. 2004).

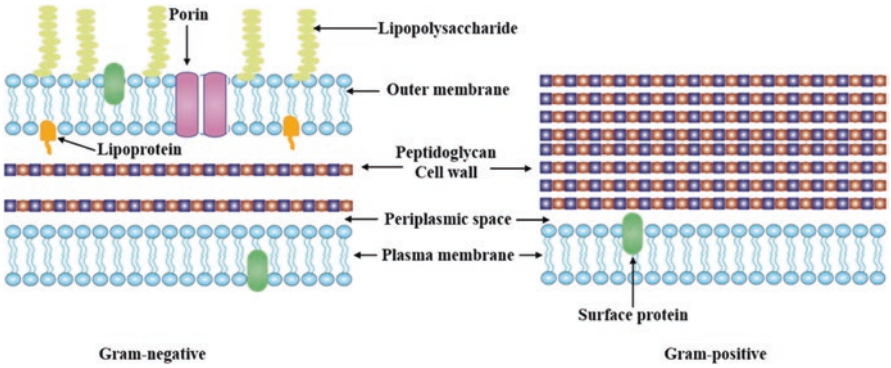

Fig. 1.4 Illustration of the cell wall structures in Gram-positive and Gram-negative bacteria

Hence, the prevention of the access of the AMPs into an orbital distance that allows an electrostatic interaction with the bacterial surface was hindered based on the amount of the produced CPS by the *K. pneumonia* rather than altering the surface chemical or serological characteristics of the LPS (Campos et al. 2004).

1.7.2 Alterations of Cell Wall and Membrane Mechanism

Manipulations of the bacterial envelope are significant objectives in bacterial cells against AMPs (Zasloff 2002). These alterations include repulsion of the AMP through modifications of the anionic net charge of the cell membrane and alteration of the membrane fluidity (Hancock and Rozek 2002; Peschel 2002).

The anionic constituents in the cell membrane include the phosphatidylglycerol (PG), the phosphatidylserine (PS) and the di-phosphatidylglycerol or cardiolipins (CL). Moreover, the anionic constituents in the cell wall are the lipopolysaccharide (LPS) and lipoteichoic acid (LTA) (Rashid et al. 2016). On the other hand, most AMPs are amphiphilic molecules consisting of a cationic moiety with a net charge between +2 and +9 due to specific amino acids such as arginine and lysin (Pushpanathan et al. 2013). This cationic part develops an electrostatic attraction towards the negatively charged heads of the phospholipids in the bacterial membrane and the hydrophobic portion penetrating the inner hydrophobic section of the bacterial membrane (Zasloff 2002). Therefore, bacterial ionic charge alteration ought to reduce the interaction level between the AMP and the bacterial membrane and even resist the existence of the AMP in the vicinity of such membrane. The ionicity of the bacterial membrane can be disrupted or masked by adding positive amino acids to the surrounding environment via a multiple resistance factor protein (MprF) (Nawrocki et al. 2014). MprF is lysyl-phosphatidylglycerol synthetase located in the membrane which uses L-lysin to manipulate the anionic charge of the membrane by synthesising aminoacylated-phosphatidylglycerol and transforming it

to the outer surface of the membrane, which, in turn, reduces the affinity of the membrane to AMPs, including the cathelicidin LL-37 and alpha-defensins (Ernst et al. 2009; Nawrocki et al. 2014). It is worth noting that the MprF mechanism of action is yet to be fully understood. However, it is known to have two required domains for full functionality, the C-terminal used for the lysinylation and the N-terminal employed for the exposure of the lysine on the surface (Ernst et al. 2009).

The *DltABCD* is another electrostatic repulsive pathway utilised by different pathogens against AMPs. The electrostatic forces of the bacterial envelope alongside other characteristics, for example, elasticity and porosity, are governed by the polyanionic network of the teichoic acid (TAs), which is made up of the composites of cell wall teichoic acid (WTA) and the lipoteichoic acid (LTA) (Neuhaus and Baddiley 2003). Scientists have reported that esterification of this matrix network by incorporating D-alanine through *Dlt* operon or (D-alanyl-LTA) pathway can modulate the properties of the envelope in many Gram-positive bacteria (Neuhaus and Baddiley 2003). Hence, the formation of the D-alanyl esters is found to be another enzymatic mediation that leads to ionicity masking the bacterial envelope and, consequently, results in AMPs' resistance in Gram-positive pathogens like *Bacillus*, *Enterococcus*, *Lactobacillus*, *Listeria*, *Staphylococcus* and *Streptococcus* species (McBride and Sonenshein 2011; Nawrocki et al. 2014).

In an attempt to further elucidate the impact of D-alanylation of the bacterial cell wall on the susceptibility to the AMPs, Peschel et al. explored the insensitivity to AMPs in mutated *Staphylococcus xylosus* and *Staphylococcus aureus* species (Peschel et al. 1999). They reported that the bacteria lacking the D-alanine esters in the teichoic acids of the cell wall were the most sensitive to the AMPs' panel, including the human defensin NHP1–3, tachplesins, magainin II, bacterial gallidermin, mammalian protegrins and nisins (Peschel et al. 1999).

Manipulation of the microbial cell wall and membrane constituents such as teichoic acids and phospholipids exerts a significant effect on the fluidity of the bacterial envelope, which, in turn, challenges the effectiveness of the AMPs (Peschel 2002). It has been speculated that the resistance of specific pathogens towards AMPs after D-alanylation of the envelope's matrix is due to the changes in LTA's configuration, which leads to an increment in the density and the rigidity of the bacterial cell wall, rather than the changes in electrostatic forces (Saar-Dover et al. 2012). By utilising atomic force microscopy, the researchers showed a drastic increase in the cell wall rigidity in wild-type group B *Streptococci*, which was escalated by 20-fold compared to the control group and, subsequently, reduced the wall's flexibility and permeability to the peptide (Saar-Dover et al. 2012).

1.7.3 Efflux Mechanism

Although the bacterial interactions with the surrounding environment are directly aligned with the bacterial cell surface features and conditions, in some cases, they are mediated via cytoplasmic/periplasmic cell components. The ABC transporter, or

the ATP-binding cassette, is a coupling system where ATP hydrolysis takes place as a driving force for an efflux mechanism that uptakes/reuptakes solutes from the cytoplasmic zone across the periplasmic space to the outer surface of the cell's environment (Davidson and Chen 2004). ABC transporters are eukaryotic and prokaryotic features that consist of four distinct subunits, including two hydrophobic membrane-crossing subunits and two hydrophilic nucleotide-binding domains that generate the required energy for the active transport (Davidson and Chen 2004). ABC transport systems play a critical role in a wide range of cell functionalities by utilising specific binding proteins with high affinity for different substrates, including sugars, amino acids, vitamins, metals and ions (Davidson and Chen 2004; Davidson et al. 2008). Hence, ABC transporters can play a significant role in supporting some virulence determinants of bacteria. For example, ejecting substrates against AMPs, such as capsular polysaccharides, and presenting them on the bacterial surface can lead to electrostatic charge manipulation inhibiting the AMPs' attraction to the bacterial envelope as discussed above (Liston et al. 2018).

Over the past 25 years, more studies were focused on mutagenesis of bacterial phenotypes associated with bacterial virulence, aiming to elucidate the significance of the membrane efflux pump concerning bacterial resistance to AMPs and virulence enhancement. Specifically, Jerse et al. tested the impact of the mutated multitransferable resistance efflux system (*mtrCDE*) on the viability of *N. gonorrhoea* in the lower genital tract of female mice (Jerse et al. 2003). Jerse's team confirmed the mutual relationship between the multidrug resistance efflux system and the survival rate of the bacterium. They reported that *mtrCDE*-deficient gonococci were more susceptible to different additives such as increased levels of progesterone which might exert an antibacterial effect (Jerse et al. 2003). Five years later, these results were confirmed through another study that investigated the effect of LL-37 on the manipulated gonococci. Shafer et al. reported that the loss of the *mtrCDE* increased the susceptibility of *N. gonorrhoea* to LL-37 (Shafer et al. 1998).

1.7.4 Biofilms

Biofilm formation is a different bacterial mechanism to combat AMPs' action beyond the intrinsic mechanisms of the individual bacterial cell to inhibit the access or bioactivity of AMPs. These cellular actions include pumping AMPs out of the cell, breaking them down, diverting them to falsely produced surface targets and refusing their entry by changing the membrane. A biofilm is an organised group of bacterial cells in which cells become glued to each other due to a self-produced slim matrix composed of exopolysaccharides contributing to the adherence of the embedded cells to a live/lifeless surface, and this bacterial community can work collaboratively to form an organisational protection (Band and Weiss 2015) (Jolivet-Gougeon and Bonnaure-Mallet 2014). The construction of the biofilm yields a cell-to-cell communication process named quorum sensing, which depends on the density of the bacterial community. It is regulated on the molecular level involving the

autoinducers and signalling molecules that control different features and functionalities such as biofilm formation, sporulation and bacterial virulence (Ashima et al. 2013).

Biofilms are of the most efficient features that contribute to bacterial resistance mechanisms against antimicrobial agents, including AMPs (Costerton et al. 1999), as they form a durable physical barrier around bacterial cells that prevents the infusibility of the AMPs towards the bacterial community zone (Costerton et al. 1999). Another possible reason for reducing biofilm susceptibility to antimicrobial agents can be directly related to their metabolic efficiency living in a cooperative protected community (Lazăr and Chifiriuc 2010). This structural way of living is manifested by a lower metabolic rate and, consequently, by a lower level of nutrient consumption, including uptake of existing AMPs below the minimum inhibitory concentration. Adding to that, some of the cells within the community can experience spatial deprivation in nutrient resources and other stress factors which might force them to develop a new mutated phenotype with less susceptibility to AMPs (Costerton et al. 1999; Jolivet-Gougeon and Bonnaure-Mallet 2014).

To sum up, bacteria differ in their susceptibility to AMPs which results in various mechanisms of action against bioactive molecules. Knowing the molecular signalling of the bacterial antagonism to AMPs with the appreciation of these molecules' physicochemical and biological features may provide new ways to combat antimicrobial-resistant and highly virulent bacteria.

1.8 De Novo Designed AMPs as Potential Therapeutics

Studies focusing on high-throughput discovery and design of new AMPs, as alternative solutions for conventional antibiotics, were successful in the past decade. Scientists developed various screening and/or design techniques to generate innovative bioactive AMP analogues, whereon they have made remarkable progress in advancing novel technologies.

The outputs of protein screening assays are forked between cell-based and computational approaches, aiming to optimise the AMPs' pharmacokinetics/dynamics, based on the current understanding of the mechanisms by which bacteria can circumvent the immune system. However, the persistent challenge of these different assays is preserving the therapeutic activity of the bioactive peptide with minimal adverse reactions. In the proceeding of this section, we present a couple of in silico approaches, including the Resonance Recognition Model (RRM) (Cosic 1994) and the Quantitative Structure-Activity Relationships (QSARs), (Taboureau et al. 2006) aiming to offer some insight into the recent advances in the field.

The RRM model is a physico-mathematical concept that employs digital signal processing approaches (Fourier and Wavelet transforms) in the translation of the peptide's physicochemical features in the linear motif that determines the nature of the protein's biological activity. It is based on the charge movement across the protein's backbone that occurs at different energy levels depending on the sequence and

the type of amino acids. The conductivity of the charge results in a resonated electromagnetic energy that transfers from the protein to the target molecule (Cosic 1994; Ćosić et al. 2006). The RRM methodology which is performed over two complementary stages, which include (i) conversion of the amino acid series into a numerical series and (ii) analysis of the resulting numerical sequences using a digital signal analysis approach. Translating the amino acid sequence into a numerical series is performed via the quantification of the electron-ion interaction potential (EIIP) value which represents the average energy level of the valence electrons for each amino acid. Subsequently, the following process is to apply the obtained numerical series to the digital signal analysis. Finally, the parent protein's/peptide's investigated bioactivity can be determined throughout the characteristic frequency of the digital signal analysis. The approach identifies the primary amino acid or the so-called hot spot that contributes to the bioactivity based on the prominent frequency shown on the characteristic frequency spectrum (Cosic 1994; Pirogova et al. 2011; Hu et al. 2013). For the last 10 years, the RRM approach has been utilised to design novel bioactive small molecular weight peptides, which proved to possess the intended bioactive therapeutic effects (Pirogova et al. 2009; Istivan et al. 2011; Almansour et al. 2012).

Another approach is the quantitative structure-activity relationship (QSAR) method, which is based on the correlation between the desired biological activity, the therapeutic agent's toxicity and the physicochemical properties of an array of molecular atoms (Taboureau et al. 2006; Jenssen et al. 2008). It involves different physicochemical characteristics, named descriptors, presented by the AMP, such as amphiphilicity, hydrophobicity, helicity and surface area. These physicochemical properties govern the electrostatic attraction between the AMP and the envelope of the bacterial cells (Jenssen et al. 2005; Jenssen et al. 2007). Manipulating these characteristics through alteration of specific amino acids does not necessarily contribute further to the antibacterial effect, but it might reduce an undesired side effect of the peptide, for example, the impact on the erythrocytes (Taboureau et al. 2006). Hence, the QSAR approach is a regression module that attempts to establish a consistent correlation between the variations in the molecular characteristics' values and the bioactivities for various compounds to rationalize the design of new chemical entities (Cherkasov and Jankovic 2004; Taboureau et al. 2006). Currently, QSAR analysis provides virtual screening for rapid design of in silico bioactive molecule libraries. Moreover, it can be utilised for data mining for novel AMPs (Cherkasov and Jankovic 2004).

1.9 Summary of the Current Knowledge on Antimicrobials Primes a Promising Upshot of Nanotechnology

The changeability and flexibility in the relationship between humankind and microbes have maintained the coexistence status quo since the early days of our history. Thus, up to a hundred years ago, this coexistence was balanced under the coevolution laws. Nevertheless, disruption evolved to this equilibrium after

discovering antibiotics arguing in favour of the predominance of the modern human species as the winners over infectious diseases. Indeed, lack of knowledge concerns the microbial kingdom amongst different groups motivated by acquisitive instincts, such as pharmaceutical companies and health professionals, which has led to the emergence of stubborn resistance against the conventional antibiotics or the "magic bullet", warning us of a challenging time to come.

The search for new classes of atypical therapeutic molecules associated with specific pharmacodynamics and fewer side effects has been upheaved (Leader et al. 2008). Peptides are one of the leading categories of nonconventional molecules that demonstrate an outstanding remedial potential across various medical conditions, including metabolic disorders, degenerative neuronal diseases, cancer and infectious diseases. Over 200 proteins and 100 peptides have been introduced to the pharmaceutical market across numerous indications (Muheem et al. 2016) during the last three decades. They exert their pharmacological effects via specific cellular pathways resembling natural molecular signalling. However, they are utterly sensitive molecules to physicochemical and biological degradations, which prevent them from reaching their target sites at the relevant bioavailability. Poor luminal permeability, high cytosolic metabolism, gastric degradation and the first hepatic clearance of such molecules have resulted in the dismissal of most of these biotherapeutics from being translated into medicines (Gupta et al. 2013; Muheem et al. 2016). Moreover, most of the currently used peptides and proteins are formulated in a parenteral form that requires high-cost cold chains for storage and transport.

Pharmaceutical scientists and biophysicists have contributed remarkably to the area of conventional drug delivery systems (DDSs) in the last 70 years. They have developed controlled drug delivery systems that proved to be successful in the medical and pharmaceutical industries. However, nonconventional and larger molecules such as peptide-based molecules have presented very challenging physicochemical properties and yet have a comprehensive and sophisticated design of a controlled drug delivery system to carry AMPs and deliver them orally for acute microbial infections and vaccinate the human body for prophylaxis.

Smart hybrid nanogels alongside other nano-delivery vehicles possess unique physicochemical properties that manifest in various dynamic changes such as transformation into different sizes and shapes in response to environmental stimuli. These changes make such materials promising co-therapeutic candidates for different medical conditions such as cancer, metabolic disorders, rheumatoid arthritis, neurodegenerative diseases and infectious diseases. We can manipulate such characteristics for ideal application and crossover of usage in different medical and pharmaceutical applications. The options for researchers in terms of various designs to diversify existing drugs and medical conditions are unlimited. However, accomplishing such a complex task requires multidisciplinary collaborative work between biophysicists, chemists, material scientists, biologists, drug discovery scientists and clinicians.

1.10 Drug Delivery System Strategies for AMPs

Researchers have been focusing on customised drug delivery systems by which the AMP is delivered directly and actively to a specific target site. This strategy ought to minimise the unnecessary exposures of healthy cells to the AMP and increase its bioavailability in the vicinity of the target site. However, actualising these ultimate grails was faced with the size of the delivery system as a significant challenge. The size of the targeted drug delivery system must correlate with the cellular and the molecular size of the target site while it is enduring the physicochemical and biological impediments along the way in the complex biological system, which is discussed in the following section.

1.11 Nano-drug Delivery Systems for AMPs

Convoluted physicochemical properties of the different colloidal nanomaterials (˂1000 nm) or the so-called nanoparticles, made of various substances such as inorganic metals, hydrogels, lipids, inorganic polymers or self-assembled peptides/amino acids, empower them to become highly sophisticated chrono-spatially controlled drug delivery systems. They provide the desired pharmacokinetics and pharmacodynamics to many AMPs by bypassing several physicochemical and biological impediments such as degradable enzymes, proteases and membranous barriers between the different physiological compartments. Moreover, manipulation of the physicochemical properties of these colloidal vehicles can be reflected in their structural functionality. For example, controlling the surface lipophilicity and charge furthers the device's chrono-spatial controlled drug release system and lowers the required dose and the frequency of administration (Makowski et al. 2019; Pinilla et al. 2021). Consequently, it increases the bioavailability of the AMPs and improves the anticipated therapeutic window by lowering their toxicity profile (Bozzuto and Molinari 2015; Sánchez-López et al. 2020; Nwabuife et al. 2021). Nevertheless, alterations to the size and shape that lads to surface deformities of the colloidal particle can impact their interactions with the surrounding environment and other biomolecules (Jeevanandam et al. 2018).

Based on the aforesaid, stability, homogeneity and consistency of distribution of the nanoparticles at the target site are the critical attributes for assessing their quality, safety and efficacy. Nevertheless, these elements can be impacted by the physicochemical characteristics of the nanoparticle device and the surrounding medium. Thus, these properties must be inspected and characterised meticulously during the validation process of every potential drug delivery system. Hence, alongside the conventional and routine analytical and characterisation methodologies such as scanning and transmission electron microscopy and different spectroscopic analyses that are typically employed in material sciences, there are a couple of critical

techniques, including dynamic light scattering (DLS) technique and electrokinetic test that must be employed (Makowski et al. 2019).

DLS, also known as photon correlation spectroscopy (PCS), is a well-established and commonly used powerful tool to study the diffusion behaviour of the colloidal system by quantification of the diffusion coefficient and the hydrodynamic radii that are based on the size and the shape of the particles (Stetefeld et al. 2016). It allows for the measurement of the particle size in a limited size range lower than 1 nm, and also it permits the estimation of the distribution of the colloidal system in a submicron region based on measurement of the Brownian motion of the tested particles (Caputo et al. 2019). Hence, DSL methodology provides an estimated degree of homogeneity between the sizes of the colloidal particles, which is crucial for the dosing uniformity.

The surface charge is a different pivotal characteristic that affects the stability of the colloidal system. Each dispersed particle is an aqueous solution surrounded by a shell-like electrical double layer named EDL (Fig. 1.5). The surface charge of the particle forms the bordering layer of the particle. This charged layer attracts the oppositely charged liquid ions that exist in the continuous aqueous phase forming the outside layer of the shell. The potential difference between the external surface of the EDL and the aqueous solution is termed electrokinetic potential or the so-called zeta potential (Salopek et al. 1992). Generally, tiny particles are inclined to aggregate and form a cluster of particles due to Van der Waals attractive forces in the vicinity of zero zeta potential (approximately between −30 and 30 mV) (Makowski et al. 2019). Hence, the estimation of the zeta potential is a critical anchor in colloidal chemistry by which scientists can anticipate the potential stability of the colloidal system, that is, sustainable suspension versus flocculation.

Just as importantly, the drug release from the nanosystem is a crucial element in the pharmacodynamic process. Hence, modelling the AMPs' diffusion kinetics from the nano-vehicle is an imperative practice required to determine the therapeutic window of the used bioactive ingredient. Finding a general model to describe the diffusion behaviour of a therapeutic agent from the various nanosystems, so far, is unachievable due to the complexity of these systems and the number of

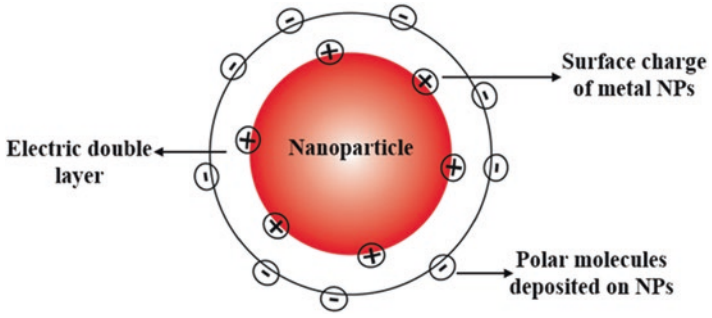

Fig. 1.5 Schematic illustration of the electrical double layer (EDL) formed around the positively charged surface of a nanoparticle

physicochemical factors involved in the process. Having said that, the two primary general theoretical rate expressions include Noyes-Whitney dissolution law and Fick's law of diffusion, in addition to over ten well-known models (Barzegar-Jalali 2008; Aulton et al. 2013) that can be utilised to study and model the different diffusion kinetics of various nanosystems based on their unique composites and characteristics.

With a view of shining some light on the current practice in the nanobiotechnology field concerning the delivery vehicles of AMPs, the following discussion depicts some examples of different nanoparticles representing the most common categories of nano-delivery systems that have been developed in recent years. These nano-delivery systems include inorganic metal, an inorganic polymer, hydrogel, lipid and self-assembled peptides/amino acid-based devices.

1.11.1 Inorganic Nanomaterial: Metal Nanoparticles (MNPs)

Deficient pharmacokinetics of AMPs could conceivably result from a high rate of degradation by different proteases or sub-absorption and transporting to the target site (Saghazadeh et al. 2018). The amalgamation of AMPs with MNPs' carriers ought to enhance their absorption into the bloodstream, prolong their half-life, reduce the required dosage and frequency of administration and lessen their toxicity (Chenthamara et al. 2019).

MNPs can be fabricated in various shapes such as cubes, rods, cones, stars and spherical forms, which are the most common shapes (Jeevanandam et al. 2018). However, notwithstanding the different forms of MNPs, the principal concept in the MNP shape is the surface-to-volume ratio (Makowski et al. 2019). The nanosized scale characteristic provides such particles with a conspicuously sizeable surface-to-volume ratio that can adsorb a significant number of molecules on a small number of employed particles that enhance the bioavailability of the active ingredient and, subsequently, the therapeutic efficacy at the target site while maintaining lower toxicity levels of excipients (Patra et al. 2018). Moreover, due to the nanosized property of such particles, they can be the ideal sensitive agents to detect diminutive target sites on the cellular and molecular levels (Sun et al. 2008). Furthermore, unlike most nanomaterials that are utilised as DDSs, metal nanoparticles offer, exclusively, few unique and vital physical properties such as electrical conductivity, specific optical behaviour and high thermal stability in addition to their chemical durability (Khan et al. 2019; Coetzee et al. 2020).

Formulation of the nanostructure of metal particles can be obtained through various chemical methods. Wet chemical techniques have been used widely in the synthesis of MNPs by using excess reducing agents such as sodium borohydride ($NaBH_4$) or trisodium citrate ($Na_3C_6H_5O_7$) in an aqueous solution (Panigrahi et al. 2004). However, there are an array of various chemical and biological techniques used for the synthesis of nanosized materials. To demonstrate one point, after the screening of different microorganisms, including bacteria, fungi and yeasts,

scientists have reported the ability of some of these microorganisms to reduce gold ions onto nanoparticles of different sizes and shapes through unknown cellular mechanisms (Gericke and Pinches 2006).

Metal nanoparticles can be tailored to certain functionalities and interactions with the surrounding environment. For example, it is attainable to change the surface charge of the metal nanoparticle by attaching different functional chemical groups to promote the interaction with specific markers such as the negatively charged phospholipids on the cell membrane of the target cells (Fig. 1.6) (Abbasi et al. 2021). At the same time, conjugation of nanoparticles with different functional groups permits the MNP-AMP complex to cross biological membranes. Moreover, manipulating the surface structure of MNPs can be utilised to promote the so-called active targeting mechanisms (Attia et al. 2019). For instance, attaching specific biomolecules or ligands to the surface of the metal nanoparticle allows it to become uniquely interactive with a specific cell or microorganism. Furthermore, attaching biocompatible polymers such as polyethylene glycol (PEG) masks the exposure of the nanoparticles to the reticuloendothelial system leading to an increment in the circulation time and the stability of the MNPs (Fratoddi 2017).

The stability of MNPs in different culture media is questionable due to their chemical interactions with several salts, whereby changes in structure (size) and surface charge are feasible (Pavlin and Bregar 2012). Consequently, the aggregation of multiple particles is becoming inevitable, which results in inhomogeneous biodistribution. Concurrently, in the biological system, the instability and clearance of the MNPs depend on the particle size. MNPs exceeding 200 nm are recognised earlier than smaller ones by the immune system and cleared out through phagocytosis. Nevertheless, the circulation lifetime for MNPs below 200 nm is longer, and they are eliminated by the spleen, followed by particles that are scaled down below 5 nm that cleared out by the renal system.

For the scope of this study, we present a couple of MNPs which include gold (Au) and silver (Ag) as representative models, which have been explored widely in

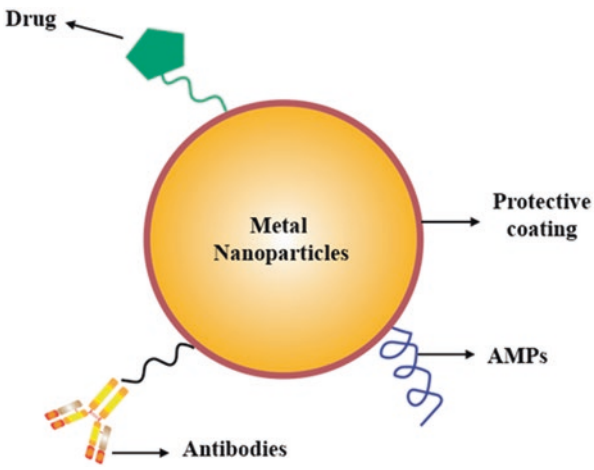

Fig. 1.6 Schematic illustration of a metal nanoparticle conjugated with various functional molecules

recent years as DDSs for AMPs. Cationic MNPs such as Au can form an electrostatic bonding with the negatively charged phospholipids in prokaryotes' cell membrane, leading to its disintegration and crumbling (Makowski et al. 2019). Moreover, MNPs can penetrate the cell and exert their anti-cellular activity by targeting intracellular molecules and different physiological processes by releasing their ions in the vicinity of the cytoplasm (Makowski et al. 2019). These ions can interfere and disrupt the electron transport chain known as the respiratory chain process leading to inhibition in ATP production and initiate the accumulation of reactive oxygen species (ROS) and consequently stimulate the oxidative stress mechanism resulting in bacterial death (Rai et al. 2009; Niikura et al. 2013). Victorious nanocarriers with optimised size such as MNPs can penetrate the bacterial cell membrane after crossing the epithelial cells and reaching the cell's apical surface, waiting to be transported to the basal side. The dissemination of the MNPs into the cell occurs through the so-called transcytosis mechanism by which they are transported through the interior of the cell via an uptake mechanism, namely, endocytosis with the likelihood of withdrawal via exocytosis process, degradation by lysosomes or deposition into a specific cellular organelle, after releasing the load inside the cytoplasm (Reinholz et al. 2018). Several studies have shown that manipulation of the size or the surface of the MNP impacts exocytosis kinetics and efficacy. For example, PEGylated gold NPs were exocytosed significantly, faster than positively charged (cationic) AuNPs (gold nanoparticles) (Oh and Park 2014). In a separate study whereby Chithrani and Chan tested the exocytosis rate of transferrin-coated gold NPs with a size range from 14 to 100 nm, the smaller NPs were exocytosed faster and at higher volume/time compared to the bigger sizes (Chithrani and Chan 2007).

In light of the above discussion, MNPs can be tailor-made to eradicate bacterial infection by enhancing their intrinsic antimicrobial properties (Sánchez-López et al. 2020). Thus, the anticipated outcomes from the amalgamated MNP-AMP complex were set so far out.

The antibacterial activity of the conjugated complex of MNP-AMP was found to be significantly augmented (Makowski et al. 2019). Cationic AMPs possess a high affinity to the negatively charged bacterial cell membrane (Hancock 2001; Scorciapino et al. 2017; Pal et al. 2019). Pal and the group have presented even more hostile activities adopted by AMPs against the bacterial membrane through molecular dynamic simulation technique. They have shown that the bacterial lysis via the barrel-stave mechanism formation in the bacterial membrane bilayer was mediated through a hydrophobic collapse mechanism that was initiated by the physical attraction between the hydrophobic moieties of the cationic peptide named Odorranian-A-OA1, which was conjugated with AgNPs (silver nanoparticles) from one side and the Gram-negative *E. coli* cell membrane from the other side (Pal et al. 2019). Conjugation of AMPs with nanoparticles at the very least leads to a higher concentration of the AMP at the target site. However, it has been reported that binding peptides to a metal surface such as silver enhances the growth and stability of the AgNPs (Zeng et al. 2007; Pal et al. 2016). The high affinity of specific amino acids such as Arg, Cys and Met to silver stabilises the nanoparticle structure (Poblete et al. 2016); subsequently delivering the AMPs to the target cells will be prolonged.

Mohanty et al. have demonstrated the effect of conjugating the AMPs NK-2 and LLKK-18 with biogenic AgNPs produced by *Alstonia macrophylla* and *Trichoderma* sp. against *Mycobacterium smegmatis* and *M. marinum* as a combination in comparison to the effect of the MNP alone. They have found that combined AgNPs with the NK-2 or LLKK-18 have a significantly higher effect on the mycobacteria than the solo MNP without causing a notable cytotoxic or genotoxic effect on mammalian cells (Mohanty et al. 2013). Furthermore, Lee et al. have tested the effect of the AMP HPA3P^{His} loaded onto a gold nanoparticle-DNA aptamer against *Vibrio vulnificus* infection in mice (Lee et al. 2017). Lee and the group reported a full recovery of the infected mice after administering the complex AuNP-Apt-HPA3P^{His} intravenously. The AuNP-Apt-HPA3P^{His} has led to a total inhibition of the *V. vulnificus* in different organs, compared to the control group that died within 40 hours of being infected (Lee et al. 2017).

Based on the above discussion, MNPs present great potential as DDSs that can deliver AMPs for various applications and types of pathogens. However, such potential requires more collaborative work between scientists and clinicians to improve therapeutic outcomes and reduce the toxic effects that have been reported for some of MNPs, such as silver, on mammalian cells and the environment (Kulkarni and Muddapur 2014).

1.11.2 Hydrogel-Based Nanoparticles: Nanogels

Presently, the knowledge of hydrogels and their potential usage as responsive, intelligent controlled-release drug delivery systems is significantly rich. Hydrogel is a cross-linked hydrophilic polymer with a three-dimensional network that is capable of swelling and retaining significant amounts of water throughout its fabric, hence providing a degree of flexibility like natural tissues (Ahmed et al. 2013; Ahmed 2015; Caló and Khutoryanskiy 2015; Akhtar et al. 2016; Nassar et al. 2021). Simple physical or chemical reactions produce these cross-linked hydrocolloid systems. They are defined as diluted systems that might be categorised as weak or strong based on their rheological behaviour in their steady-state phase (Lauren et al. 1980). Hydrogels can be produced in different physical forms, including slabs, microparticles, nanoparticles, coatings and films. As a result, they can be commonly used in clinical practice and experimental medicine for a variety of applications, including biosensors, tissue engineering and regenerative medicine, separation of biomolecules or cells and hydrogel-based drug delivery devices that have become a significant area of research interest. Biopolymers, including hydrogels, can be manipulated in size, architecture and composites, leading to different physicochemical characteristics. Subsequently, they differ in responsiveness and functionality that determine the type of drug delivery system they control (Li and Mooney 2016).

These developments are due to an extensive effort worldwide by biophysicists and pharmaceutical scientists over the past few decades. They have delivered a fundamental understanding of polymeric science by establishing various critical

concepts concerning the rheological behaviour and pharmacokinetic modules of such biomaterials (Korsmeyer et al. 1983; Peppas and Korsmeyer 1986; Peppas and Sahlin 1989; Siepmann et al. 1999; Kasapis 2006; Kasapis 2008). Advances in peptide screening and optimised in silico design of small molecular weight protein/peptide analogues in addition to developments in hydrogel system design for controlled drug delivery are likely to drive the commercialisation of AMPs as alternative antimicrobial medicines to improve human well-being.

1.11.2.1 Nanogels

Nanogels are one of the major three subdivisions of the hydrogels family, including macroscopic hydrogels, microgels and nanogels. Nanogels are designed at nanoscale sizes ranged between 10 and 100 nm depending on the requirements of the route of administration and the targeted delivery site of the active pharmaceutical ingredient (API) (Li and Mooney 2016). Nanogels, like hydrogels, have attracted the attention of drug delivery scientists by far and away for the sake of their versatile properties. They gained the reputation by being fastidious in their responsiveness to environmental stimuli such as pH, temperature and organic metabolites leading to the diffusion of the entrapped cargo at different and specific target sites (Soni and Yadav 2016). Fundamentally, they are three-dimensional hydrogels with the capacity of holding large amounts of water while sustaining their structural integrity (Ahmed et al. 2013; Zhang et al. 2016).

Furthermore, they are spherical with tunable physicochemical characteristics such as size, softness, porosity, electrostatic charges and chemically manipulative surface through alteration of its lipophilic properties (Soni and Yadav 2016; Soni et al. 2016). This structural flexibility and tunability allow for customised nanogels as exclusive controlled drug delivery systems in various formulations (e.g. oral and subcutaneous) for specific target sites at which they are subject to specific time-dependent stimuli (Li and Mooney 2016). Case in point, nanogels can be designed in an injectable form to deliver compatible APIs, including AMPs systemically due to their ability to infiltrate out of the small blood vessels via fenestrations across the endothelium towards different tissues (Li and Mooney 2016). The customised delivery via nanogel system is designed based on specific manipulation of the nanogel surface. They can be designed pharmacodynamically to interact with the surface of the targeted cell (Zhao et al. 2014). For example, few studies have successfully managed to deliver cytotoxic agents by utilising micellar nanogels directly to the tumour cells whereby a higher concentration of the APIs was achieved in the vicinity of the cancer cells and simultaneously lower drug availabilities were recorded nearby the normal cells (Qiu et al. 2014; Zhao et al. 2014).

Furthermore, nanogels can be utilised to optimise the stability, solubility and absorption of various AMPs. It is conceivable to enhance the solubility of hydrophobic drugs in an aqueous solution by encapsulating them within amphiphilic nanogels (Zhao et al. 2014). Moreover, such encapsulation can protect sensitive substances such as bioactive peptides and nucleic acids from chemical and

biological enzymatic degradation throughout the delivery process via the bloodstream and other biological compartments (Soni et al. 2016).

Nanogels are uniquely defined by their morphology which consists of two main parts: (1) the inner micellar compartment, which holds and protect the AMP until its release, and (2) the corona shell that carries the ligands for specific targeting or self-protection (Fig. 1.7) (Alexis et al. 2008; Moritz and Geszke-Moritz 2015).

Hence, the protection of the nanogel and subsequently the entrapped AMP within the physiological system (bloodstream and other body compartments) and the enhancement of the functionality of the outer shell of the nanogel are achievable via a specific design of the corona. To illustrate, grafting the hydrophilic polyethylene glycol (PEG) onto the surface of the nanogel provides a receptor-mediated drug delivery through PEG-conjugated agonists. This type of conjugation minimises the non-specific interactions with other proteins and reduces the adsorption to biodegradable enzymes leading to a reduction in the clearance rate of the nanogel (Otsuka et al. 2003; Romberg et al. 2007).

Nanogels are synthesised through chemical crosslinking or physical interactions between two low molecular weights of monomers (Soni et al. 2016). Chemical crosslinking between the different polymers/monomers is carried out via covalent bonding between the interactives, whereas the attractive physical forces between these polymers/monomers can be established due to relatively week forces such as hydrophobic attractions or ionic interactions (Soni et al. 2016). Nevertheless, conventional chemical synthesis through radical polymerisation provides nanogels with the required architectural structure, including the core-shell and the nanogel hollow (Chiang et al. 2012).

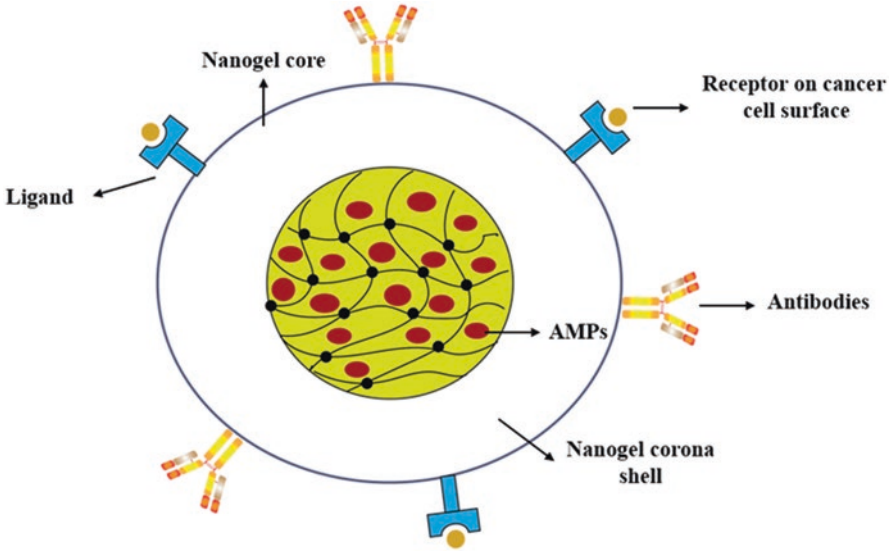

Fig. 1.7 Schematic representation of the nanogel morphology conjugated with different functional molecules

Nanogels become highly sophisticated delivery systems after a unique tuning of their size, morphology and specific chemical manipulations leading to unique functionalities. Specific responsive morphological features concern different environmental stimuli such as pH, temperature, electromagnetic field, light, glucose, electrolytes and other metabolites and result in the so-called intelligent nanogels; however, it can be a convoluted chemical process. This complexity includes using different initiators that activate specific chemical reactions, allowing different functional groups to incorporate the structure (Sanson and Rieger 2010). Recently, the accumulated scientific data concerning stimuli-responsive polymers has been remarkably increased due to the advancement in polymerisation techniques. These new technologies, such as the atom transfer radical polymerisation (ATRP) and the reverse addition-fragmentation chain transfer polymerisation (RAFT), provide an excellent tolerance towards the addition of new chemical motifs (Jochum and Theato 2013). Generally, the stimuli-responsive behaviour is a downstream cascade of events initiated by either an indigenous environmental parameter in the vicinity of the nanogel such as pH or an exogenous stimulus including light or electromagnetic field (Soni et al. 2016). These stimuli initiate conformational changes in the nanogel structure based on the alteration in the balance between the surface hydrophobicity and the lipophilicity (Li and Mooney 2016; Soni et al. 2016), resulting in changes in the swelling ratio of the hydrogel and the expulsion of the trapped material (Eichenbaum et al. 1998).

Temperature-responsive nanogels were popular in drug delivery applications (Jochum and Theato 2013). Their type of response was studied intensively by introducing the lower critical solution temperature (LCST) concept, a unique chemical characteristic. Below, the LCST nanogels show complete miscibility in solution; however, above the LCST, clear phase separation occurs (Jochum and Theato 2013). For example, poly(N-isopropyl acrylamide) (PNIPAM) shows lower solubility in aqueous solution within the range of temperature between 30 and 35 °C due to macromolecular transition from hydrophilic to hydrophobic moiety around its LCST (Schild 1992). Hence, grafted nanogel with PNIPAM loaded with an AMP can be triggered externally to initiate the macromolecular transition back to hydrophobic nature, leading to the release of the drug. Hyaluronic acid (HA) is another example of a stimuli-responsive nanogel. HA is a common signal molecule in the human body, and it is ubiquitous in the extracellular matrix. Moreover, HA is targeted by hyaluronidase (HAdase), a highly expressed enzyme in metastatic cancer and lymph nodes. Based on that, nanogel made of HA was utilised to encapsulate indocyanine green (ICG) to be released post enzymatic stimuli-responsive mechanism in cancer diagnostic imaging (Mok et al. 2012).

Although the nanoscale size of nanogels enhances their diffusion through biological membranes and across the blood vessel endothelial linings with an average fenestra between 50 and 100 nm across most physiological compartments (Alexis et al. 2008); however, they are considered as foreign bodies to our immune system. Hence, manipulating the outer shell of the nanogel can reduce the attraction to digestive enzymes and natural immune peptides, leading to a long-circulating time in the different body compartments. On the other side, the clearance of nanoparticles from the biological system is performed via spleen and renal filtration or

through macrophagic phagocytosis, determined by the particles' size. Hence, particle size below 20 nm will be diverted to renal filtration, whereas larger particles up to 200 nm can be squeezed through the spleen (Moghimi et al. 1991; Zhang et al. 2012; Soni et al. 2016). Thus, phagocytosis, which targets larger sizes (0.5–10 μm), can be markedly halted as a leading clearance mechanism against nanogels by reducing the size below 0.5 μm (Alexis et al. 2008).

1.11.3 Lipid-Based Nanomaterials: Nanoliposomes and Nano-micelles

The name liposome is a combination of two Greek words *lipos*, meaning fat, and *soma*, meaning body. Liposomes were discovered and identified by Alec Bangham et al. in 1965 (Liu et al. 2016). Nanoliposomes are nanoscaled spherical liposomes. Generally, they are unilamellar chambers that consist of single amphiphilic bilayers made of cholesterol or nontoxic phospholipids (Fig. 1.8) (Akbarzadeh et al. 2013; Pinilla et al. 2021). Larger vesicles that can be tumid up to the micrometre range are, typically, made of multiple lipid bilayers (multilamellar vesicles). The spherical architecture of nanoliposomes offers an aqueous core as a suitable vacancy to lodge, protect and deliver sensitive cargos such as AMPs from the surrounding environment (Pinilla et al. 2021), which are diversified, based on their physicochemical properties, between hydrophobic, hydrophilic, anionic and cationic AMPs (Scorciapino et al. 2017; Magana et al. 2020).

Moreover, the phospholipid-based membrane of the liposome exhibits a unique platform for specific organic molecules that can protect the vesicle from different

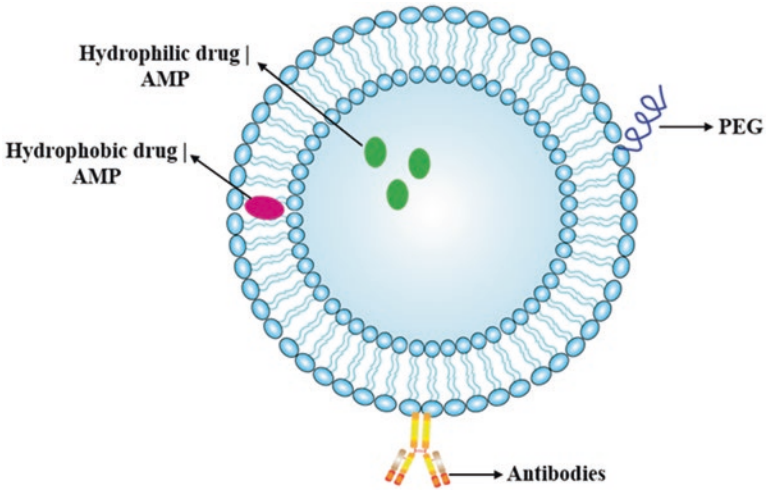

Fig. 1.8 Schematic illustration of the structural and functionalised liposome

physical, chemical and biological injuries and provide customised guidance systems targeting specific delivery terminals (Manikkath et al. 2020). Furthermore, utilisation of liposomes for drug delivery systems has been proven to be utterly compatible and biodegradable in the biological system and significantly low in their cytotoxicity profiles (Akbarzadeh et al. 2013).

In light of the aforesaid information, these state-of-the-art properties depict liposomes as intelligent drug delivery systems that were significantly utilised commercially for various therapeutic agents, including AMPs (Liu et al. 2016). Nevertheless, apprehension and portrayal of the diversified physicochemical characteristics of liposomes that have been increased remarkably in recent years (Patil and Jadhav 2014) are imperative in demarcating their suitability and fitness as DDSs for the wide range of distinctive AMPs.

Liposomes are categorised concerning their function/response, such as ligand-triggered, controlled-release, exogenous/endogenous stimuli release and stealth. Moreover, the architecture of different liposomes is defined by the number and the size of the vesicles' lamella (core). The completion of the different architectures occurs during the designing process based on the delivery material and the target site of the liposome. Furthermore, the degree of the fluidity of liposomes is another crucial feature that must be considered during the engineering process as the balancing point between the stability of the liposome and the diffusion of its load at the target site (Monteiro et al. 2014). Liposomes' different groups and characteristics are controlled via their constituents and the employed technologies for their formulation. For this chapter's scope, we will briefly discuss the conventional and the atypical formulation techniques; moreover, we will expound on some of the different customised functionalities only summarily, and further details may be found in the cited literature.

The formation of liposomes is a process that toes the line of the fundamental concept in pharmaceutical sciences, at which point oil and water can form a homogeneous mixture or the so-called oil-in-water emulsion by lowering the surface tension of the aqueous phase (Aulton et al. 2013). Based on that, the conventional technologies that are used in the formulation of liposomes can be achieved either by (i) forming a bulk of a yield via transferring a mass of organic phase of phospholipids into an aqueous (continuous) phase or (ii) depositing the organic phase on the surface of the aqueous solution forming a film leading to the deposition of liposome vesicles into the bulk of the continuous phase after reaching the critical micelle concentration point (CMC). This process is purported as a self-assembled mechanism at which a circular biological membrane is formed due to the phospholipids' crowding of immiscible hydrocarbon chains due to their mutual hydrophobic attractions in aqueous solution (Shohda et al. 2015; Liu et al. 2016). Hindering of the hydrophobic moiety of the phospholipids within the membranous structure while revealing the hydrophilic polar heads of these phospholipids to the aqueous solution ought to increase the solubility and stability of the formed liposomes. Dynamically, the stability of the emulsion system is achieved by lowering the high energy status associated with the edges of the hydrophobic chains exposed in aqueous solution to a preferred conformational energy associate with the self-assembled hydrocarbons

shielded from the water phase (Patil and Jadhav 2014). In completing the formulation process of the vesicle, the final size and the lamellarity of the formed liposome can be determined later through a wide range of typical and atypical techniques.

In general, inputting a more considerable amount of the used phospholipid into the system can lead to the size expansion of the vesicle. Nevertheless, fragmentation of that size is achieved through different dynamics. Moreover, allowing the bilayer edges to merge faster can lead to multilamellar vesicles and vice versa. For example, applying electric fields during the formulation process can reduce the hydrodynamic flow, causing a decline in the bilayer synthesis, and subsequently, unilamellar vesicles merge as the final product (Patil and Jadhav 2014; Rideau et al. 2018).

The formulation technique's nomination depends on the size and the lamellarity of the vesicle that is required. For example, preparing giant unilamellar vesicles (GUVs) (>1 μm) can be achieved via hydration of a dried phospholipid film by employing different conventional methods. We can utilise the "gentle hydration of phospholipid film" method to produce a single GUVs made of a closed lipid bilayer membrane of diameter greater than 1 μm (Shohda et al. 2015). This method is known as the "natural swelling" method, whereby the formation of the liposome occurs spontaneously after hydration (Rodriguez et al. 2005), and it is the preferred technique for obtaining GUVs of charged phospholipids (Patil and Jadhav 2014). Furthermore, utilising this technique to prepare liposomes allows the formation of the GUV at ionic strength close to the physiological level after further improvement by Akashi et al. (Akashi et al. 1996).

Another technique used to formulate the GUVs is called the "electro-formation" method. It is a very similar technique to the gentle formation methodology. However, applying an electric current to the aqueous medium enhances the liposome formation from the dried phospholipid film (Rodriguez et al. 2005). Hence, an electromagnetic field is generated in the vicinity of hydrating solution after depositing the phospholipids on the surface of the electrodes, leading to the formation of a few to tens of micrometre liposomes with an improved rate of the unilamellar formation (Rodriguez et al. 2005).

Coalescence of small vesicles is another type of procedure to obtain GUVs. For instance, spontaneous clumping of small vesicles after prolonged incubation in suspension can lead to fusion between them and the formation of GUVs (Oku and Macdonald 1983). The fusion of small liposomes may be induced by various routes, including subjecting them to freeze-thaw cycles in highly concentrated solutions with electrolytes (Chavanpatil et al. 2006). In 2015, Motta et al. had developed a generic methodology by which GUVs were based on the free detergent coalescence of organic material in aqueous solution (Motta et al. 2015). The idea of this new method is based on the fragmentation of proteo-liposomes triggering the formation of proteo-GUVs. Hydration of dried small liposomes with purified water supposedly induces an osmotic shock causing the liposomes to burst before triggering the resealing process into GUV (Motta et al. 2015).

The giant unilamellar vesicles can be used as precursors to create large unilamellar vesicles (LUVs) and small unilamellar vesicles (SUCs) that fall within the nanoscale range. Nevertheless, these nanoliposomes can be, also, produced via

specific conventional methods. The "reverse-phase evaporation" is a traditional preparation method that involves the hydration of phospholipids after their dissolution in an organic phase such as chloroform or ethanol. Inputting water into the organic solution throughout vigorous mixing results in the water-in-oil emulsion, transformed to an aqueous suspension containing LUVs after the evaporation of the organic phase (Deamer and Bangham 1976). Another technique used to produce LUVs is the detergent dialysis methodology (Patil and Jadhav 2014). In this methodology, the solubilisation of phospholipids in an aqueous solution occurs by utilising the amphiphilic property of detergents. Then stabilisation of the phospholipid-detergent nano-micellar system's formed complex is enhanced by reducing the surface tension between the phospholipid micellar membrane and the aqueous solution. Elimination of the detergent from the micellar system increases the surface tension to a higher level, resulting in crowding of smaller phospholipid micelles leading to LUVs (Helenius et al. 1977).

SUVs' formation can be achieved by injecting organic solvent with dissolved phospholipids into an aqueous phase (Nwabuife et al. 2021). The idea behind this technique is to force a self-assembly mechanism of phospholipids in an aqueous solution after dissolving them in an organic vehicle (Lasic 1995). When the organic solvent such as ether or ethanol is injected into a more significant amount of aqueous solution, the concentration of the organic phase is dissolved and diluted in the aqueous solution leading to a drastic reduction in their concentration below the critical dissolving forces that required to maintain the solubility of the phospholipids. Subsequently, the phospholipids are released into the aqueous solution and forced to self-assemble, forming SUVs (Lasic 1995).

Generally, liposomes provide great potential for a targeted and controlled drug delivery system, primarily attributed to the flexibility in size, lamellarity and charge alterations (Nwabuife et al. 2021). As has been mentioned earlier, the reduction in the size and lamellarity of the MUVs (>1 μm) is one of the methods that is employed to formulate SUVs (20–100 nm) and LUVs (>100 nm) (Yu et al. 2018). Sonication, homogenisation and membrane extrusion are common techniques used to downsize the MUVs to SUVs and LUVs (Patil and Jadhav 2014; Nwabuife et al. 2021).

Fabrication of multilamellar vesicles (MLVs) is achievable by utilising different conventional methods such as hydration of phospholipid film of stacked bilayers under strong hydrodynamic flow for few hours (Carugo et al. 2016). Moreover, the reverse-phase evaporation method, which is utilised to produce LUVs, can also fabricate MLVs. Providing higher concentrations of phospholipid content in the aqueous phase yields a higher fraction of MLVs than LUVs (Pidgeon et al. 1987).

Hydration of pro-liposomes is another conventional method that can be employed to formulate MLVs with the encapsulated bioactive agent. Pro-liposomes are dry and stable liposomes that consist of phospholipids with the encapsulated bioactive ingredient. They can be formed by drying an organic solution containing the phospholipids and the cargo, such as AMPs (Patil and Jadhav 2014), transformed to MLVs upon their dispersion in an aqueous solution (Nekkanti et al. 2015).

Since the commercial production of liposomes has been levelled up strikingly in the pharmaceutical industry and beyond, the range of liposome preparation

techniques has been advanced significantly. Amongst these methodologies are the heating method, spray-drying, freeze-drying, supercritical reverse-phase evaporation and several modified ethanol injection techniques that are highly qualitative and engaging (Koynova and Tenchov 2015). Aside from that, in the early 1980s, an interesting multidisciplinary methodology called "microfluidic technology" has been introduced for different research purposes in various fields such as biochemistry, physics, biotechnology, engineering and nanotechnology. Microfluidic technology has been utilised for multiple drug screening in different body organs to the cell level. Nowadays, it is an atypical leading technique used to produce liposome-based DDSs for various conventional drugs, gene therapy and bioactive compounds, including AMPs (Carugo et al. 2016; Damiati et al. 2018; Dong et al. 2019).

The microfluidic formulation methodology is a nonconventional technique that provides unique advantages in the synthesis of drug delivery vesicles in terms of efficiency, homogeneous geometry and reproducibility over the conventional bulk methods that have been discussed above. It is a cost-effective technology that enables the fabrication of highly stable and uniformed vesicles at a fast rate with remarkable efficiency of encapsulation (Damiati et al. 2018). The microfluidic technology can be performed through different methods based on a hydrodynamic device that is consisted of microchannels and chambers to control the flow behaviour of a small volume of fluids and produce liposomes within the range of 50–75 nm (Dong et al. 2019), while, precisely, controlling the lipid hydration process as it has been reviewed extensively by Yu and the co-authors in Yu et al. (2009).

Structurally, liposomes can carry hydrophilic and lipophilic molecules; hence, they are suited for amphiphilic compounds such as AMPs. However, the uppermost role of the liposome in such an engaging process with the AMP is to permit and maintain its encapsulation within a stable and intact architecture. Moreover, maintaining the coexistence between the encapsulated AMP and the liposome is primarily controlled by the membrane's fluidity. Generally, the fluidity of the biological membrane is regulated by the relative proportions of cholesterol to the constituents of the phospholipids (Charalampous 1979). Higher ratio levels of the phospholipids tend to increase the fluidity of the biological membrane leading to stability reduction, which can be counteracted by increasing cholesterol (Cooper 1978; Chabanel et al. 1983).

Material scientists could utilise different lipid-based materials with an amphiphilic property to formulate liposomal lamellar through self-assembly processes. Nevertheless, the uppermost aim is complete control over the fluidity (stability) of the membrane and the diffusivity of the incorporated AMPs. Huang and co-authors have reviewed a wide range of studies that dealt with the so-called amphiphilic mesogens, which can form lyotropic liquid crystals (LLCs) (Huang and Gui 2018). These LLCs are amphiphilic molecules formed through the self-assembly process in aqueous solutions producing unique physicochemical and internal structural properties (Boge et al. 2016). They have usually formed throughout various phases: the lamellar phase, the reversed bi-continuous cubic phase and the reversed hexagonal phase. The different yielded structures of these variable phases have presented different diffusion coefficients to a wide range of loaded cargos that were also

managed to be released in a sustained manner. Moreover, they have shown varied responsiveness to different levels of pH and temperature, in addition to their abilities to load various sizes of cargos (Huang and Gui 2018).

Boge and co-researchers have examined the ability of a couple of lyotropic liquid crystalline (LC) structures incorporating (i) cubic glycerol monooleate in water and (ii) hexagonal glycerol and oleic acid in water, to carry different AMPs separately and maintain their antimicrobial effect while the LC structure is preserved (Boge et al. 2016). The results showed the antibacterial effectiveness of two of the tested AMPs, including AP114 and DPK-060, preserved in the cubic LC structure, whereas they noticed a reduction in the antibacterial effect of the LL-37. Moreover, the hydrophobic peptide (AP114) has induced an increment to the negative curvature of the cubic LC system. Conversely, the polar peptide (DPK-060) has reduced the negative curvature, while the LL-37 has not changed the LC phase. Interestingly, none of the peptides has affected the hexagonal LC phase; nevertheless, their antibacterial effect was significantly reduced (Boge et al. 2016).

The selection of the right combination of the liposomal constituents that are physiochemically compatible with the loaded AMP is another crucial aspect of liposomal formulation (Thapa et al. 2021). Nevertheless, scientists went beyond that by advancing liposomes to collaborate with AMPs and act synergistically, mounting the antimicrobial effect. Malheiros et al. used liposomes made of phosphatidylcholine (FC) and the cationic lipid named 1,2-dioleoyloxy-3-trimethylammonium propane (DOTAP) to encapsulate bacteriocin (Malheiros et al. 2016). The results showed that the encapsulation of the bacteriocin in the FC/DOTAP had advantaged the antibacterial activity of the bacteriocin in goat milk compared to the activity of the free bacteriocin. This synergistic effect between the liposome and the AMP might be attributed to the cationic effect of the DOTAP against the bacterial membrane (Malheiros et al. 2016).

Enriching the DDS with different selective physicochemical features as we know now can be advantageous in fighting microbial infections. Thus far, the hydrophobic attraction has been proven to be one of the effective mechanisms against bacterial cell integrity (Tian et al. 2015) and can be utilised to stabilise the AMPs and reduce their toxicity effects by confining them away from interacting with mammal cells (Chen et al. 2019). Hence, the inclusion of an augmented hydrophobic feature to a lipid-based vesicle might be an appealing choice for some AMPs for superior efficiency and stability. Based on this, utilising the concept of the micellar-based vesicle, a closed lipid monolayer with a polar core and hydrocarbon chain presenting the hydrophobic potential on the surface or a hydrophobic core with a polar surface (Fig. 1.9), has become one commonly used DDS as an alternative to the bilayer liposome.

Groo and co-researchers tested the effect of micellar nanosystem on the stability of an AMP called AP138 (Groo et al. 2018). They have developed reverse micelles incorporating lipid solution forming lipid nano-capsules (LNCs) by using the phase inversion process. The encapsulation rate of the AP138 into the complex of AP138-RM-LNCs was 97.8% which allowed them to conduct a comparison study, whereby they have tested the stability and the antibacterial effectiveness of the encapsulated

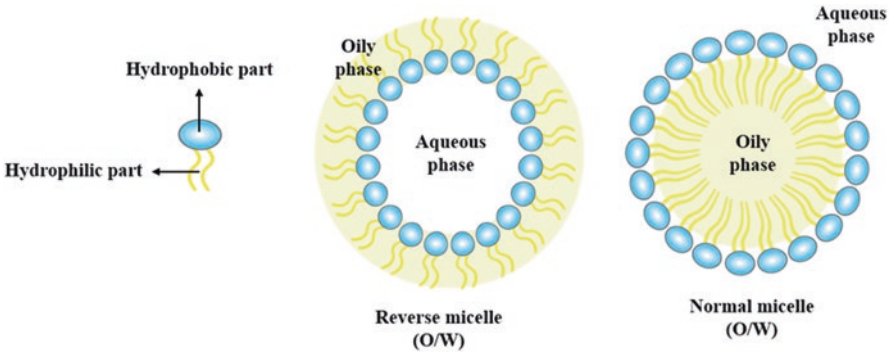

Fig. 1.9 Schematic illustration of revers versus normal micelle

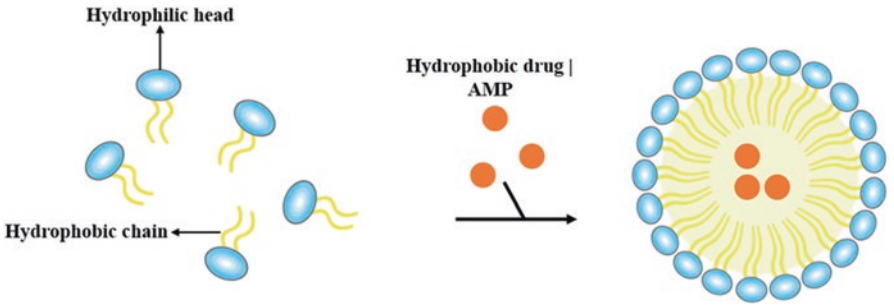

Fig. 1.10 Schematic illustration depicting the formulation of a micelle loaded with an AMP

peptide in comparison to the free AP138. They have shown that the encapsulated peptide (similar to the schematic illustration in Fig. 1.10) has demonstrated a significant resistance against trypsin compared to the free AP138; moreover, the antibacterial activity of the encapsulated peptide was preserved against *Staphylococcus aureus*, including MRSA (Groo et al. 2018). In short, micellar formulations loaded with antimicrobial peptides in Fig. 1.10 have already been applied into some dental applications due to their ability to bind to certain minerals integrated into teeth surfaces and to deliver encapsulated AMPs over a prolonged time against the formation of *Streptococcus mutans* biofilms (Carmona-Ribeiro and de Melo Carrasco 2014).

1.11.4 Inorganic Polymer-Based Nanomaterial: Nanofibres

Nanofibres are polymeric structures with the size of around 100 nm made of fibre that can be fabricated from inorganic materials such as ceramics, metal and metal oxides. Mixed metals such as Ba, Cu, Fe, Mn, Mo, Ni, Sb, Si, Sn and Ti and their oxides like CeO_2, CuO, Fe_2O_3, MnO_2, $NiCo_2O_4$, SnO_2 and TiO_2 culminate in various

considerable types of synthesised nanofibres with captivating properties (Barhoum et al. 2019). Nanofibres, like most nanomaterials, exhibit physical properties such as high surface area, high porosity with small mesh size and high pore density over conventional fibres (Sousa et al. 2020). Nevertheless, the morphological properties and homogeneity of the system are hard to control during fabrication (Barhoum et al. 2019). Generally, nanofibres can be employed in different areas in the medical and pharmaceutical fields, such as wound dressings, tissue engineering scaffolding and controlled-release drug release systems (Sousa et al. 2021; Topcu et al. 2021).

In the proceeding chapter, we endeavour to unravel the latest status of drug delivery research which concerns the formulation of various AMPs via nanofibres as one of the widely explored strategies in recent years. However, it is imperative to briefly consider the different methodologies employed in the synthesis of nanofibres and their general compatibility with AMPs.

Polymeric fibres with a diameter range between nanometres and micrometres are complex biomaterials that can be fabricated through various methods such as electrospinning, dry spinning, laser spinning and gel spinning (Frenot and Chronakis 2003; Sousa et al. 2021). Electrospinning is a nonconventional methodology that can produce an inorganic polymer fibre in the nanometre diameter. Moreover, it is one of the current advanced primary methods to deliver AMPs (Sousa et al. 2021) and, hence, is of interest in this review.

Electrospinning or the electrostatic wiring technique, in essence, is based on a combined electric and hydrodynamics, whereon an electric force is applied to a polymer solution resulting in a formation of a continuous hydrofibre upon its release into the air. Precisely, the electrospinning process is involving the application of high voltage to a needle that is attached to a filled syringe with a polymeric solution (Fig. 1.11). The polymeric solution is set for rotation supported by an electrode after its ejection from the needle by a high electric field developed between the needle and the collector (Frenot and Chronakis 2003). That is to say, an electric charge

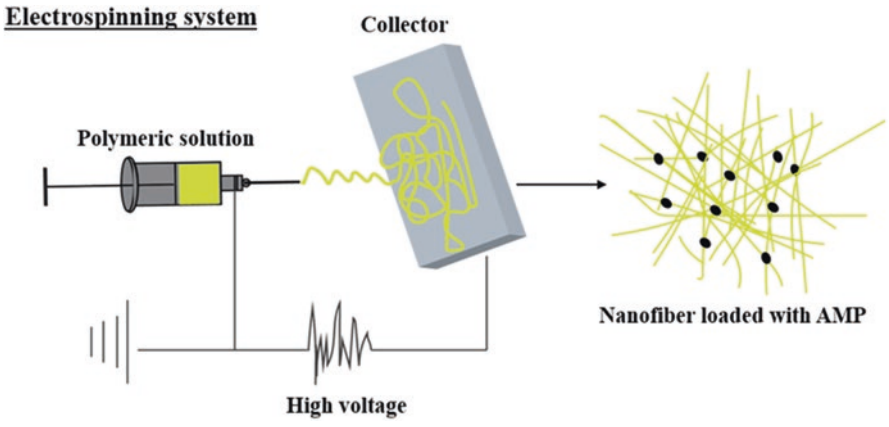

Fig. 1.11 Schematic representation of a traditional electrospinning system producing nanofibre loaded with AMP

develops on the surface of the first droplet coming out of the tip of the needle leading to higher surface tension. When the applied electrical field between the needle and the collector overcomes the surface tension of that first droplet, the ejection of the solution occurs, leading to instability during its traveling through the air to the collector, which results in a whipping-type flying (Sousa et al. 2021). During this traveling time and distance, the solvent evaporates, and the solidification of the polymer takes place, leading to a longer and thinner nanofibre (Shahriar et al. 2019).

Various exemplary physical and morphological characteristics of the constructed functional electrospun nanofibres (nanotubes/nanowires) such as high surface-to-volume ratio, high density of porosity and hollow diameter are subject to various elements, including the molecular weight and the concentration of the polymer; viscosity, conductivity and surface tension of the solution; the applied electric potential; the gauge and the angle of the fixed needle; the distance of the needle from the collector; and the feeding and releasing rate of the polymer (Frenot and Chronakis 2003; Peng et al. 2015; Ren et al. 2015; Shahriar et al. 2019; Sousa et al. 2020).

The incorporation of the AMPs into the electrospun nanofibre can be performed in different ways. In situ technique is a commonly used method to load AMPs into the drug delivery system by introducing them into the solution before forming the nanostructure (Truskewycz et al. 2021). Differently, AMPs can be adsorbed to the surface of the generated nanofibres, whereby they are actively attached to the polymeric system (Dart et al. 2019). The attachment of AMPs to the nanofibre's surface can be obtained through (i) immersion of the nanofibre into a solution of the dissolved peptide until reaching a saturation point. This type of attachment occurs without generation of covalent bonding between the AMP and the surface (Sousa et al. 2021), (ii) grafting the AMP onto the surface via "graft-to" methodology by creating a covalent bonding that results in a stable and long-lasting attachment via a direct grafting of the AMP onto the surface of the nanofibre (Costa et al. 2011) or through "surface-initiated" strategy (Sousa et al. 2021). The "graft-to" process involves activation of the surface of the fibre through UV radiation and oxidation, which activates different functional groups such as carboxylic acid, amines, aldehydes or thiols leading to covalent bonding with the AMP (Felgueiras and Amorim 2017). In comparison, the "surface-initiated" strategy involves the attachment of the AMPs mediated by initiators covalently immobilised at the surface (Green et al. 2011).

In doing so, it is possible to create a compatible loaded vehicle with the biological system that can release the loaded AMP at the target site according to the chronospatial plan. However, to achieve the desired kinetics and the pharmacological effect of the AMP, it is essential to choose the appropriate combination of the solvent and the rest of the excipients based on the physicochemical characteristics of the employed AMP. Eriksen et al. have investigated the effect of the physicochemical properties of different molecules loaded into nanofibre material on their diffusion kinetics (Eriksen et al. 2013). They have tested the diffusion properties of a synthetic AMP named fluorescein-labelled inverse-Crabrolin (iCR-fluor) in comparison to the diffusion kinetics of tetracycline hydrochloride, where both were

incorporated into the electrospun poly(ε-caprolactone) (PCL) nanofibres. The results showed that the release of iCR-fluor was only 40% compared to 85% of the tetracycline diffusion rate. Furthermore, the distribution density of the iCR-flour was less uniformed within the fibre compared to the distribution density of the tetracycline in the same nanofibre (Eriksen et al. 2013). The offered clarification by Eriksen and the group was based on the argument that the larger molecules of the iCR-flour were insoluble enough in the polymeric mix, which led to an integration of the AMP in the nanofibre structure followed by a significantly low rate of discharge from the fibre (Eriksen et al. 2013).

Just as significantly, unlike most of the nano-drug delivery systems, nanofibres can be utilised as a platform of more than one bioactive agent to be released either simultaneously or sequentially as each one of the bioactive agents is required along with the chronological development of the targeted medical conditions such as skin wound infections (Homaeigohar and Boccaccini 2020). Ahire and Dicks (2015) have successfully incorporated the antibiotic nisin and the 2,3-dihydroxybenzoic acid (DHBA) into nanofibre made of poly(D, L-lactide) and poly(ethylene oxide) that was diffused onto a biofilm formed by MRSA. They have demonstrated an apparent synergistic effect between the AMP and the DHBA against bacterial biofilm formation (Ahire and Dicks 2015). Furthermore, the different types of AMPs or bioactive agents can be loaded in different DDSs such as liposomes before incorporating them into the nanofibre for further protection (Xu et al. 2005). Alternatively, two or more syringes can coaxially produce multiple layers that result in a protective type of core-shell nanofibre (Calamak et al. 2017). Han et al. (2017) investigated the effect of the hygroscopic outer layer on the lifespan of the antimicrobial activity of the AMP nisin implanted in multiple syringes (Han et al. 2017). They found that nisin in a triaxial fibre membrane provides a more durable and sustainable antimicrobial activity that lasted over 7 days with over 99.99% of kills (4 log kills) compared to the coaxial fibre, which showed the exact duration of activity but with just 2 log cell death. The single nanofibre showed weaker antibacterial activity than the co- and obviously the triaxial, which lasted only for 1 day (Han et al. 2017).

1.11.5 Organic Polymer-Based Nanomaterials: Self-Assembled Peptides

Self-assembly is the process in which a spontaneous association of individual molecules or particles into a complex or functional structure through soft (non-covalent) chemical and physical interactions (Yadav et al. 2020). Peptides and proteins are amongst the first biological molecules, including carbohydrates, lipids and nucleic acids, to demonstrate the self-assembly phenomenon behind the cellular structure's integrity (Misra et al. 2021). Hence, inventive material scientists were easily swayed to utilise this phenomenon in designing nano-blocks made of short peptides or amino acids for advanced DDSs. Researchers were invigorated by the diversified physicochemical properties of peptides and their inherited biocompatibility and

biodegradability. Dipeptides and tripeptides have been identified to carry motifs with all the required information to compound sophisticated self-assembled nanostructures with unique functional characteristics such as mechanical rigidity, semiconductivity, piezoelectricity and visible luminescence (Gazit 2015). Moreover, manipulating the self-assembled characteristics is attainable easily through a different assortment of the attached amino acids or by attaching different functional groups to the chain (Misra et al. 2021; Caruso et al. 2014). Nevertheless, peptides that are crafted from canonical amino acids are challenged by various issues concerning their stability and intramolecular interactions (Caruso et al. 2014).

Peptides, in general, are made of alpha-amino acids, and as such, they were called alpha-peptides which have a brief lifespan due to their high susceptibility to enzymatic degradation (Boto et al. 2018). Moreover, alpha-peptides are short chains, making it hard for them to form a stable secondary structure (Hecht and Huc 2007). Therefore, molecular engineering of durable DDSs for AMPs made of self-assembled peptides is still a convoluted process that requires a great deal of appreciation of the physicochemical characteristics of these small proteins. Notwithstanding, stable secondary structures made of alpha-helix, beta-sheet, beta-turn and beta-hairpin are determined by the sequence and the nature (canonical vs. non-canonical) of the amino acids and, eventually, play a pivotal role in the self-assembly process (Gopalan et al. 2015). Moreover, a prime arrangement for these secondary structures through soft chemical and physical interactions such as Van der Waals forces, hydrogen bonding or hydrophobic attraction can generate supramolecular design such as nanotubes, wormlike micelles, vesicles, fibrils and spherical micelles (Fig. 1.12) (Misra et al. 2021).

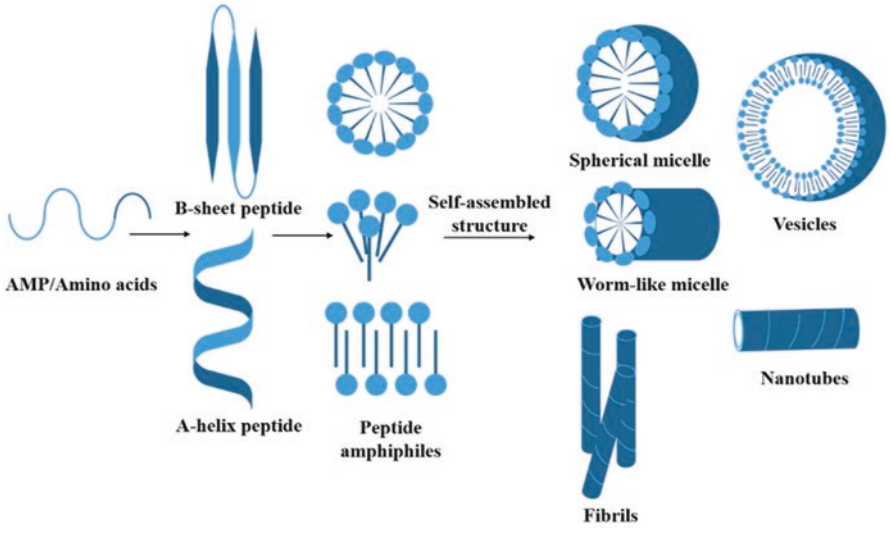

Fig. 1.12 Schematic illustration of a self-assembled antimicrobial peptide forming various functional structures

This section will summarily discuss some of the different strategies adopted to counteract the significant elements upholding the poor quality of self-assembled peptides as a nano-DDS. Also, we will briefly shed the light on recent advances of this nanotechnology concerning the delivery of AMPs.

The instability of the natural alpha-peptides is an inherent property of the homochirality of the L-(alpha)amino acid enantiomer. It has been found that incorporation of the non-coded enantiomer (D-(alpha)amino), which possesses the same chemical and physical properties of the L structure but is rarely found in nature (in some venoms and antibiotics), enhances the peptide's stability against proteolysis and prolongs its shelf-life significantly (Misra et al. 2021; Lu et al. 2020). Lu and co-workers have synthesised derivatives of a cationic AMP named Pep05 via the substitution of L-amino acid residues with the D structures, for example, D-arginine and D-lysine. They have revealed that the stability of the peptide made of the D structures of amino acids was enhanced significantly against trypsin and plasma proteases compared to the natural Pep05 (Lu et al. 2020). Moreover, formulation of a stable and self-assembled supramolecular structure or the so-called foldamer can be achieved through peptides comprising beta-amino acids (alpha-amino acids with an additional carbon atom) as an alternative to the natural alpha-amino acids (Gopalan et al. 2015). These beta-amino acids are regarded as "unnatural", identified in mammals, living organisms and plants (Griffith 1986; Yasumoto and Satake 1998). Daura and co-researchers have found that beta-peptides are markedly chemically diversified and exhibit a more extraordinary secondary structural motif, resulting in higher resistance to enzymatic degradation (Daura et al. 1997).

Formulation of a unique functional architecture such as a vesicle, or a vesicular nanofibre, through the peptide's self-assembly mechanism depends on the peptide's amphiphilic nature and the specific secondary structure of that peptide (Rughani and Schneider 2008). For example, beta-hairpin and beta-sheet secondary structures lead to the formation of the fibrillar bilayers and higher-order laminates (Dong et al. 2007; Rughani and Schneider 2008). These secondary structures can be facilitated by combining D-proline with L-proline or glycine in the constructed peptide (Misra et al. 2021; Mahalakshmi and Balaram 2006).

In closing the formulation part, the advancement of the supermolecular assembly of peptides has experienced enormous input in recent years through the introduction of different alterations such as the incorporation of 3,4-dihydroxyl-L-phenylalanine, dehydro amino acids and 2-amino isobutyric acid to the peptide molecules, in addition to the introduction of gamma and δ-peptides and their hybrid analogues which have been reviewed in great details by Misra et al. (2021).

Another topic to highlight here is the toxicity which is the major hurdle in using AMPs as they may exert their cytotoxic activity against mammal cells upon interaction with the cell membrane (Hollmann et al. 2018). The cytotoxic effect of AMPs on mammal cells is preliminary based on configurational measures of the peptide that contribute to selective interactive forces with the eukaryotic cells (Ebenhan et al. 2014). Integration of such AMPs into self-assembly structures forming supramolecular nanofibres ought to alter their configurations, hindering their toxic effects by preventing them from interacting with eukaryotic cells (Chen et al. 2019). Chen

and co-researchers have utilised the AMP melittin as a self-assembled natural model. They reported a dramatic change in its conformation when presented on the nanofibre surface, and consequently, the permeability of the bacterial and the mammalian cell membrane was modified (Chen et al. 2019). Precisely, positioning the melittin on the nanofibre's surface has impacted the degree of freedom of the hydrophobic residues, which, in turn, has reduced its hydrophobic attractions to the lipids in the mammalian cells. Improving the selectivity of AMPs and enhancing the targeted cell responsiveness are examples illustrating the significance of the role of peptide self-assembly on the functionality of AMPs (Tian et al. 2015).

Finally, confining AMPs within a rigid supramolecular polymeric scaffold could introduce great features to them in terms of responsiveness, controlled release, enhancement of stability and lifespan and reduction of toxicity. Moreover, the peptide self-assembly engineering technique could be one of the promising methodologies in nanotechnology to promote AMPs for different therapeutic applications.

1.12 Conclusions

The relationship between humankind and microbes went through many hurdles since the early days of coexistence; however, it was balanced up to a hundred years ago under the coevolution laws. This equilibrium was disrupted after discovering antibiotics arguing for the predominance of the human species that won the war over an infectious disease. However, the lack of fundamental knowledge of the microbial world and the main aim of pursuing financial benefits led to the emergence of stubborn resistance against the indiscriminate and massive assault of the "magic bullet," the antibiotics, teaching us a valuable lesson about other life forms on this planet. Over time, prokaryotes and eukaryotes have gone through countless evolutionary processes that support their survival and mutual relationships. AMPs are one of the yields of these protective mechanisms that have been developed, and we can take advantage of their availability to communicate precisely and gently with microbial infestations. They can be utilised as an alternative treatment for conventional antibiotics; nevertheless, they are too sensitive to handle complex biological systems abundant in physical and biological impediments. Advances in biomaterial sciences over the last 50 years allow the design of sophisticated biocompatible, biodegradable and chronospatial, nano-controlled drug delivery systems to carry AMPs and deliver them bioactively to the molecular level of the targeted microorganism cell at different physiological compartments of the eukaryotic biological system for effective remediation and self-healing.

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Chapter 2
Current Approaches and Prospects of Nanomaterials in Rapid Diagnosis of Antimicrobial Resistance

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Abstract Antimicrobial resistance (AMR) is one of the major global concerns that threatens both public health and the healthcare system. Albeit AMR in the microbes could emerge naturally, misuse or inappropriate use of antimicrobials plays a major role in resistance evolution. The global epidemic of AMR necessitates radical interdisciplinary solutions to better detect antimicrobial susceptibility and manage infections. Rapid diagnostics that identify drug-resistant pathogens determine antimicrobial susceptibility and distinguish between viral and bacterial infections can aid in initiating appropriate treatment for antimicrobial stewardship. Moreover, rapid diagnostics could also mediate epidemiological surveillance, as emerging resistant pathogens and their transmission can be monitored. In this chapter, we summarize different technologies applied for the development of rapid diagnostics for AMR and antimicrobial susceptibility testing (AST). We briefly discuss conventional diagnostic approaches followed by a comprehensive discussion on current approaches for detection of AMR and AST. While useful, most of these technologies take several hours to days to determine AMR in clinical samples and require extensive user inputs. Merits of incorporating nanomaterials in biosensor platform for improving AMR detection are further highlighted. We conclude the chapter by discussing the challenges that impede their adaption for point-of-care application and give insights into the future directions to overcome current challenges.

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2.1 Introduction

Antimicrobial resistance (AMR) has emerged as a major global health concern that has the potential to change the entire healthcare landscape (Organization 2014). Common infections are no longer easily treatable due to the emergence of resistance in the causal microbes. Today, AMR is associated with a rise in patient morbidity and mortality rates, prolonged hospital stays, surgical complications and economic losses from loss in productivity. In 2014, a report attributed 700,000 deaths across the globe to AMR infections; this figure is estimated to reach an annual toll of ten million by 2050 (Humphreys and Fleck 2016; O'Neill 2014). AMR is also projected to cause a reduction of 2–3.5% in the world's gross domestic product by 2050, an amount equivalent to US$100 trillion (Ahmad and Khan 2019; Allcock et al. 2017; Humphreys and Fleck 2016; O'Neill 2014). Factors, including but not limited to misuse and overuse of antimicrobials and lag in the development of new antimicrobials, have contributed to the emergence of resistance in microbes (Shanmugakani et al. 2020; Singer et al. 2016). Evidence suggests that 50% of the antibiotics prescribed in the hospitals are either unnecessary or inappropriate (Fleming-Dutra et al. 2016). The criteria to prescribe antibiotics is rather consistent throughout most of the Europe and USA, and depends on empiric pathogen identification (IDn) and susceptibility testing, whenever possible. The most acute infections reported in patients are either urinary tract infections (UTI) or respiratory tract infections (RTI). Rapid detection of such infections with high precision in outpatient care could effectively aid in impeding the AMR pathogen transmission by enabling prompt isolation of the patient and initiating appropriate treatment (Maurer et al. 2017). It could also aid in rapid IDn of pathogen aetiology and their antimicrobial susceptibility profile with high accuracy, which is crucial to initiate appropriate antimicrobial stewardship. Quick adaptation and optimization of antibiotic therapy specific to the infectious microbe, typically within the first 6–12 h of infection, could improve patient outcomes to a great extent (Burnham et al. 2017). One way to achieve this is through the development of better diagnostics that can enable the prediction of microbial resistance and susceptibility profile in a rather impartial way. Albeit this is appealing, technological advancements turn into profits only when they are inexpensive, and clinicians apply systematic dissemination and analysis of the results.

Advancements in optics, electronics, microfluidics, nucleic acid amplification (NAA) and hybridization techniques and biosensor technology have contributed significantly to the development of novel antimicrobial susceptibility testing (AST) approaches. However, reports on these innovations do not appropriately associate their results with the practical requirements of point-of-care (PoC) testing.

Often in these reports, the need of a clinical microbiology laboratory, the requirement of time and resources for culture enrichment and pure culture preparation are overlooked causing ambiguity in estimating the overall cost of AST (Doern 2018). Owing to this, the transfer of new successful AST approaches from lab to field has

been extremely slow. In order to overcome these issues, the right questions should be asked when considering a new AST technology: '(i) Whether the technology is generally applicable or target/infection specific? (ii) What is the capacity i.e., how many microbes/agents per hour can be processed? (iii) Has the technology been validated against reference methods? (iv) Has there been any reference installation? (v) Has the technology been scientifically corroborated?, and (vi) When will the technology be available in the market?' (Kahlmeter 2016; Vasala et al. 2020).

While it is difficult to find complete answers to the aforementioned questions, herein we try to address them in the best way possible. We introduce the need for AST development and discuss the current and emerging diagnostic approaches for rapid detection of AMR. Emphasis is laid on the methods and technologies that can be performed near patients and have rapid turnaround time (TAT). Furthermore, the potential merits of incorporating nanomaterials in such diagnostic platforms have been outlined. We conclude the chapter by addressing the challenges that impede the adoption of technologies at PoC level and hinder their commercialization. Figure 2.1 shows schematic illustration of the techniques and methods covered in the AST and AMR detection.

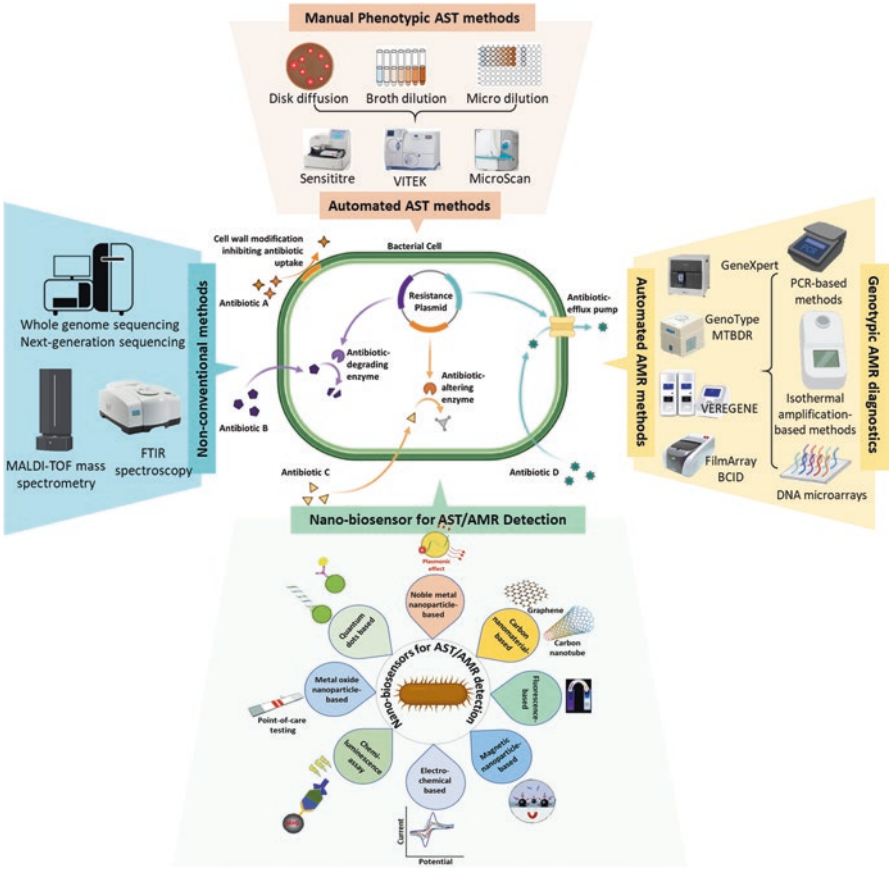

Fig. 2.1 Schematic illustration summarizing diagnostic methods and techniques for antimicrobial susceptibility and AMR detection

2.2 Current Diagnostic Tools for AST and AMR

2.2.1 Phenotypic Methods

Bacteria can acquire AMR by several mechanisms such as presence of an enzyme that can modify or destruct the antibiotic molecule; decrease in permeability to restrict the influx of drug molecule or increase in its efflux; global cell adaptations such as cell wall synthesis and membrane homeostasis; and changes in target site by mutation, enzymatic alterations and complete replacement or bypass of the target sites (Munita and Arias 2016). The overwhelming resistance mechanisms and the wide range of antimicrobials have made the process of AST extremely complex.

In current clinical practice, pathogen IDn is typically the preliminary step and is performed by culturing samples on chromogenic agar followed by isolating pure colonies. Once the pathogen IDn is established, only then the AST is carried out. The phenotypic methods detect antimicrobial susceptibility based on changes in the phenotypic characteristics of the organisms such as growth, expression of proteins, and their enzymatic activities in the presence of antimicrobials. The classical phenotypic methods used for AST include broth dilution, agar dilution, and disk diffusion. These methods are based on the continual exposure of the microbial isolates to a group of antimicrobials to determine their minimum inhibitory concentration (MIC) and offer breakpoint interpretation. MIC is defined as the lowest concentration of antimicrobials required to effectively impede the visible growth of pathogens. These phenotypic tests work irrespective of the resistance mechanism and are the gold standards for informing antimicrobial treatment decisions. However, performing standardized susceptibility tests and analysing their results in the best way possible are constant challenges for the guiding agencies such as the Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST). Considering a typical healthcare setting does not have access to advanced technologies, the whole process of isolation and IDn requires several days. Furthermore, these tests are user-intensive and typically performed manually, which could lead to human errors as observed in the case of the microdilution method (Reller et al. 2009; Váradi et al. 2017). In view of this, several automated phenotypic methods have been developed and discussed in the following section.

2.2.1.1 Automated Phenotypic AST Systems

Numerous automated systems such as VITEK® 2 (bioMérieux), MicroScan WalkAway (Siemens Medical Solutions Diagnostics), Sensititre ARIS™ 2X (Trek Diagnostic Systems), BD Phoenix™ (Becton Dickinson Diagnostic Systems), PhenoTest® BC Kit (Accelerate Diagnostics, Inc.), etc. have been approved by the Food and Drug Administration (FDA) for clinical applications (Pancholi et al. 2018;

Syal et al. 2017). These systems typically allow automation of broth dilution systems that employ ready-made cassettes or panels containing positive controls and increasing concentration of antibiotics. They can analyse a large group of microorganisms in a single run through their extensive database and a few of them can even allow bacterial IDn. In these platforms, bacterial growth is monitored by either change in colour, turbidity, or fluorescence (Reller et al. 2009). VITEK® 2, an improved version of VITEK systems, measures the turbidity of the inoculum in liquid culture by measuring transmittance at each time point. An increase in sample turbidity correlates with a decrease in transmittance values, which in turn is used to plot growth vs. time to determine MIC. It allows 30–240 assays to run simultaneously and generates results within 4–10 h (Reller et al. 2009). Conversely, the MicroScan WalkAway system measures the fluorescence of the samples to determine MIC and requires half of the time needed by VITEK systems (Rhoads et al. 1995). Fluorescence measurement does not directly correspond to bacterial growth but instead to their enzymatic activity. Like the MicroScan WalkAway system, Sensititre ARIS™ 2X also measures fluorescence and generates MIC post 18–24 h of incubation. BD Phoenix™ system incorporates a redox indicator to improve the sensitivity towards bacterial growth and requires 6–16 h to generate the MIC. PhenoTest® BC Kit incorporates real-time imaging for pathogen IDn (Pancholi et al. 2018). It, however, suffered from the FDA recall in 2018 due to a high number of false-positive results with *Staphylococcus aureus* (FDA 2018) and was later improved by coupling it with Accelerate Pheno™ system. The improved system can generate MIC in ~7 h and is suitable to work with positive blood cultures (Humphries and di Martino 2019; Pancholi et al. 2018). A number of new equipment that can facilitate AST automation continue to emerge. The latest in the series is an Automated Plate Assessment System (APAS®) from the Clever Culture System AG that is claimed to be the only FDA-cleared artificial intelligence technology for automated culture plate reading (https://cleverculturesystems.com). This equipment employs an intelligent imaging and machine learning software to automate the imaging, analysis and interpretation of culture plates following incubation.

While automation has certainly improved the TAT and reduced manual errors encountered in conventional phenotypic methods, certain limitations remain to be addressed. For instance, the improved automated methods take somewhere between 4 and 24 h to generate the susceptibility profile, which is not rapid enough to inform timely treatment decisions. Regardless of this, phenotypic testing is routinely used by clinical microbiology laboratories (CMLs) to obtain susceptibility profiles for bacteria irrespective of their site of infection. Table 2.1 discusses various phenotypic methods for rapid detection of AST. It is estimated that genotypic methods might change the way antimicrobial treatment decisions are taken; however, phenotypic AST will continue to be a part of routine CML's practice due to its innate flexibility, simplicity, and low cost. Figure 2.2 shows timeline of development/modification of phenotypic and genotypic methods for AST and AMR diagnosis.

Table 2.1 Rapid phenotypic and genotypic AMR/AST diagnostics techniques

Technology	Description of techniques	Target AST/ AMR	Turnaround time (TAT)	Mode of monitoring	Advantages	Limitations	References
1.1 Phenotypic methods							
MALDI-ASTRA	Enzymatic-based degradation of antimicrobials from the resistant microorganism	β-Lactam antibiotics	30–180 min	Mass/charge ratio	Applied for both pure culture isolates and positive blood culture	The culture medium should be devoid of the respective amino acid that is used as a heavy marker in the analysis	Vatanshenassan et al. (2018)
Carba NP test	In vitro hydrolysis of imipenem by bacterial lysate which is detected by a change in the pH using phenol red	Carbapenem	2 h	Visual	The test showed very high sensitivity and specificity for carbapenemase-producing carbapenem-resistant organisms	Cannot be applied for the detection of other AMR	Nordmann et al. (2012)
Adenylate kinase (AK)	Quantification of adenylate kinase after post antimicrobial treatments	Methicillin	5 h	Bioluminescence	Applied directly to the clinical specimens	Cannot be applied for the detection of other AMR. Require pre-enrichment of pathogens	Pierce et al. (2017)
Later flow immunochromatography	Paper-based test for detection of target proteins using the antigen-antibody interaction	Carbapenem	Less than 30 min	Visual	No need for any specialized equipment	NS	Koczula and Gallotta (2016)

Multiplex immunochromatographic assay	Detect carbapenemase enzymes from carbapenem-resistant organisms	Carbapenem	15–45 min	Visual	Applied directly to the positive clinical specimens and can be applied to PoC testing	NS	Koczula and Gallotta (2016)
Latex agglutination	The antimicrobial resistance proteins are visualized by agglutinating the latex particles conjugated with monoclonal antibodies	Methicillin	20 min	Visual	Have very less TAT and can be applied for PoC diagnostics for AMR	Cannot be applied for the detection of other antimicrobial resistance	Tasse et al. (2016)
Nanotube assisted microwave electroporation (NAME)	Fluorescence-based detection of the target region of the single pathogen	Wide range of antimicrobials	Less than 90 min	Fluorescence	Single bacterial cell growth rate and AST were determined within TAT of less than 30 min	A wide range study on different pathogens and AST is needed	Gao et al. (2019)
Single-cell morphological analysis (SCMA)	Microfluidic-based determination of single-cell AST of pathogens	Wide range of antimicrobials	4 h	Optical microscope	Morphological changes in the single bacterial cell and AST are monitored individually	Expertise requirement for image analysis and data interpretation	Choi et al. (2014)
Droplet-based fluorescent antimicrobial susceptibility test (drop-FAST)	The single bacteria are encapsulated in picolitre-sized droplets, and their growth is monitored using the fluorescence method	Wide range of antimicrobials	1 h	Fluorescence	NS	Further improvement and automation for multiple antimicrobial/ pathogens are needed	Kaushik et al. (2017)

(continued)

Table 2.1 (continued)

Drug susceptibility testing microfluidic (DSTM) device	Microscopically evaluates drug susceptibility based on cell number and shapes of antimicrobial-treated and control cells	Wide range of antimicrobials	3 h	Microscopic	NS	Requires expertise for post-experimental analysis	Matsumoto et al. (2016)
1.2 Genotypic techniques *1.2.1 PCR based*							
Real-time PCR	Quantitative PCR technique used for the quantification of nucleic acids during reaction itself	NS	1.5 h	Fluorescence	The PCR products can be detected during the reaction itself. Used for the detection of a wide range of pathogens and AMR	High cost, limited multiplex target detection and non-availability of kits for all the RG detections	Smiljanic et al. (2017)
PCR dipstick chromatography	PCR performed with biotin and single tag-linker-labelled polymer	NS	1–1.5 h	Naked eye	It offers multiplex detection of up to eight targets in a single reaction. DNA denaturation is not necessary	Detect single RG and could miss the other genes involved in the resistant mechanism	Shanmugakani et al. (2019)
Line probe assay	Reverse hybridization of PCR amplicons to single-stranded, membrane-bound probes on the strip extraction and amplification	NS	5 h	Naked eye	Requires denaturation of PCR products. Comparatively longer TAT than other nucleic acid-based technologies	Determinant genotypic markers are not always correlated with the phenotypic resistance	Bloemberg et al. (2017)
Microarray	Microarray-based hybridization of PCR amplicons	NS	5 h	Fluorescence	Detect several pathogens and their target RGs in many samples	Higher cost and expertise requirement	Card et al. (2013)

Microfluidic quantitative PCR (MF-qPCR)	Quantitative PCR using a very small volume of samples	NS	4 h	Fluorescence	Detect RGs directly from environmental samples	Require preamplification step for higher sensitivity	Sandberg et al. (2018)
1.2.2 Isothermal amplification-based							
Loop-mediated isothermal amplification (LAMP)	Isothermal amplification technique which uses six primers for detection of single targets	NS	40 min	Turbidity	Highly reliable at poorly resourced laboratories with less cost and expertise requirements and highly specific; primer design is complex and tolerant to biological substances	Complex primer design and unable to perform multiplex application	Schoepp et al. (2017)
Helicase-dependent amplification (HDA)	Use DNA helicase enzyme to uncoil the DNA	NS	2 h	Charge-coupled device image	Detect multiple targets within a single reaction. Simple primer design, robust to biological substances and no initial heating step	Purification of DNA is required Expensive enzymes	Cao et al. (2013)
Rolling circle amplification (RCA)	Amplification of short DNA/RNA primer to form long single-stranded DNA/RNA using a circular DNA template and DNA polymerase	NS	3 h	Fluorescence	Both gel electrophoresis and fluorescence-based detection of amplicons are feasible	Primer is complex, RNA amplification is complex and it works only with a circular nucleic acid template	Chen et al. (2014)

(continued)

Table 2.1 (continued)

Recombinase polymerase amplification (RPA)	The detection of target genes by using recombinase, DNA polymerase and single-stranded protein	NS	1 h	Fluorescence	Multiplex detection of more than one target in a single reaction. Simple primer design, extremely quick (20 min), no initial heating step and robust to biological substances Possible to use fluorophore and quencher for real-time detection within 1 h	Cannot be applied directly to clinical samples	Nelson et al. (2019)
Multiple cross displacement amplification (MCDA)	Ten primers are used for amplification of single target with polymerase having strand displacement activity	NS	1 h	Visual	Can be performed at normal ambient temperature (37 °C)	Difficult to apply multiplexity. Cannot be applied to direct clinical samples	Wang et al. (2015)
1.2.3 Sequencing-based techniques							
Whole-genome sequencing (WGS)	Genome sequencing	Wide range of antimicrobials	1–3 days	Bioinformatic tools	Detailed understanding of multi-antimicrobial resistance, mechanisms, transmission modes and molecular epidemiology could be achieved	Less accurate than other phenotypic techniques Higher cost and expertise requirement	Kim et al. (2017)
Next-generation sequencing (NGS)	Genome sequencing	Wide range of antimicrobials	1–3 days	Bioinformatic tools	Raid and ultra-high-throughput sequencing	Bulky and expensive sequencing instruments are required, longer TAT	Boolchandani et al. (2019)

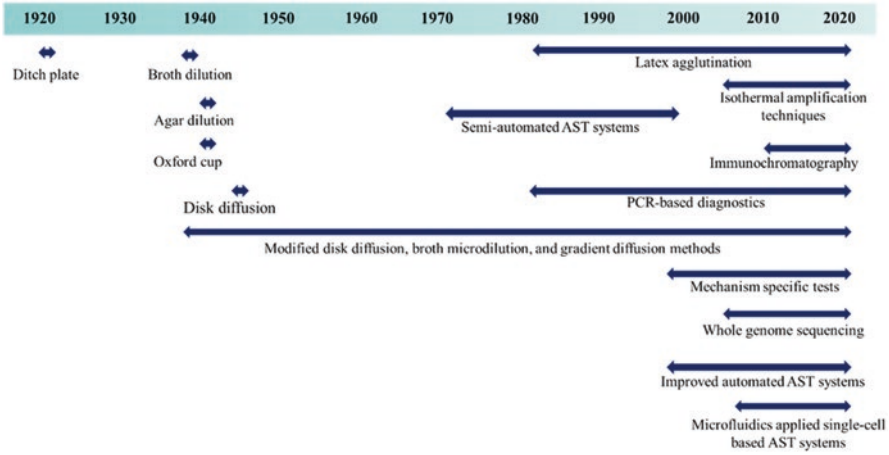

Fig. 2.2 Timeline showing development/modification of phenotypic and genotypic methods for AST and AMR diagnosis since the discovery of the first antimicrobial to the currently existing rapid diagnostics. (Adapted with permission from Ref. (Shanmugakani et al. 2020))

2.2.2 Genotypic Methods

Genotypic or molecular methods are one of the most indispensable methods for screening AMR in microorganisms. These methods offer several advantages over phenotypic methods by allowing multiplex detection of targets with accurate characterization and identification of AMR genes without requiring pure colony isolates (as polymicrobial cultures can be used) (Leonard et al. 2018; Maugeri et al. 2019). They also present suitable alternatives for the taxonomic units for which susceptibility breakpoints are yet to be established. Furthermore, they offer the flexibility of quickly adapting to newly discovered R-factors- small pieces of DNA, usually plasmids, containing AMR genes (Kaprou et al. 2021; Vasala et al. 2020). While these merits make genotypic methods stand out, they also suffer from certain limitations for AMR detection. For instance, genotypic methods do not define the level of resistance (MIC) and can only identify specified targets due to which a new variant of specified resistance gene (RG) or a newly evolved RG can be easily missed. Furthermore, the higher cost, non-availability of specific target resistance markers, poor affinity of the resistance markers in polymicrobial samples, new mutations, and non-characterized resistance mechanisms further hinder their universal acceptance over phenotypic methods (van Belkum et al. 2020). Nevertheless, incessant advances in the field have helped genotypic methods gain a position in standard diagnostics.

Current genotypic methods take advantage of advancements in NAA and hybridization techniques, and enable simultaneous IDn of pathogen and AMR (Pulido et al. 2013). In addition, these methods allow rapid and sensitive detection of RGs that confer bacteria with the ability to survive in antibiotics and continue their growth. Availability of the target RGs in various databases has exploded due to the

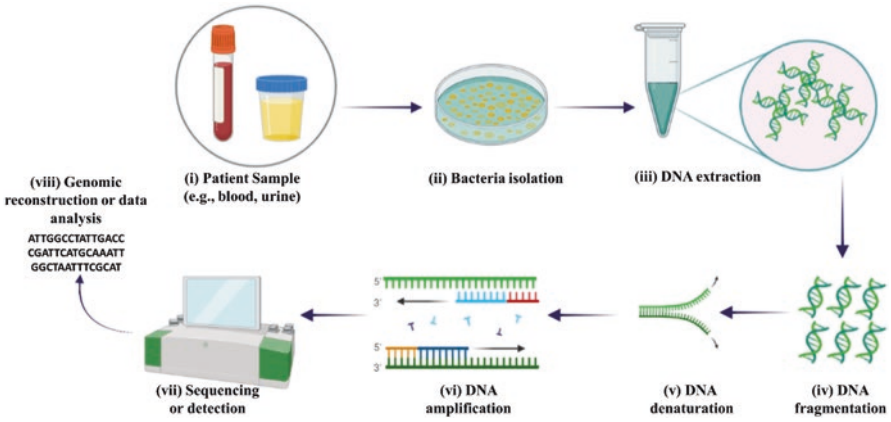

Fig. 2.3 Typical procedure of genotypic methods to predict AMR in clinical samples. (i) Patient sample collection, (ii) culturing the sample on agar for bacterial colony isolation, (iii) DNA extraction by cell lysis and (iv) DNA fragmentation, followed by (v) denaturation to separate the double strands, (vi) specified target amplification and (vii) labelling or reading of antibiotic resistance genes. Finally, (viii) the data analysis is followed by statistical analysis to predict the antibiotic susceptibility profile. (Adapted with permission from Ref. (Leonard et al. 2018)

expansion of whole-genome sequencing (WGS) and subsequent cost reduction of next-generation sequencing (NGS) technologies (Waseem et al. 2019). The following sections briefly discuss techniques based on NAA such as PCR (polymerase chain reaction), isothermal amplification (IA) and their automation for commercial application in CML settings. It further navigates sequencing-based approaches for AMR detection. More detailed information on these technologies can be found in published reviews (Anjum et al. 2017; Boolchandani et al. 2019; Dunne Jr et al. 2017; Ellington et al. 2017; Shanmugakani et al. 2020). Figure 2.3 depicts the typical procedure followed by genotypic methods to predict AMR in clinical samples. Patients' sample such as blood or urine is collected and cultured on agar for bacterial colony isolation. The isolated cells are then lysed to extract DNA followed by its fragmentation, denaturation, and amplification through PCR-based or IA approaches. To generate an AST profile from the amplified DNA targets, NAA assays must be accompanied by analytical methods such as sequencing, fluorescence detection, and mass spectrometry. Table 2.1 discusses various genotypic methods for rapid detection of AMR.

2.2.2.1 PCR-Based Techniques

The PCR-based techniques rely on the sequence-specific amplification of nucleic acids (NAs). With increased knowledge of the genetic basis of AMR, PCR-based techniques have been widely used to detect RGs to a variety of antimicrobials in different bacterial species (Maurer et al. 2017; Pashazadeh et al. 2017). Many previous studies have reported PCR-based approaches for the detection of

methicillin-resistant *Staphylococcus aureus* (MRSA) (mecA), vancomycin-resistant *Enterococcus* sp. (vanA and vanB genes) and many other Gram-negative bacteria for identifying cephalosporinase- and carbapenemase-encoding genes (bla IMP, bla VIM, AmpC, bla KPC, etc.) (He et al. 2020; Smiljanic et al. 2017). Although PCR-based techniques are more rapid and sensitive than phenotypic methods, their application in rapid AST is limited due to the requirement of DNA extraction from isolated strains and prior knowledge of the DNA sequences to be amplified (Pulido et al. 2013; van Belkum et al. 2020; Vasala et al. 2020). Importantly, they are typically designed to detect a limited set of preidentified genes, which in the context of AMR does not cover the rapidly evolving novel RGs. Moreover, the detected resistance may not always correlate with phenotypic resistance as it depends on the level of gene expression (Vasala et al. 2020). Because of this, advancements in the PCR-based assays to develop real-time reverse transcription PCR (RT-PCR) (Donà et al. 2016; Menezes et al. 2013), digital (Košir et al. 2020; Whale et al. 2016) and multiplex (Cetinkaya et al. 2014; Sigmund et al. 2020) PCR assays have played a major role and solidified its ground in clinical practice of detecting AMR. For instance, the RT-PCR technique offers multiple advantages over conventional PCR such as relatively rapid response with high precision, low sample volume requirement, and monitoring of the PCR products during the reaction itself (Smiljanic et al. 2017; He et al. 2020). However, the higher cost, limited multiplexing capabilities, requirement of technical expertise, and non-availability of kits for all the RGs limit their applications for clinical AMR testing. In view of this, quantitative reverse transcription-PCR (qRT-PCR) has been developed, which can monitor bacterial growth in the presence of antimicrobials by quantifying the bacterial DNA copies in less than 6 h (Ellappan et al. 2018; Seborova et al. 2019). It can additionally assess the expression level of RGs after exposure to varying concentrations of antimicrobials, thereby providing rough MIC values (Vasala et al. 2020). However, these systems are too expensive and require separate kits for DNA extraction and PCR. In addition, the higher false-positive rates limit their application for routine CML tests.

The application of conventional PCR for reliable detection of RGs directly from the clinical specimens is challenging as it can compromise the sensitivity and lead to longer TAT. In view of this, digital-PCR (dPCR) platforms have been designed to partition the bulk samples into minute PCR reaction volumes to increase the effective target concentration in each reaction. The dPCR is a more rapid and sensitive technique than the conventional PCR (Hu et al. 2017; Mao et al. 2019). However, the requirement of separate kits for DNA extraction and PCR makes them expensive and limits their application in resource-limited CMLs. Advancements in sequencing technologies have paved a way for high-throughput quantitative PCR (HT-qPCR), which is comparatively fast and cost-effective than the conventional qPCR (Waseem et al. 2019). HT-qPCR can simultaneously detect a large number of antimicrobial RGs stemming from various sample types (Waseem et al. 2019). Owing to the small sample volume requirement and short processing time, the microfluidic integration with NAA-based AMR detection has drawn much attention in recent years. The microfluidic quantitative PCR (MF-PCR) has been developed for the rapid detection of different RGs directly from the environmental samples within a TAT of 4 h

(Sandberg et al. 2018). Similarly, multiplex PCR-based microfluidic platforms have shown excellent sensitivity and specificity for vancomycin- and β-lactam-RGs from the perianal swab specimens (Walker et al. 2016). Although these platforms are rapid and sensitive, they suffer from high cost and expertise requirements, and in some cases, target preamplification limits their application at routine CMLs.

2.2.2.2 Isothermal Amplification-Based Techniques

The IA is performed using a heating block within a short period than PCR-based methods. Unlike conventional PCR, the IA is performed at one specific temperature and does not require a thermal cycler instrument (Shin et al. 2019). Further, these techniques offer multiple advantages over conventional PCR technique such as high sensitivity, rapid response, shorter TAT, and better affordability. The IA-based techniques reported so far for AMR diagnosis include loop-mediated isothermal amplification (LAMP), helicase-dependent amplification (HDA), rolling circle amplification (RCA), recombinase polymerase amplification (RPA), and multiple cross displacement amplification (MCDA).

The LAMP technique uses six primers to amplify a single target and forms multiple amplicons of different sizes. It can be performed in a heating block in less than 40 min, and the results can be interpreted with the naked eye by visualizing the turbidity. Although the LAMP technique has a complex primer design, they offer greater specificity than other PCR techniques (Kaprou et al. 2021). However, the difficulty in deploying multiplexity and high false-positive rates with real biological samples are some of the limitations that need to be addressed.

The HDA technique uses DNA helicase and polymerase together to unwind the double-stranded DNA and amplification, respectively. Like PCR techniques, the HDA uses two primers and forms a single amplicon, which can be determined using fluorescent probes when performed in a real-time PCR machine (Barreda-García et al. 2018). This technique is known for detection of MRSA directly from the blood cultures and can be deployed for multiplex detection (Cao et al. 2013).

The RCA technique employs a circular DNA template and DNA/RNA polymerase to amplify short DNA/RNA primers. The amplicons can be determined by gel electrophoresis or fluorescence-based detection similar to conventional PCR techniques (Ali et al. 2014; Xu et al., 2020). Although this technique detects RGs directly from the clinical specimens, longer TAT is the major limitation for its regular clinical application. To address this, Xu et al. (2020) developed a novel RCA and CRISPR-Cas12a-integrated dual aptamer-based platform for the rapid and sensitive detection of MRSA. The aptamers specific to a membrane protein shared by MRSA and *S. aureus* and penicillin-binding protein (PBP2a) were deployed for the selective detection of MRSA directly from clinical blood specimens. Moreover, the RCA- and CRISPR-Cas12a-assisted trans-cleavage enabled the aptamer-based protein recognition into quantifiable fluorescent signals with a linear detection range of 10^2–10^6 CFU mL^{-1} and an LOD (limit of detection) of 10^2 CFU mL^{-1} with a 20 min of response time.

The RPA technique exhibits rapid amplification of the target sequence at relatively low temperature (between 37 and 42 °C) and requires relatively simplified instrumentation than that of conventional PCR. It employs DNA recombinase for strand exchange at cognate sequences, single-stranded DNA binding proteins and a DNA polymerase for amplification. The rapidity, portability and low cost, along with the simplicity of operation, make RPA suitable for any global setting (Nelson et al. 2019). One of the major limitations of this technique is that it cannot discriminate between live and dead bacteria, which may result in higher false-positive rates.

The MCDA is a unique technique that uses DNA polymerase and ten primers to amplify the target sequence under isothermal conditions (60–65 °C). It is a simple, rapid, highly specific technique that amplifies the DNA sequence with high efficiency and sensitivity (Chen et al. 2019). While useful, its inability to perform multiplex analysis and detect RGs directly from clinical specimens limits the wider application of this technique (Wang et al. 2015). To this end, Wang et al. (2018) developed an advanced MCDA technique by combining it with lateral flow biosensor for multiplex detection of both *Staphylococcus aureus* and its target RGs (*mecA*). The technique showed 100% specificity for *S. aureus* with a detection limit of 100 fg DNA/reaction *nuc* and *mecA* genes in the pure culture and 10 CFU mL^{-1} for the bacterial cells from the blood samples. Additionally, the technique enabled visual detection of target genes directly from the blood samples, thereby serving as a PoC diagnostic platform.

Among all the IA techniques, the LAMP, RCA, and HDA offer an additional advantage of eliminating the step of template denaturation (Kaprou et al. 2021; Karami et al. 2011). Moreover, the higher simplicity, selectivity, reproducibility, and lower costs of LAMP and RPA techniques provide them with an advantage of application in resource-limited settings (Kaprou et al. 2021; Zou et al. 2020). More recently, the integration of IA techniques with microfluidic devices and downstream fluorescence capture allowed even more rapid and sensitive use of LAMP and RPA for antimicrobial RG detection (Kalsi et al. 2019). However, the microfluidic integrated LAMP still suffers from poor specificity and high false-positive rates (Zou et al. 2020).

2.2.2.3 Automated Genotypic Methods

A broad range of commercial diagnostic tools are available for AMR detection in routine healthcare clinical systems. These systems are suitable for routine clinical application due to rapid response, high sensitivity and selectivity and ease of use to analyse targets in real clinical samples. The Xpert® MTB/RIF (Cepheid, Inc., USA) is an automated RT-PCR-based assay for the detection of *M. tuberculosis* and its resistance to rifampicin (RIF) within a TAT of 2 h directly from the clinical sputum specimens (Pooran et al. 2019). While rapid, the Xpert® MTB/RIF requires a continuous power supply, regular maintenance, annual calibration, higher costs, and expertise for the module operation, which limits its application in resource-limited settings. Despite these limitations, in 2011, the World Health Organization (WHO)

endorsed this technique in countries with high tuberculosis (TB) burden (Qin et al. 2015).

The GenoType MTBDR*plus* VER 2.0 (Hain Lifescience GmbH, Germany) employs DNA strip technology to directly detect *M. tuberculosis* and its resistance to RIF and isoniazid antibiotics, simultaneously from the pulmonary specimens with a TAT of 5 h. Interestingly, the result can be visualized by comparing specific bands on the DNA strip (Lanzas et al. 2016). Similar to GenoType MTBDR*plus* VER 2.0, the GenoType MTBDRsl VER 2.0 (Hain Lifescience, GmbH) also detects AMR of *M. tuberculosis*, however against clarithromycin, fluoroquinolones and tetracycline antibiotics (Gardee et al. 2017).

Gene Xpert® MRSA NxG (Cepheid) is an FDA-approved culture-independent diagnostic technique that employs qRT-PCR for rapid detection of MRSA RGs (*spa*, SCC*mec* and *mec*A) directly from the positive blood samples with a TAT of 1 h (Afshari et al. 2012). The test reported a 100% sensitivity and 99.4% specificity for MRSA detection. Another similar technique is BD GeneOhm™ MRSA ACP (BD Diagnostics, USA) which employs RT-PCR for MRSA detection directly from the clinical specimens with a TAT of ~3 h (Grabsch et al. 2013).

VERIGENE® BC-GP and VERIGENE® BC-GN (Luminex, USA) are also FDA-approved automated rapid sample-to-answer, hybridization-based techniques for the detection of common Gram-negative and Gram-positive RGs (Poole et al. 2018). Unlike other PCR techniques, VERIGENE® does not require target gene amplification and requires only ~5 min of hands-on time and ~2.5 h of run time. Although precise and rapid, this platform requires Gram staining to select appropriate assay and is not fully automated, which prevents its deployment for PoC testing. FilmArray® BCID (BioFire Diagnostics, USA) is also an RT-PCR-based diagnostic technique for the multiplex detection of different pathogenic bacteria and their RGs directly from the positive blood samples (Salimnia et al. 2016). The main advantage of FilmArray® BCID is that it performs NA extraction, multiplexed nested PCR, and melt curve analysis within 1 h.

While these technological innovations are commendable, more work is required to evaluate their true potential in clinical settings. Several other RG targeting platforms, typically PCR-based, continue to emerge; it would be out of the scope of this chapter to review all of these. More details on these platforms can be found in Maugeri et al. (2019), Shin et al. (2019) and Vasala et al. (2020).

2.2.3 Sequencing-Based Approaches

2.2.3.1 Whole-Genome Sequencing

Advancements in sequencing techniques have led to the emergence of whole-genome sequencing (WGS) for AMR diagnosis. The WGS enables the detection of resistance against all known antimicrobials and genes conferring resistance to them. It also investigates novel resistance mechanisms, transmission modes and molecular

epidemiology of AMR pathogens (Boolchandani et al. 2019). Apart from AMR detection, the WGS can also be used for determining the susceptibility profile of fastidious, viable non-culturable bacteria (Haas et al. 2016). Although the WGS predicts the AST of pathogens towards different antimicrobials, the accuracy compared with the conventional phenotypic methods needs to be improved (Shanmugakani et al. 2020).

While the WGS platforms are portable, require less laboratory space and can be used for on-site sequencing; their high cost, complex methodology, longer analysis time, and inability to determine MIC limit their application in routine AMR diagnosis (Kaprou et al. 2021; Oniciuc et al. 2018; van Belkum et al. 2020). The WGS-based AMR detection is more complicated when there is altered expression of intrinsic genes and the genetic variation leading to the AMR is unknown (Ellington et al. 2017; van Belkum et al. 2020). Furthermore, the currently available AMR databases only focus on the identification and characterization of protein-coding RGs due to which other potential mechanisms responsible for AMR such as genomic changes or de novo mutations in ribosomal RNA genes and regulatory elements get ignored (Vasala et al. 2020). In addition to this, the current bioinformatic cleaning system in WGS often omits the direct repeats and insertion in the plasmids, thereby overpassing the plasmid-mediated AMR outbreaks and transfer (Ellington et al. 2017).

2.2.3.2 Next-Generation Sequencing

Next-generation sequencing (NGS) is a rapid, ultra-high-throughput sequencing technology that is used to determine the order of nucleotides in the entire genome or targeted region of DNA/RNA. This developing technology complements traditional culture-based methods for clinical and surveillance applications and provides opportunities for quick and sensitive detection of AMR in cultivable and non-cultivable bacteria (Ajbani et al. 2015; Boolchandani et al. 2019). Contrary to phenotypic tests which provide an antimicrobial susceptibility profile, the NGS can reveal not only the molecular basis of AMR but also characterize the novel resistance mechanisms (Kaprou et al. 2021).

Pyrosequencing is one of the early successes for NGS owing to high reliability and robustness towards detection of resistance-associated mutations in *M. tuberculosis* with 96–100% sensitivity (Ajbani et al. 2015). In another study, a very high sensitivity (83–100%) and specificity (73–100%) were observed for detecting resistance in *M. tuberculosis* against RIF (rifampicin) and fluoroquinolones (Govindaswamy et al. 2018).

Although NGS is a high-throughput and reliable technique, its high cost, complex workflow, the requirement of expertise and enriched samples limit its application in routine clinical testing. The possibility to inform clinical decisions based on NGS information is still under evaluation (Ellington et al. 2017). Further detailed information on sequencing-based AMR diagnostics can be found in published

reviews (Boolchandani et al. 2019; Kaprou et al. 2021; Oniciuc et al. 2018; van Belkum et al. 2020).

2.3 Nano-biosensors for Rapid Detection of Antimicrobial Resistance

Biosensors are rising as sensitive, selective, and affordable analytical tools that can detect and quantify target analytes (Pashazadeh et al. 2017). As shown in Fig. 2.4, a typical biosensor consists of three elements: a biological receptor (e.g., nucleic acids, antibodies, enzymes or whole cells) which specifically recognizes the target species; a transducer, which captures the response to a biological recognition event and translates it into a quantifiable signal; and a signal processing and display component (Rai and Jamil 2019). In recent years, various biosensors such as mechanical, optical and electrochemical (EC) have been developed to monitor AST. To do this, these biosensors monitor changes in microbial metabolism, pathogen movement, and heat production against specific antimicrobials (Vasala et al. 2020). Mechanical (bio)sensors analyse changes in the mechanical properties of the sensor in the presence and absence of antimicrobials (Reynoso et al. 2021). For instance, asynchronous magnetic bead rotation and microcalorimetry can sense the changes in the rotational frequency and dynamic heat flow patterns

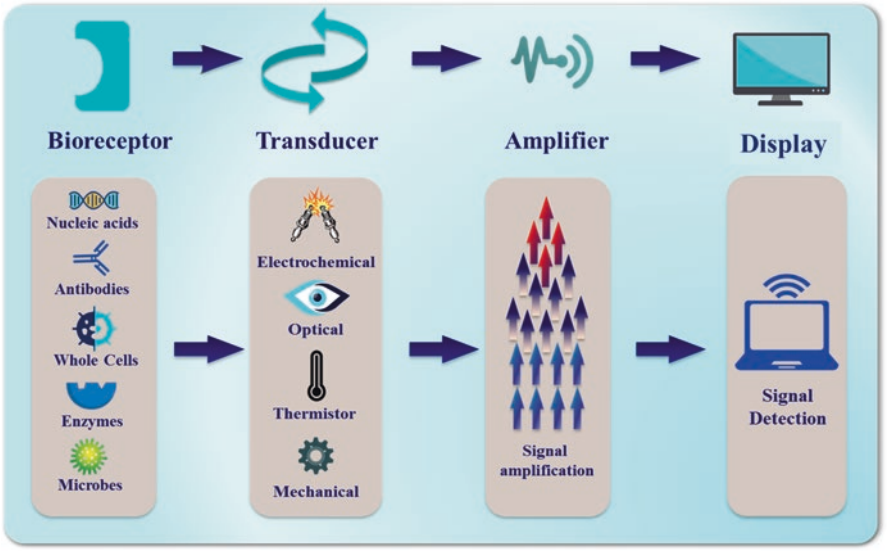

Fig. 2.4 Schematic representation of the various components of biosensors and their detection system

against antimicrobials for estimating real-time antimicrobial susceptibility profiles (Reynoso et al. 2021; Sinn et al. 2011). Although these sensors are rapid and sensitive, they have not yet provided convincing clinical demonstrations (Butini et al. 2018; Vasala et al. 2020). The optical biosensors sense the changes in the bacterial growth and metabolism and expression of proteins and enzymes against specific antimicrobials. So far, colorimetry, fluorimetry and surface-enhanced Raman scattering are the most popular optical biosensors reported for AST and AMR diagnostics (Majdinasab et al. 2019; Reynoso et al. 2021). Each optical method has its unique feature for biosensing applicability and performance, which is determined by the type of recognition elements, sensor composition and structure, measurement procedure used, and the type of specimen (Majdinasab et al. 2019). The EC biosensors monitor the interaction between recognition element and target bacteria or its components such as RGs, proteins or enzymes, to generate a measurable EC signal which is used to determine the susceptibility profile against specific antimicrobials (Simoska and Stevenson 2019; Zhou et al. 2021).

Recently, the integration of nanotechnology in the aforementioned biosensor platforms has led to efficient device integration, sensing unit fabrication, interfacing, packaging and performance (Sharma et al. 2021). Nanoparticles (NPs) are small particles ranging between 1 and 100 nm in size and can be classified into different categories depending on their shape, size, or other properties. They exhibit unique physico-chemical properties such as optical, mechanical, electrical and thermal due to their nanoscale dimensions (Arshad et al. 2021; Ray et al. 2017). Owing to these properties, a variety of nanomaterials including metals, metal oxides, carbon- based nanostructures, quantum dots, etc. have been incorporated in biosensor platforms (Majdinasab et al. 2019; Zhou et al. 2021). The inherent properties of NPs, including but not limited to higher surface area, controllable size and morphology, and ease of surface modification have endowed nano-biosensor platforms with low cost, rapid TAT, excellent sensitivity and specificity for AST (Arshad et al. 2021; Yuan et al. 2018). More information on such nano-biosensors has been compiled in Table 2.2.

2.3.1 Optical Nano-biosensors for the Rapid AMR/ AST Detection

2.3.1.1 Colorimetric Nano-biosensors

The colorimetric biosensor platforms enable ease of result interpretation by visualizing the change in colour with the naked eye or with simple instrumentation in the case of quantitative analysis (Reynoso et al. 2021)). The unique surface plasmon resonance (SPR), high catalytic activity, ease of surface modification, biocompatibility, and high molar extinction coefficient (1000 times higher than organic dyes) of certain metal NPs (e.g. Ag and Au) have led to the development of various

Table 2.2 Nanodiagnostic platforms for AST/AMR

Nanomaterial	Mode of detection	Principle	Target microbe(s)	Detection target	Antibiotic	LOD/MIC	TAT	Reference
2.1 Optical methods								
AuNPs	Naked eye	The non-enzymatic hydrolysis of the β-lactam ring caused pH-induced aggregation in the gold NPs	*Klebsiella pneumoniae* and	Carbapenemase	Carbapenem	10^5 CFU mL^{-1}	2.5 h	Santopolo et al. (2021)
Graphene quantum dots (GQDs) and gold NPs	FRET (fluorescence energy transfer)	The target gene amplification with NP-conjugated capture probes brings GDQs and gold NPs into proximity and changes the fluorescent intensity of the solution	*Staphylococcus aureus*	*mecA* gene	Methicillin	1 nM	NS	Shi et al. (2015)
SiO2 NPs	Fluorescence	Bacteria-activated polyelectrolyte dissociation from NPs	*Staphylococcus aureus*	MRSA bacteria	Methicillin	10^7 CFU mL^{-1}	NS	Zhao et al. (2017)
AuNPs	PCR colorimetry	Target gene hybridization with NP-conjugated complimentary probe	*Staphylococcus aureus*	*mecA*	Methicillin	100 ng	NS	Abd-El-Hady et al. (2014)
AuNPs	Colorimetry	The target gene detection by DNA-modified gold probe leads to plasmon band red shift of the NPs, enabling visually detectable colour change	*Staphylococcus aureus*	Genomic DNA	Methicillin	66 pg μL^{-1}	NS	Storhoff et al. (2004)
AuNPs	MCDA	The MCDA product amplicons were detected by using gold NP-based lateral flow biosensor	*Enterococcus faecalis*	*Ef0027* gene	NS	10 fg per reaction system	70 min	Chen et al. (2019)

colorimetric nano-biosensors for rapid and sensitive detection of AMR (Gill et al. 2019; Majdinasab et al. 2019). A simple colorimetric AST nano-biosensor was developed by Nath et al. (2008) to investigate the susceptibility profile of *E. coli* against ampicillin. The principle behind the developed platform was based on the inhibitory effect of ampicillin on bacterial growth and metabolism which led to concanavalin A (ConA)-induced clustering of dextran-coated AuNPs (gold nanoparticles). This led to a large red shift in the SPR band of the AuNPs resulting in visible colour change. The developed platform could successfully determine the MIC and AST profile for *E. coli* within an overall readout time of 3 h. While rapid, the inability of the developed platform to monitor the SPR band shift in the turbid or opaque media limited its application in direct clinical specimens. In view of this, a similar platform with dextran-coated iron oxide NPs was developed for the AST of *E. coli* and *Shigella sonnei* against ampicillin (Kaittanis et al. 2008). The inhibitory effect of ampicillin on bacterial growth and carbohydrate metabolism appeared as a change in the degree of ConA-induced clustering of dextran-coated NPs. This clustering of NPs led to the change in the magnetic relaxation time which was used to determine the MIC and AST within a TAT of 2.5 h. In addition to this, the author reported that replacing dextran-coated iron oxide NPs with silica-coated iron oxide NPs further reduced the response time by 40 min. Although these designs performed AST within a short TAT, they failed to justify the resistance mechanisms and/or the resistance-determining factors. More recently, a rapid, affordable and quick alternative to disk diffusion test involving AuNP-conjugated optical fibre biosensor was developed (Fig. 2.5a) to establish the susceptibility profile of *Pseudomonas aeruginosa* (cephalosporin-resistant bacteria) against third-generation cephalosporins (Nag et al. 2020). The cephalosporin-mediated cell lysis decreased the localized refractive index (RI) around the NPs, thereby decreasing the absorbance of the evanescent wave of the optical fibre. Thus, the changes in the localized RI value led to the determination of MIC of 10 μg mL^{-1} within TAT of 2 h and a linear range (LR) of 0.5–10 μg mL^{-1}. Recently, Punjabi et al. (2020) developed a simple paper-based PoC device by incorporating core-shell chitosan NPs layered with starch-iodine complex to identify β-lactam-resistant bacteria. The hydrolysis of β-lactam rings by β-lactamase breaks the starch-iodine complex causing a structural change in the core-shell NPs, which appears in the form of visible colour change (blue to white). The device exhibited high specificity and selectivity towards β-lactam antibiotics and resulted in an LOD of 10^5 CFU mL^{-1} within TAT of 30 min.

Apart from establishing antimicrobial susceptibility profiles, nano-biosensors have also been employed to detect resistance markers like RGs, proteins and enzymes. In one such study, the peroxidase (POx) mimicking activity of AuNP was investigated for the rapid detection of MRSA (Fan et al. 2020). The AuNP-loaded GO was functionalized with resistance protein-specific aptamer for the selective detection of PBP2a, which is a vital determinant of the broad-spectrum β-lactam antibiotic resistance in MRSA strain. The developed biosensor could detect PBP2a in 30 min with an LR of 20–300 nM and an LOD of 20 nM.

Owing to their spectacular optical properties, NPs have also found their application in PCR-based AMR diagnostic platforms to obtain colorimetric output. For

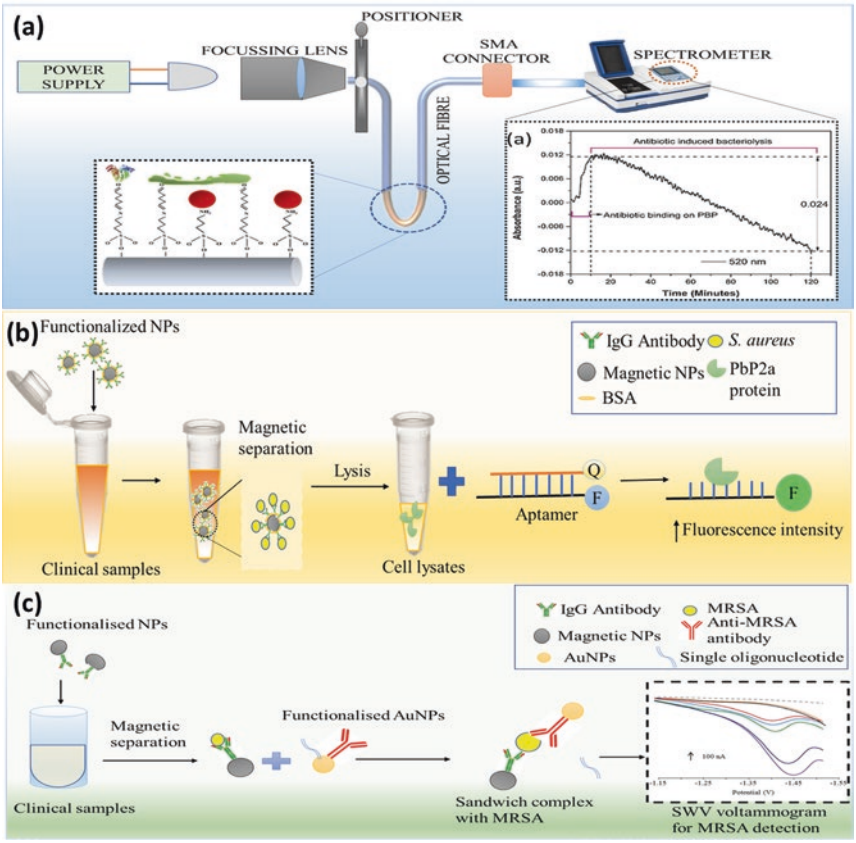

Fig. 2.5 Illustration of different nano-biosensors employed for AMR detection. (**a**) Colorimetric-based detection of cephalosporin-resistant bacteria based on changes in the absorbance of the evanescent wave of the optical fibre. (Adapted from Nag et al. (2020)). (**b**) PbP2a-specific aptamer-based fluorescence detection technique for detection of MRSA. (Adapted from Qiao et al. (2018)). (**c**) A square wave voltammetry-based immunosensor for the detection of MRSA. (Adapted from Cihalova et al. (2016))

example, Chan et al. (2014) used AuNPs for colorimetric detection of PCR amplicons of MRSA directly from the clinical specimen. To do this, the sensor probe was designed by functionalizing AuNPs with *S. aureus* 23S rRNA- and *mecA*-specific oligonucleotides. After completion of PCR reaction, the amplicons were hybridized with the sensor probe, which caused a change in the SPR band of AuNPs. This change in spectra was visualized in the form of visible colour change, that is, red to blue. Although interesting, the high false-positive rate encountered during direct specimen analysis is the major limitation that needs to be addressed before deploying it for commercialization. Similarly, the streptavidin-biotinylated probes conjugated with NPs were used to hybridize with the LAMP amplicons for MRSA detection (Nawattanapaiboon et al. 2015). The changes in the SPR band of AuNPs,

before and after hybridization with the target amplicons (*mecA* gene), were observed with the visible colour change of the AuNPs. A multicomponent NA enzyme (MNAzyme)-AuNP platform was designed to detect the *mecA* gene after RPA reaction (Abdou Mohamed et al. 2021). After the IA, the amplicons were denatured and blocked to prevent rehybridization. In the absence of the target gene, the linker DNA remains intact with the MNAzyme strand, causing AuNPs to aggregate (blue colour). However, in the presence of target gene, upon hybridization with the targets, specific MNAzyme breaks the linker DNA rendering AuNPs in monodispersed states (reddish-pink colour). The platform showed 100% specificity and 86% sensitivity for *mecA* gene detection with direct clinical application.

2.3.1.2 Fluorometric Nano-biosensors

The fluorometric biosensors are more sensitive (up to 1000 times higher) and offer better LOD than colorimetric biosensors. Owing to the higher surface-to-volume ratio, biocompatibility and ease of surface modification, NPs have been employed for accelerating signal transduction, enhancing signal intensity and easy signal read-outs in fluorescence-based assays (Yuan et al. 2018). For instance, Chan and Chen (2012) developed a simple fluorescent sensing platform for sensitive and selective detection of both non-MRSA and MRSA using human serum albumin (HSA)-stabilized Au nanoclusters (AuNCs). The HSA-AuNCs showed special affinity towards both non-MRSA and MRSA and produced reddish photoluminescence after 2 h of incubation. This platform offered a selective detection of non-MRSA and MRSA in the mixture containing other AMR bacteria such as pan drug-resistant *Klebsiella baumannii* and vancomycin-resistant *Enterococcus faecalis* with an LOD of 10^6 CFU ml^{-1}.

The large molar extinction coefficient and tunable extinction spectra of some NPs enabled their application for fluorescence quenching in various biosensor platforms. A graphene oxide (GO)-based fluorescence sensing platform was developed to detect MRSA by targeting its RGs (*mecA* gene) (Ning et al. 2016). In this assay, an FAM (carboxy-fluorescein fluorophore)-labelled complementary probe was used to obtain the fluorescent signal upon target hybridization. The platform enabled target gene detection with an LR of 0.5–40 nmol L^{-1} and an LOD of 0.5 nmol L^{-1}. However, the target gene isolation and amplification and fluorescence tagging limit its application in resource-limited clinical laboratories.

The magnetic nanomaterials such as iron oxide NPs offered an additional advantage of concentrating and separating the target RGs or DNA from untargeted NAs that could significantly improve the sensitivity of nano-biosensors (Cihalova et al. 2017; Pramanik et al. 2017). For instance, Pramanik et al. (2017) developed a red/blue fluorescent carbon dot (CD)-attached magnetic nano-biosensor for the selective identification of MRSA and multidrug-resistant *Salmonella* DT104. To do this, the fluorescent NPs were conjugated with anti-*Salmonella* antibody or anti-MRSA antibodies for the fluorescent imaging of the resistant pathogens directly from the blood samples. Owing to broad absorption bands, long fluorescence time and large

molar extinction coefficient, the quantum dot (QD) NPs have also been deployed as alternatives to other fluorophores for biosensing application. Recently, Cihalova et al. (2017) developed a multiplex fluorescence-based biosensor to detect MRSA and *Klebsiella pneumoniae* by taking advantage of magnetic and fluorescent properties of the NPs with an LOD of 10^2 CFU mL^{-1}. In this case, the target genes were initially amplified and allowed to hybridize with magnetic NPs and cadmium telluride quantum dot (CdTeQD)-conjugated complementary probes forming a sandwich complex. The sandwich constructs were detected by measuring the fluorescent intensity at 598 and 695 nm for MRSA and *K. pneumoniae*, respectively. The major limitations of these platforms include complex primer design, longer TAT, and higher costs limiting their application in resource-limited clinical settings. To address these limitations, an aptamer-based fluorometric platform was developed by Qiao et al. (2018) for the sensitive detection of PBP2a directly from infected clinical plasma and spiked nasal swab samples (Fig. 2.5b). The assay has an overall testing time of 2 h with the detection limit of 1.38×10^3 CFU mL^{-1} in spiked nasal swabs. Although these fluorescence-based platforms are rapid and sensitive, further studies should focus on the development of facile, rapid and PoC testing platforms by deploying different NPs and molecular recognition elements (MREs) (antibodies, aptamers and enzymes).

2.3.2 Electrochemical Nano-biosensors

The electrochemical (EC) biosensors exhibit tremendous potential in terms of simple instrumentation, high sensitivity, miniaturization and low cost. Reports suggest that the incorporation of NPs in these biosensor platforms can potentially increase their sensitivity and signal amplification to a great extent (Ma et al. 2015; Yang et al. 2017). In most cases, for the detection of AST/AMR, the biological targets bind with the target antibody or to the complementary NA sequence, and the perturbations in the current or voltage signals are measured. In one such report, an electrochemical impedance spectroscopy (EIS)-based nano-biosensor was developed by incorporating a ZnO nanorod array decorated with vancomycin-functionalized silver NPs (AgNPs) for MRSA detection. The specific recognition of Gram-positive bacteria by vancomycin antibiotics has led to the selective detection of *Staphylococcus aureus* with an LOD of 330 CFU mL^{-1} (Yang et al. 2017). Similarly, a reduced graphene oxide (rGO)-modified glassy carbon electrode (GCE) was developed for the detection of MRSA-DNA by EIS (Wang et al. 2011). In this, the rGO was conjugated with target complementary ssDNA, and a difference in the electrode's EIS between prior and after target hybridizations was used to determine the target DNA concentration with an LOD of 100 fM. Although this platform is ultrasensitive, it requires additional (3-aminopropyl)triethoxysilane (APTES) coating on GCE to increase the stability and target binding efficiency.

The square wave voltammetry (SWV) is also an EC-based technique that measures the change in the current flow with time by keeping constant electric potential

at the reference electrode. The sensitive scanning even with nanomolar analyte concentration offers an additional advantage over the traditional cyclic voltammetry (CV). Recently, a sandwich-type square wave voltammetric (SWV) sensor was designed by conjugating magnetic NPs and AuNPs with immunoglobulin G (IgG) and anti-MRSA antibodies, respectively (Cihalova et al. 2016). The IgG-magnetic NPs conjugate specifically bind and separate the MRSA from unreacted components, and the functionalized anti-MRSA-antibody-AuNPs conjugate form a sandwich with MRSA captured on magnetic NPs (Fig. 2.5c). The sensor probes selectively detected MRSA strain with an LOD of 2×10^4 CFU mL^{-1} and showed flexibility for other bacterial detections depending on the antibodies used.

Similar to SWV, cyclic voltammetry (CV) is another EC-based technique that measures the change in the current flow as a function of constant potential. The CV measures the changes in the current flow as a result of target detection. Watanabe et al. (2015) had developed a highly sensitive CV-based EC system for MRSA detection. In this case, the two complementary sequences were conjugated with both magnetic and AuNPs, respectively. After target hybridization, the ferrocene-labelled AuNPs generate an electric signal proportional to the target DNA concentration. The targeted DNA was detected with an LR of 10–166 pM and an LOD of 10 pM. Albeit these hybridization-based EC techniques offered high sensitive detection of MRSA, they cannot determine the resistant determining factors such as RGs, proteins or enzymes. In view of this, an ultrasensitive and specific differential pulse voltammetric (DPV)-based EC biosensor was developed by Liu et al. (2014) to detect the MRSA RGs (*mecA*). In this case, the working electrode was modified with dual labelled AuNPs with capture probe and alkaline phosphatase to detect *mecA* gene fragments from MRSA with an LR of 50–250 pM and TAT of 2 h. Although the EC nano-biosensors have been observed to be more sensitive than optical biosensors in the context of AST and AMR detection, future studies should focus on the development of PoC AMR diagnostic platforms by exploring novel nanomaterials and bioreceptors.

2.4 Challenges and Future Prospects

Several challenges are encountered in AST and AMR detection; however, the main challenge is to obtain rapid (typically within minutes or hours rather than days as observed in the case of standard phenotypic and genotypic methods) and reliable results by employing cost-effective, user-friendly techniques. To this end, the development of automated systems has certainly helped in reducing the TAT for AST analysis and overcoming manual errors encountered in the conventional diagnostic approaches. Despite this, their high cost and a typical requirement of pure colonies isolated by an expert in a well-established CML setting limit their application in resource-limited settings.

In the last few years, nano-biosensors have emerged as one of the most promising alternatives for rapid AMR/AST diagnosis owing to their remarkable features

such as rapid TAT, high sensitivity (quantitative and qualitative), low cost, ease of miniaturization, user-friendly application and portability. In addition to this, nano-biosensors also offer high selectivity towards target analytes without requiring exhaustive pretreatment steps, which is crucial for PoC testing application. Despite their excellent potential and pressing demand in the market, most biosensors are still in the early stages of development as they fail to meet the validation requirements. A key challenge impeding the validation process is the detection of a low concentration of target analyte in the presence of a high concentration of interfering molecules. To surmount this challenge, enrichment of colonies (where the initial bacterial concentration in a blood specimen is around 1–100 CFU/mL) and amplification steps have become a necessity in analysing AMR in real samples.

Evidence suggests that the integration of different technologies could aid in overcoming the need for enrichment steps. For instance, incorporation of magnetic beads or NP conjugated with MREs specific for target bacteria could help in its preconcentration, thereby overcoming the interferences coming from non-target biological entities (Cihalova et al. 2017; Pramanik et al. 2017). Moreover, integration of simplified NAA techniques such as LAMP and RPA, which require inexpensive instrumentation and minimum sample pretreatment and allow miniaturization, could further benefit nano-biosensors. Despite these merits, multiplexing approaches are rather less explored for these IA techniques than that for PCR. Very recently, clustered regularly interspaced short palindromic repeat (CRISPR)-based methods have been reviewed for their potential of NA detection (Bonini et al. 2021). This approach represents innovation in developing powerful tools for rapid, precise, sensitive and selective detection of genes conferring AMR to microbes. Furthermore, integration of microfluidics with biosensor platforms and transducer miniaturization could aid in the deployment of robust, compact, multiplexed and user-friendly systems, thereby increasing their acceptance by consumers and industrial collaborators. As amazing as it sounds, miniaturization is not easily attainable and requires a high level of standardization, as the specimens need to represent similar growth states and culture density. Automation of dispensing reagents could help in overcoming this issue to a great extent. Therefore, it would be imperative to focus on the integration of current biosensing platforms into inexpensive, portable, fully automated devices to develop widely accepted PoC testing platforms.

In addition to this, consolidated efforts between interdisciplinary teams from backgrounds in chemistry, biology, engineering, medicine and healthcare could help in surmounting the challenges in scalable and reproducible nanomaterial synthesis, sensor design and engineering, sensor stability and real biological sample analyses. Despite the accumulation of numerous studies on the onset of pathogenic infections, only limited reports have investigated quantitative, real-time analyses using nano-biosensor platforms. Future studies should focus on integrating complex biological samples along with laboratory standards to validate their robustness and reproducibility.

Another major challenge impeding the application of nano-biosensors in AMR diagnosis is pathogen IDn due to poor selectivity towards target bacteria. Incorporation of chemo- and biomimetic recognition elements such as molecularly

imprinted polymers, aptamers and peptides in the biosensor platform could not only improve its selectivity but also enhance its stability. For instance, aptamers are considered an excellent alternative to antibodies for bacterial IDn due to their high selectivity and stability, low production cost and ease of manufacturing.

In view of the growing global concern of AMR, the development of a better, rapid and robust biosensor platform could certainly play a crucial role in keeping the issue in check. This, in the future, undoubtedly requires the fabrication of more efficient diagnostic platforms along with the development of adequate MREs to enable precise IDn of pathogens, particularly in those settings where the nature of the threat is rather not evident.

2.5 Concluding Remarks

The classical phenotypic AST based on disk diffusion and broth dilution are likely to remain the core technologies in clinical diagnostics. While they are slow and require pure colonies, these are currently highly effective techniques for AST and MIC determination. It is likely that the molecular testing will eventually evolve towards syndrome-oriented multiplexed detection of pathogens including genomic AST; commercial competition will increase, prices per test will go down and in the end, all tests will be of the 'sample-in-result-out' format.

The nucleic acid amplification-mediated detection of RGs will play an important role in clinical microbiology laboratories and will continue to do so. The advancement in molecular diagnostics has improved the result interpretation strategies from agarose gel electrophoresis to real-time visualization, but still further improvements of different facets are required to efficiently apply them for the PoC diagnosis of AMR. The dipstick and line probes have been developed for PCR-based techniques to implement them at the PoC level. However, these molecular techniques still need improvement with multiplexing, ease of use and most obviously accuracy, precision and speed.

However, the accuracy compared with the conventional phenotypic AST approach needs to be improved. Also, these techniques require rigorous quality assurance and quality control in addition to detailed clinical evaluation and health economic studies. The nano-biosensors offered several advantages including simple instrumentation, high sensitivity and low cost. However, these designs are still in the developmental stage, and vigorous research should focus on sensor probe development, miniaturization and automation for PoC AST/AMR diagnostics directly from the clinical specimens.

Last but not the least, AST and AMR diagnostics have a large dependence on regulatory approval due to their relevance for detection under clinical settings. The regulatory approval processes are lengthy and often cost-restrictive for newly found start-ups and innovation hubs. Thus, there has to be a clear commercial incentive to develop biosensors and relevant technologies for AST and AMR diagnostics. Natural events such as the recent emergence of COVID-19 have placed greater

emphasis on improved molecular testing, and we have seen rapid development and regulatory approval of relevant sensor technologies for strain-specific pathogen detection. AMR and AST are definitely some of the biggest challenges currently faced by the mankind, and we are hopeful that it is not too far in the distant future that we will see a plethora of activities that will lead to regulatory-approved technologies for rapid detection of AMR and AST under clinical settings.

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Chapter 3
Nanomaterial-Mediated Delivery of Antimicrobial Agents: 'The Nanocarriers'

Pramod Barathe, Sagar Reddy, Kawaljeet Kaur, Varsha Shriram, Rohit Bhagwat, Abhijit Dey, Sandeep Kumar Verma, and Vinay Kumar

Abstract Antimicrobial resistance (AMR) emergence has entangled the cure of health-related diseases with the existing medicines. Though several potent and novel antimicrobial agents have been identified in recent past, their safe and effective delivery is yet to be achieved fully. Nanotechnology has emerged as the continual and practical solution in the delivery of antimicrobial therapeutics using nanotechnology-based drug carriers (nanocarriers) and signifies the correlation between biological and physical sciences, by employing it in the variety of branches like nanomedicines and nanomaterial-based drug delivery approaches. Owing to their tiny size and large surface area, nanocarrier is the hotspot in the nanotechnology world. In the recent reports, biocompatibility, cost-effectiveness, controlled drug release, deep penetration, target specificity and sustainability of nanocarriers have revealed their ideal role in the drug delivery system. In this chapter, we discuss about the various nanomaterials and antimicrobial agents employed in the delivery of antimicrobials such as metals, peptides, drugs and plant resources to target drug

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determinants such as efflux pumps, cell membrane permeability, biofilms and quorum sensing in the drug-resistant bacteria with their applications in the clinical trials.

Keywords Antimicrobial resistance · Nanocarriers · Antimicrobial agents · Biosafety · Efficacy

3.1 Introduction

Since earlier civilizations, natural products have been widely employed as potent therapeutics against numerous ailments. Based on historical learning, modern medications are thus mostly developed from medicinal plant resources (Veeresham 2012). Natural substances exhibiting various molecular backgrounds provide a starting point for the development of new medications. Various approaches such as natural product-based drug development and drug delivery have been developed to cure enormous diseases caused by drug-resistant bacteria (Atanasov et al. 2021). However, incompatibility issues, availability restrictions and tedious purification techniques (Siddiqui et al. 2014) somewhat restrict their full potential therapeutic usages, and thus newer technologies are required to address these and other issues for the development of efficient drug/antimicrobial delivery systems. Nanotechnology has been demonstrated to bridge the gap between the physical and the biological sciences by employing nanomaterials in a variety of sectors, including nanomedicines and nanomaterial-based drug delivery approaches (Patra et al. 2018). Employing nanocarriers as an effective drug delivery system has lately gotten a lot of press because of their capacity to identify and cure diseases caused by drug-resistant bacteria (Yeh et al. 2020).

Antimicrobial resistance (AMR) or antibiotic resistance (ABR) is a condition where microbes or bacteria show resistance against commonly used antimicrobial drugs, especially antibiotics. The AMR has become a major public health threat making it challenging to cure health-related diseases with existing medicines (Lee et al. 2019b). The World Health Organization (WHO) has recognized AMR and multidrug-resistant (MDR) bacteria as major global public health threats humanity is facing (WHO 2021) owing to their colonizing abilities both domestically and globally (Hall et al. 2020), though there are several successful attempts of identifying potent and novel antimicrobial agents in recent years. However, effective and safe delivery of potent antimicrobial agents has emerged a major hurdle. The use of nanomaterial- and nanotechnology-based drug carriers (nanocarriers) that can carry nano- or other antimicrobial therapeutics is emerging as a sustainable and practical solution (Krishnamoorthi et al. 2021). These approaches use nanoscale materials for the delivery of antimicrobials including natural products to their target tissues (Yeh et al. 2020). Various antimicrobial agents including metals, peptides, drugs and plant resources possessing different inhibitory mechanisms have been owned in

nanocarriers. Nanocarriers or nanomaterial-based antimicrobial systems in MDR microorganisms have been shown to inhibit various drug resistance determinants such as efflux pumps (EPs), cell membrane, biofilms and quorum sensing (Baptista et al. 2018) showing their potential clinical applications.

In this chapter, we discuss the nanomaterials emerging as a nanocarrier for delivering wide-ranging antimicrobial agents. We describe the various types of nanomaterials and antimicrobial agents with their inhibitory mechanisms that act as the major components in the delivery of antimicrobials such as drugs, metals, peptides and plant resources. Further, we highlight the delivery of antimicrobial agents via nanomaterials to target the major bacterial drug resistance determinants (cell membrane, EPs, quorum sensing, biofilm formation). The potential applications of nanocarriers in clinical trials have been also discussed herein.

3.2 Nanocarriers as Emerging Drug Delivery Systems

The significance of nanocarriers as drug delivery systems was discovered around a century ago for the delivery of therapeutic drugs and other natural agents to the site of microbial infections (Patra et al. 2018). Nanocarriers are defined as the nanoparticles that can be employed to carry antimicrobial agents or other chemical agents to the target location for their effective treatment of infections caused by the pathogenic microbes including drug-resistant bacteria (Chamundeeswari et al. 2019). Nanocarriers mainly consist of many small-sized nanoparticles (1–100 nm range) such as nanomaterials, dendrimers, lipid-based nanoparticles and liposomes that effectively transport the antimicrobial agents to the target tissue (Lombardo et al. 2019). The property of enhanced stability, improved drug serum solubility, pharmacokinetics, sustainability, longer systemic circulation duration and reduced toxicity make them excellent choice as drug delivery systems (Zhang et al. 2010). Further, deep penetration abilities of nanomaterials into the host cells, controlled drug release and endocytosis for treating drug-resistant pathogens make them ideal drug carriers with their potential clinical applications against wide range of infectious diseases (Fatima et al. 2021). To enhance the pharmacokinetics and therapeutic effects of drugs, antimicrobial agents are loaded into nanomaterials via adsorption, chemical conjugation and physical encapsulation (Patra et al. 2018).

Nanocarriers are designed in a wide range of materials with different chemical compositions to transport diverse bioactive compounds in a regulated, systemic and targeted manner, making them highly effective drug delivery agents (Manju and Sreenivasan 2010). Various nanomaterials such as metal, non-metals, semiconductors, quantum dots, dendrimers, biopolymers and organic and inorganic nanomaterials have been successfully used as nanocarriers in medical applications. Interestingly, the organic nanomaterials including liposomes, ferritin and micelles have been reported to enhance the drug bioavailability and thus improved antimicrobial activity (Yetisgin et al. 2020). Besides, other metallic and non-metallic nanomaterials combined with drugs and other antimicrobial agents have also been

widely used for the drug delivery applications (Mba and Nweze 2021). Recently, nanostructured lipid-based carriers (NLCs) have emerged as novel drug delivery systems for the delivery of chemotherapeutic agents because of their excellent physical stability, good drug-loading capacity and biocompatibility (Haider et al. 2020). The development of amoxicillin- and clarithromycin-loaded magnetic nanostructure lipid-based carriers (AMO-CLR-Fe_3O_4@NLCs) with enhanced and prolonged drug delivery with 3.13 μg/mL minimum inhibitory concentration (MIC) value against *Staphylococcus aureus*, *Bacillus subtilis* and *Bordetella pertussis* resulted in deterioration of bacterial cell morphology and ultimately led to cell death (Sharaf et al. 2021). Nano-drug carriers are also being explored in diagnosis and treatment of brain infections (Barani et al. 2021). *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Neisseria meningitidis*, *Haemophilus influenza* and *Listeria monocytogenes* are found to invade the brain causing bacterial infections in the endothelial barrier and inflammation in meninges called meningitis (Al-Obaidi and Desa 2018). The intranasal route for delivering the drugs to the brain for overcoming the blood-brain barrier and central nervous system (CNS) is considered the most viable method. The nanocarriers for drug delivery are transferred to the brain via receptor-mediated transcytosis (Sharma et al. 2021). In vivo and in vitro studies on levofloxacin-/doxycycline-loaded solid lipid nanoparticles against bacterial meningitis (Abdel Hady et al. 2020), gentamicin-loaded polymeric nanoparticles against *Pseudomonas aeruginosa* (Abdelghany et al. 2012), ansamycin-loaded polymeric nanomaterials (Nair et al. 2020b) and ofloxacin-loaded nano-transfersomes against bacterial meningitis (Eid et al. 2019), recombinant protein OmpAVac-loaded chitosan-modified poly(lactic-co-glycolic acid) (PLGA) nanoparticles against *E. coli* K1 in neonatal meningitis-infected mice (Zhang et al. 2021) and bacitracin A and brain-targeting peptide (BTP)-loaded polymeric nanoparticles against *Pneumococcal* meningitis (Hong et al. 2018) have been investigated as drug delivery systems for their biocompatibility, controlled drug release and longer systemic circulation duration for treating brain bacterial infections.

Considering the phototoxicity and low tissue penetration, light-responsive nanomaterials are emerging with potential drug design and light-triggered controlled drug delivery systems mostly useful in photothermal therapy (PTT) and photodynamic therapy (PDT) (Zhao et al. 2019; Tang and Wang 2021). Liu et al. (2021a) developed rough carbon-iron oxide nanohybrids (RCF) for near-infrared (NIR) synergistic antibacterial therapy, resulting in increased RCF bacterial adhesion and PTT in methicillin-resistant *Staphylococcus aureus* (MRSA), proposing a facile strategy to construct antibacterial agents for designing drugs and medical applications. Further in vivo studies in MRSA rat wound models showed enhanced synergistic antibacterial effects revealing their potential role in treating drug-resistant bacterial infections (Liu et al. 2021b). Wang et al. (2018) developed *Staphylococcus aureus*-pre-treated macrophage-membrane-coated gold nanocage (Sa-M-GSNC) drug delivery system, where macrophage membrane receptors were used to achieve specific bacterial-targeted delivery under near-infrared (NIR) laser irradiation in infected mice, and this resulted in better bacterial adherence, effective delivery and retaining in infection site with prolonged blood circulation and system

biocompatibility. Other than light-responsive nanomaterials, some of the alternative strategies such as pH-responsive nanomaterials, enzyme-responsive nanomaterials and redox-responsive nanomaterials are also used as drug delivery systems (Devnarain et al. 2021). Hassan et al. (2020) developed novel chitosan-based pH-responsive lipid polymer hybrid nanovesicles (OLA-LPHVs) as a vancomycin delivery system against MRSA biofilms leading to the easy release of vancomycin at pH 6.0 and inhibition of biofilms via damaging bacterial cell membrane and showing their potentials in treating bacterial infections. Enzyme-responsive nanogels developed from alginate/peptide ciprofloxacin conjugates with enhanced stability in dispersion and aqueous environment resulted in enzyme-triggered release of ciprofloxacin by degrading the peptide linkers against *S. aureus* (Bourgat et al. 2021). Similarly, Salamatipour et al. (2019) synthesized light-reduction-/oxidation-responsive alginate nano-hydrogels loaded with the folic acid drug by reverse emulsification-diffusion method and improved water retention capacity (WRC) under UV light that resulted in antibacterial activity against *S. aureus* and *E. coli*.

3.3 Types of Nanocarriers

3.3.1 Metal-Based

Metal nanoparticles usually have non-specific broad-spectrum bacterial toxicity mechanisms where they bind to outer membrane receptors (Yuan et al. 2018) that enhance their potencies. Metal-based nanoparticles have shown their efficacy in both Gram-positive and Gram-negative bacteria with multiple biomolecule target involved in the development of resistant strains (Slavin et al. 2017).

3.3.1.1 Silver Nanoparticles (AgNPs)

Chemical methods in the production of AgNPs include three components: a metal precursor, a reducing agent and a stabilizing agent (Singh et al. 2015). Appropriate size, shape and polydispersity of AgNPs can be achieved by monitoring experimental parameters such as precursors used in the reaction, reducing agents, reagent concentration, pH and temperature in the nucleation step during the synthesis process (Solomon et al. 2007; Dakal et al. 2016; Kumar et al. 2018b). Stabilization being the critical stage, chitosan, amine derivatives, thiols and gluconic acid have been recently used as stabilizers with polymeric compounds proven advantageous (Solomon et al. 2007). Beta-D glucose as the reducing agent has emerged as with special interest of researchers for the reduction of $AgNO_3$ and green synthesis giving $AgNO_3$ up to 10 nm mean size (Kumar et al. 2018b). Pal et al. (2019) studied antimicrobial peptide (AMP)-AgNP against MDR bacteria strains (*Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Salmonella typhi*) using combinations of AY1

(CAY1-AgNP and AY1C-AgNP) showing increased stability and antimicrobial activity. Recent investigations on tragacanth gum, N-isopropyl acrylamide and 2-(vinyloxy) ethanol-based stimuli-responsive silver nanocomposites (TGIAVE-Ag) resulted in controlled release of 5-fluorouracil against MDR bacteria (Nagaraja et al. 2021). Similarly, selective delivery of AgNP-responsive microparticles incorporated into dissolving microneedles against *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms resulted in controlled release of AgNPs and eradication of biofilms with improved antibiofilm activities in ex vivo biofilm-infected rat skin model (Permana et al. 2021).

3.3.1.2 Gold Nanoparticles (AuNPs)

Gold nanoparticles are colloidal particles consisting of gold as a core substance with good biocompatible property. The synthetic versatility of these NPs allows them to control particle solubility, stability and interaction with the environment. Further, studies on gold nanospheres conjugated with gentamicin have shown great activity against *S. aureus* than gentamicin alone (Ahangari et al. 2013). Reduction of chloroauric acid followed by agglomeration in the presence of the stabilizing agent is the basic synthesis process of all chemical, biological and physical pathways (Newman and Blanchard 2006). Pathogen-specific antibodies or photosensitizing molecules for photothermal and PDT conjugated with AuNPs have also been proven to promote antimicrobial activity (Savas et al. 2018; ElZorkany et al. 2019). Flavonoid-coated AuNPs with enhanced antibacterial effects of chrysin, kaempferol and quercetin against Gram-negative *E. coli* bacteria resulted in bacterial cell membrane penetration and their ablation, hence making them good drug delivery candidates (Alhadrami et al. 2021). Similar to this, *Punica granatum* extract delivering chitosan-gold hybrid nanoparticles (CS-AuNPs) exhibited high synergistic effects against MRSA (Hussein et al. 2021).

3.3.1.3 Ceramic Nanoparticles

Ceramic nanoparticles constitute oxides, carbides, phosphates and carbonates of metals and metalloids such as calcium, titanium, silicon, etc. The favourable property of heat resistance and chemical inertness makes them suitable for their wide variety of applications in medicine where they are structured by heat and pressure (comprising of solid core and a combination of metal/non-metal, at least two non-metallic elemental solids, at least one metal and a non-metallic elemental solid or a non-metal) (Wu and Zreiqat 2010). Depending upon architectural differences, they are further categorized into ceramic nanoparticles, ceramic nano-scaffold and nano-clay and are made up of ceramic compounds such as silica-titania and alumina (Rawat et al. 2008). Nano-scaffolds are defined as a structure that allows interactions of cells and extracellular matrices with microporosity (pore size >50 nm), whereas nano-clay resembles thin layers having a thickness of few nanometres.

These ceramic nanoparticles can be synthesized with microemulsion preparation, hydrothermal synthesis, sol-gel process, aerogel method, pechini-citrate gel method and low-temperature combustion (LCS) methods (Singh et al. 2016). In a recent study on biphasic calcium phosphate (BCP), a biocompatible and non-immune-responsive biphasic ceramic was used to synthesize silver-doped BCP/alginate (AgBA) microcluster stating their inhibitory action on *S. aureus* and *E. coli* (Nie et al. 2021). In one of the studies, the in vitro release profile of vancomycin-loaded hydroxyapatite compared with the pure vancomycin-HCl with increased antibiotic-loaded hydroxyapatite release rate and antibacterial activity (zone of inhibition 11.5 ± 0.5 mm and 15 ± 0.4 mm) in *S. aureus* and *E. coli*, respectively, when compared to antibiotic alone (Ain et al. 2020). Similarly, zirconia nanoparticle green synthesized using *L. nobilis* were found to be more effective against Gram-negative pathogenic bacteria (Chau et al. 2021). Chauhan (2021) reviewed the distinctive benefits of ceramic-based hybrid nanoparticle as a drug delivering system.

3.3.1.4 Silica Nanoparticles

Because of large surface area, ease of functionalization and biocompatibility, silica nanoparticles are commonly used in drug delivery applications. The mesoporous silica nanoparticles (MSN) are the porous variant that confers amenities and have been recently demonstrated as a powerful drug delivery tool for combating bacterial infections (Şen et al. 2018; Martínez-Carmona et al. 2018; Bernardos et al. 2019; Selvarajan et al. 2020). Synthesis of silica nanoparticles is carried out by Stober's method (Stober et al. 1968) and the microdilution method. Modifications in Stober's process have been performed to suit user-specific requirements such as usage of low-cost precursor (sodium silicate solution instead of tetraethyl orthosilicate) (Zulfiqar et al. 2016a, b). Another method, the microdilution, involves the formation of oil-in-water (O/W) micelles and water-in-oil (W/O) reverse micelles (Arturo Lopez-Quintelá 2003) stabilized using surfactants (twins or pluronics) acting as nanoreactors to synthesize nanoparticles depending upon the nanoreactor volume (Selvarajan et al. 2020). As peptides can be loaded using silica, Kwon and his team used the tandem peptide cargo made of lactoferrin and a synthetic bacterial toxin $_{D}$ $[KLAKLAK]_2$ for treating *Pseudomonas aeruginosa* infection in lungs (Kwon et al. 2017). Stewart et al. (2018) reported a lower drug release rate for a longer period compared to the initial burst release of the conventional drug formulation using co-assembly of an antimicrobial drug (octenidine dihydrochloride, OCT) and silica with the loading efficacy of 35%. Similarly, a nanoantibiotic system made of MSN loaded with levofloxacin (LEVO) was designed with anti-biofilm activity against *S. aureus* resulting in cell destruction (Pedraza et al. 2018). Further, effective penetration of LEVO-loaded MSN grafted with poly(propyleneimine) dendrimer of third generation (G3) in the cellular membrane of *E. coli* was reported with excellent anti-biofilm activity (González et al. 2018).

3.3.2 Liposome-Based

Liposomes are composed of lipids. Due to their similar structure and composition of the cell membrane, they are used for bacterial cell targeting that can carry both hydrophobic and hydrophilic antimicrobials and thus offering a wider choice of antimicrobial candidates to be loaded. Liposomes show fusogenicity property as they have a phospholipid bilayer structure which upon fusion with antimicrobials is directly available inside a bacterium. The most important factor of liposomes in in vivo investigations is the diameter, so to avoid rejection of liposomes by the reticuloendothelial system and allowing penetration through water channels in infectious biofilms, they should preferentially have a diameter in the range of 100–200 nm (Ferreira et al. 2021). Realization of biofilm targeting from the blood circulation, penetration and accumulation over the entire thickness of an infectious biofilm, associated with deep killing in the biofilm, are some of the challenges in the development of liposomal antimicrobial nanocarriers (Wang et al. 2020). Sanches et al. (2021) demonstrated the potential use of rhamnolipid-based liposomes as nanocarriers against *E. coli* and *S. aureus* with high haemolytic activity and negligible cytotoxicity (highest concentration of 1.3 mmol L^{-1}) to HepG2 cells. Liposomes have also been identified as one of the major antimicrobial agent (meropenem, PEG, triclosan, benzyl penicillin, zinc citrate) delivery systems for treating bacterial biofilm-mediated infections (Wang 2021).

3.3.3 Quantum Dots (QDs)

The ultra-small size semiconductor nanocrystals, with the average size in the range of 1.5–10 nm, are defined as the quantum dots and are synthesized from group II–VI elements in the periodic table depending on their conductive properties and high surface to volume ratios. Due to their unique physical and chemical properties of QDs such as high stability, exceptionally narrow range of emission and high quantum yield, they are used in biosensors, real-tracking, multipolar labelling and imaging (Jahangir et al. 2019; Wang et al. 2019). Polymer-functionalized QDs give QD a promising feature with higher antibacterial activity. Based on structural dimensions (spherical, pentagonal and hexagonal) and size, QDs can be tuned with the ligands and polymer, and thus modified GQDs facilitate the attachment of GQDs to bacterial membrane. For example, PEGylated GQDs exhibited 100% growth inhibition for *S. aureus* and *P. aeruginosa* following 8 h of incubation (Habiba et al. 2015). Reports on antibiotics conjugated with QDs (ceftriaxone conjugated to CdTe QDs) with increased antibiotic efficiency have demonstrated the synergistic antimicrobial effect against *E. coli* (Luo et al. 2011). Recently, gentamicin (GEN)-loaded mesoporous silica nanoparticle sealed with acid-decomposable 3-mercaptopropionic acid capped-ZnS QDs (MPA-ZnS QDs) resulted in controlled release of GEN drug against *E. coli* (strain 0157:H7) and *S. aureus* (strain ATCC:25923) (Mandani et al. 2021).

3.3.4 Biopolymeric Nanomaterials

Polymers derived from living organisms are said to be biopolymers and are made up of several monomeric units forming macromolecular polymer structures with covalent bonds. Rational selection of biopolymers is the most important challenge in controlled drug delivery systems which necessitate a comprehensive understanding of surface and bulk characteristics of biopolymers to achieve optimum therapeutic efficacy. Chitosan (CS) is the most common linear polysaccharide derived from naturally occurring chitin and is mainly extracted from crustacean shellfish and certain fungi. Chemically, it consists of N-acetylglucosamine and glucosamine joining together with the beta-1-4 linkage, giving a positive charge under acidic pH (Kumar 2000; Rinaudo 2006). Chitosan is often chemically modified at amino or hydroxyl groups to make them more effective and widen their medical applications (Rabea et al. 2003; Verlee et al. 2017; Sahariah and Másson 2017). Antimicrobial chitosan is prepared mainly via quaternarization and carboxylation to improve its solubility and antimicrobial activity with the maintenance of its biodegradability and biosafety. Essential oils such as rosemary essential oil when nanoencapsulated on chitosan/polyglutamic acid nanoparticles resulted in a significant increase of the antibacterial activity against *B. subtilis* (Lee et al. 2019a). Bacterial cellulose combined with ZnO-NPs was analysed for the healing property (Mihai et al. 2019).

3.3.5 Dendrimers

Dendrimers are synthetic polymers with a large number of exposed anionic, neutral or cationic functionalities on the surfaces formed by the branched repeating units that emerge from a focal point (Lyu et al. 2019). Carbon, nitrogen and phosphorus as central atoms of dendrimer play an important role in determining the structure, branches and cavities (Elsabahy and Wooley 2012; Kulthe et al. 2012; Fox et al. 2018). Further, the structural specificity of dendrimers allows attachment of compounds and drug molecules to the outer surfaces of dendrimers with final inclusion inside the cavities, which helps in encapsulation and conjugation (Pandurangan et al. 2016; Kim et al. 2018).

Dendrimers can be used in combination with traditional drugs, besides their structures can be formulated based on the pharmacodynamics and pharmacokinetics of the drug (Authimoolam and Dziubla 2016). In an interesting study, poly(amidoamine) (PAMAM) dendrimers conjugated with fluoroquinolones (nadifloxacin and prulifloxacin) showed enhanced antimicrobial activity and water solubility (Kuwahara et al. 2005; Cheng et al. 2007). Further studies on nanodendrimers conjugated with erythromycin significantly showed delivery of erythromycin with four times lesser minimum inhibitory concentration (MBC) against *P. aeruginosa*, 2 times lower against *S. aureus* and 16 times lower against *S. epidermis* (Xue et al. 2013).

3.3.6 Photothermally Activated Nanomaterials (PANs)

PANs are the broad spectrum of nanoparticles that convert absorbed light into heat. Resonance oscillation of the surface electron (surface plasmon) or energy of band transition gives the thermal effect to the nanoparticles. These nanoparticles produce thermal relaxation which leads to temperature increase, and their effect depends on many factors such as irradiation intensity, wavelength, the concentration of nanoparticles and photothermal conversion efficacy (Borzenkov et al. 2019). In a recent study, a chitosan-based hydrogel with embedded gold nanorods under low-power diode laser irradiation showed antimicrobial activity against both Gram-positive and Gram-negative bacteria including MDR strains (Bermúdez-Jiménez et al. 2019). Another study on the photothermal effect of phospholipid-coated gold nanorods loaded into a poloxamer 407 hydrogel resulted delivery of poloxamer 407 in ≈4.5–5 log cycle reduction of *P. aeruginosa biofilm* (Al-Bakri and Mahmoud 2019).

3.3.7 Carbon-Based Nanomaterials

3.3.7.1 Graphene-Based Nanomaterials

Graphene is the thinnest two-dimensional crystal sheet of single-layer sp^2 carbon (Goenka et al. 2014). Graphene nanomaterials are comprised of graphene oxide (GO), reduced graphene oxide, single layer, bi-layer graphene and multilayer graphene. Graphene-based nanostructures have wide applications including antimicrobial coatings, cellular targeting, biosensor, wound dressings, etc. The antimicrobial activity of GO increases after the reduction of sheet area. As GO is also a semiconductor material, hence it can be utilized for catalytic disinfection once exposed to UV-visible irradiation. Functionalization of GO with antibiotics, metallic compounds, immunoglobulins, chemotherapeutics, metallic nano-compounds and other organic/inorganic functionalities such as amine and carboxyl is comparatively easy because of its chemically reactive oxygen groups (carboxylic acid, hydroxyl and epoxy groups) (Sun et al. 2018a; Zarafu et al. 2018). Sharp edges of GO make it capable of killing bacteria through direct contact interactions. This mechanism of killing bacteria is called ‘trapping’ and ‘nanoknife’ mechanisms. DNA aptamer-conjugated magnetic graphene oxide (Apt@MGO) for rapid eradication of MRSA superbugs via generation of heat and cell death (~78%) under NIR laser irradiation considering them as biocompatible and light-activated photothermal agent for efficient ablation of MRSA (Ocsoy et al. 2021). Antibacterial activity of three-dimensional porous self-assembled graphene-based composite and VA-laden RGO-nHA composite scaffold (VA@RGO-nHA) against *S. aureus* with controlled release of vancomycin was reported using the *S. aureus*-infected bone by Weng et al. (2017).

3.3.7.2 Carbon Nanotubes (CNTs)

The size and surface area of carbon nanotubes are inversely proportional to each other which enhances the cell damage and subsequent cell death (Wang et al. 2016; Costa et al. 2020). Functionalization and modification help CNTs to improve their biocompatibility and dispersibility and to optimize their antimicrobial property (Rebelo et al. 2016). Enhanced antimicrobial activity of multiwall layer CNTs (MWCNTs) has been observed when functionalized with amino acids such as lysine and arginine. Antimicrobial activity of antibiotic ciprofloxacin can also be improved when coated with the single-wall CNTs (SW-CNTs) resulting in increased bactericidal activities against *S. aureus* and *P. aeruginosa* by 16-fold and *E. coli* by 8-fold, compared to ciprofloxacin alone (Assali et al. 2017).

3.3.7.3 Fullerenes

Fullerenes are ball-shaped molecules, C60 being the most common fullerene. Amphiphilic fullerenes are widely used as drug nanocarriers because of their biocompatibility and cage-like structure (Tan et al. 2017). Fullerene has also being used in PDT to treat drug-resistant bacteria, for instance, against *P. aeruginosa* in the form of its derivatives like fulleropyrrolidinium salts and sulfobutyl fullerene (Hamblin 2016). Photochemical activity and antimicrobial activity of fullerenes as drug carriers upon exposure to light via ROS have been studied in Gram-positive bacteria such as *Streptococcus pyogenes* (Kazemzadeh and Mozafari 2019).

3.3.7.4 Carbon-Based Nanodots

Carbon nanomaterials, such as graphene quantum dots and carbon nanodots with zero-dimensional, are celled as carbon-based nanodots (Manisha et al. 2019). Carbon quantum dots are electrically conductive materials and hence can be used with various antimicrobial materials (Miao et al. 2015). Carbon nanodots synthesized via top-down or bottom-up approaches with a diameter <10 nm have been investigated for loading ciprofloxacin hydrochloride for their antimicrobial activity. These ciprofloxacin hydrochloride-loaded carbon nanodots exhibited enhanced antimicrobial activity against both Gram-positive and Gram-negative bacteria (Thakur et al. 2014). Recent reports on enhanced antimicrobial activity of CDs via green synthesis medicinal turmeric leaves (*Curcuma longa*) against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli* and *Klebsiella pneumoniae* resulted in effective delivery of phytochemicals and reactive oxygen species (ROS) production leading to cell death (Nair et al. 2020a; Saravanan et al. 2021).

3.3.7.5 Carbon Nitride Nanomaterials

Graphite C_3N_4 (g-C_3N_4) is a metal-free photocatalyst. A study on strong mesoporous g-C_3N_4 which were manufactured with the cyanimide as raw material and silica as a template showed good inactivation of *E. coli* under visible irradiation (Huang et al. 2014). Modification of graphite 'carbonitrides' with other antimicrobial agents such as aerobic conditions (caused by photocatalytic oxidative inactivation) and under anaerobic circumstances (caused by photocatalytic reductive inactivation), co-rapping of g-C3N4 and reduced graphene oxide sheets have been reported to destroy bacteria (Wang et al. 2013). Graphitized carbonitride (g-C3N4) nanosheets with embedded AgNPs improved the generation of photoelectrons and thus proved to be effective antibacterial agents (Bing et al. 2015).

3.4 Antimicrobial Agents and Their Inhibitory Mechanisms

Antimicrobial agents destroy bacteria by interfering with their bacterial growth/survival/reproduction mechanisms. Various antimicrobial agents such as antibiotics/drugs, AMPs, phytochemicals and metal-based nanomaterials are used as or in delivery systems to treat microbial infections (Patra et al. 2018). These antimicrobial agents show specific inhibition mechanisms against bacteria as illustrated in Fig. 3.1 and Table 3.1.

3.4.1 Antibiotics

Antibiotics represent the most common antimicrobial agents that exert their effects by targeting major bacterial mechanisms such as cell wall synthesis, DNA synthesis, protein synthesis, DNA damage and mRNA synthesis and can be classified into various groups based on the mode of action (bacteriostatic or bactericidal) and their origin, route of administration, range of action (broad-spectrum or narrow-spectrum) and chemical structure (Table 3.2). β-Lactam antibiotics are the bactericidal agents that contain β-lactam ring in their molecular structures and interrupt bacterial cell wall formation by binding covalently to penicillin-binding protein (PBP) enzyme involving the terminal step of peptidoglycan cross-linking in both Gram-positive and Gram-negative bacteria (Bush and Bradford 2016) and include penicillins, cephalosporins, carbapenems and monobactams. Penicillins further can be broadly classified into four different groups: natural penicillins, aminopenicillins, extended-spectrum penicillins and penicillinase stable penicillins. Cephalosporins like penicillins are β-lactam antibiotics developed from cephalosporin C (a natural product of *Cephalosporium acremonium*). Successive modification of cephem ring structure has led to the 'generations' of cephalosporin to be divided into first, second, third, fourth and fifth generations. Carbapenems are derivatives of thienamycin from

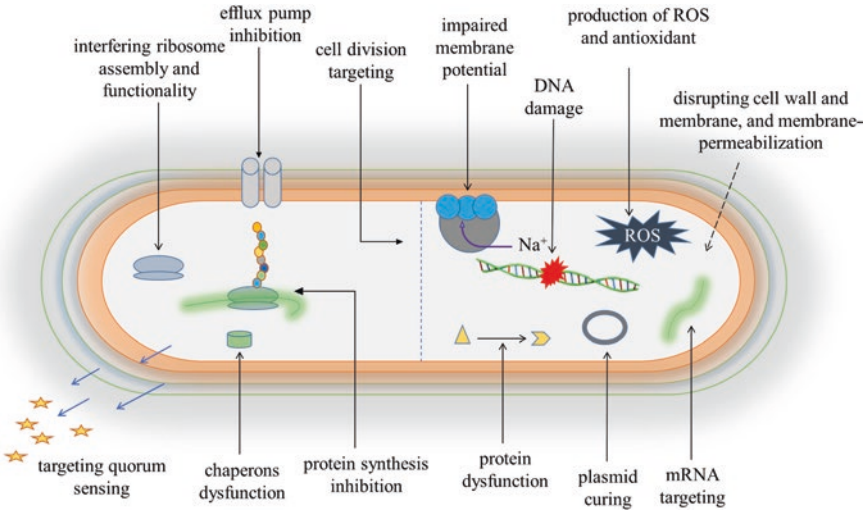

Fig. 3.1 Major mechanism of action displayed by antibiotics, AMPs, phytochemicals and metals

Streptomyces cattleya and differ from penicillins with replacement of sulphur by methylene group in a five-membered ring of β-lactams, further represented by meropenem, doripenem, ertapenem and imipenem. Monobactam is characterized by a non-fused β-lactam nucleus that differs from penicillins, cephalosporins and carbapenems including Aztreonam (Paris 2012). Aminoglycoside antibiotics are bactericidal agents structurally characterized by the presence of amino sugars attached to an aminocyclitol ring by glycosidic bond (Dasenaki and Thomaidis 2017) that include neomycin, amikacin, kanamycin, gentamicin and tobramycin (Shriram et al. 2018). Aminoglycosides inhibit protein synthesis in bacteria by irreversibly binding to the 30S ribosomal subunit, preventing the transfer of aminoacyl-tRNA to the peptidyl site, causing premature termination of the peptide chain and also increasing the frequency of mRNA misreading (Waller and Sampson 2018). Tetracyclines are usually considered as bacteriostatic antibiotics characterized chemically by a linear fused tetracyclic nucleus that inhibits bacterial protein synthesis by binding to 16S rRNA of 30S bacterial ribosomal subunit, arresting translation by interfering with the docking of incoming aminoacyl-transfer RNA (tRNA) at the acceptor site (A site) (Grossman 2016; Markley and Wencewicz 2018). Tetracycline antibiotics are broad spectrum in activity, spanning a wide range of Gram-positive and Gram-negative bacteria, obligate intracellular bacteria, protozoan parasites, chlamydia, mycoplasma, rickettsia and spirochetes and are represented by tetracycline, minocycline, demeclocycline and doxycycline. Streptogramins (pristinamycin, mikamycin, virginiamycin and quinupristin-dalfopristin) are composed of two structurally different components, A and B. A component (pristinamycin IIA, mikamycin A or dalfopristin, virginiamycin M) is polyunsaturated macrolactones, and B component (pristinamycin IB, mikamycin B or quinupristin, virginiamycin S) is a cyclic hexadepsipeptide (Schwarz et al. 2016).

Table 3.1 Different types of antimicrobial agents and their inhibitory mechanism of action against bacteria

Antimicrobial agent	Origin	Bacteria susceptible to the antimicrobial agent	Effective concentration of antimicrobial agent	Mechanism of antimicrobial action	References
Antimicrobial peptides					
1018 M	Mutant peptide of innate defence regulator (IDR-1018)	Methicillin-resistant *staphylococcus aureus* (MRSA)	32 μg/mL	Inhibition of biofilm formation, bacterial cell wall destruction, disruption of genomic DNA and regulation of the expression of ppGpp metabolism and biofilm-forming-related genes	Jiale et al. (2021)
Scolopendin 2	*Scolopendra subspinipes mutilans*	*Enterococcus faecium*, MRSA, *Escherichia coli* O157, *Salmonella typhi* and *Pseudomonas aeruginosa*	6.3–25.0 μM	Membrane permeabilization and formation of pores in the cell membrane	Lee et al. (2015)
PrAMP Bac5(1-17) derivatives	Bovine proline-rich cathelicidin	*K. pneumonia*, *E. coli* and *A. baumannii*	4 μM	Membrane permeabilization and inhibition of bacterial protein synthesis	Mardirossian et al. (2019)
P7	Derivative of cell-penetrating peptide	*Escherichia coli* ATCC25922, *Salmonella typhimurium* CMCC50013, *Staphylococcus aureus* ATCC25923, *Shigella dysenteriae* CMCC51302 and *Listeria monocytogenes* CMCC54002	4–32 μM	Pore formation, interfering with normal DNA replication and cell cycle by binding genomic DNA in *E. coli* plus decreased expression of DNA replication genes	Li et al. (2015)
Rhesus macaque θ-defensins	*Rhesus macaques*	*Staphylococcus simulant* and *S. aureus*	0.5–6 μg/mL	Membrane impairment and activation of the autolytic enzyme	Wilmes et al. (2014)
Phytochemicals					

Eugenol	Commercialized from the Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China)	Carbapenem-resistant *Klebsiella pneumoniae* (CRKP)	0.2 mg/mL	Damaged to cell membrane coupled with enhanced membrane permeability and inhibition of biofilm formation and biofilm-associated gene expression	Qian et al. (2020)
Andrographolide (Andro)	*Andrographis paniculata*	*Staphylococcus aureus*	100 μg/mL	Inhibition of intracellular DNA biosynthesis, biofilm formation and protein synthesis	Banerjee et al. (2017)
Carnosol	*Rosmarinus officinalis* L.	*Staphylococcus aureus*	5 μM	Inhibition of *S. aureus* agr (operon controlling quorum sensing in *S. aureus* called as accessory gene regulator) gene expression	Nakagawa et al. (2020)
Conessine	*Holarrhena antidysenterica*	*P. aeruginosa* PAO1 strain K767 (wild-type), K1523 (MexB deletion) and K1455 (MexAB-OprM overexpressed)	40 mg/L when used alone and 20 mg/L when combined with antibiotics	Inhibition of MexAB-OprM efflux pump in *P. aeruginosa*	Siriyong et al. (2017)
8-Epidiosbulbin E acetate (EEA)	*Dioscorea bulbifera* L.	*Enterococcus faecalis*, *Escherichia coli*, *Shigella sonnei* and *Pseudomonas aeruginosa*	25–200 μg/mL	Antibiotic resistance plasmid (R plasmid) curing	Shriram et al. (2008)

(continued)

Table 3.1 (continued)

Antimicrobial agent	Origin	Bacteria susceptible to the antimicrobial agent	Effective concentration of antimicrobial agent	Mechanism of antimicrobial action	References
Berberine and palmatine	*Berberis vulgaris*	*Pseudomonas aeruginosa*	250–1000 μg/mL and 50–100 μg/mL, respectively	Inhibition of *Pseudomonas aeruginosa* MexAB-OprM efflux pumps	Aghayan et al. (2017)
ε-Viniferin	*Paeonia lactiflora*	*Escherichia coli* O157:H7	10 μg/mL	Inhibition of bacterial biofilm formation	Cho et al. (2013)
Carvacrol and eugenol	Commercialized from Sigma Aldrich, UK	*Pectobacterium* species	250 μM	Downregulation of genes related to quorum sensing	Joshi et al. (2016)
4-Diphenylamino 3-iodo coumarin (4-DPA3IC)	Traditional drug formulations	*S. aureus*	10 μg/mL	Bacterial DNA gyrase inhibition	Sareena and Vasu (2020)
Metals					
Nickel		*E. coli*	8 μM	Inhibition of fructose-1,6-bisphosphate aldolase (FbaA), a class II aldolase through binding to the noncatalytic zinc site	Macomber et al. (2011)
Copper		*Escherichia coli* (ATCC 23724)	4–8 mM	Nonenzymatic oxidative damage of membrane phospholipids that led to the loss of membrane integrity and resulted in cell death	Hong et al. (2012)
Chromium		*Escherichia coli* MTCC 40 and *Bacillus subtilis*-industrial strain 168	1–10 μg/mL	Membrane damage caused by ROS production followed by DNA damage	Fathima and Rao (2018)
Metal complexes					

Complexes of 3-(trifluoromethyl)phenylthioureas with Cu(II)	*Staphylococcus aureus* and *Staphylococcus epidermidis*	1–4 μg/mL	Bacterial biofilm and topoisomerase inhibition	Bielenica et al. (2018)
Mixed-ligand copper(I) halide complexes bearing 4,5-bis(diphenylphosphano)-9,9-dimethyl-xanthene and N-methylbenzothiazole-2-thione	*Escherichia coli*, *Xanthomonas campestris*, *Bacillus subtilis* and *Bacillus cereus*	5–15 μl	DNA degradation, generation of ROS and bacterial membrane damage	
Metal nanoparticles				
Biosynthesized silver nanoparticles	*Escherichia coli* and *Pseudomonas aeruginosa*	4.5 and 2.7 μg/mL	Altered cell membrane permeability and membrane damage associated with ROS production	Ramalingam et al. (2016)
Zinc oxide nanoparticles	Vancomycin-resistant *S. aureus* (VRSA)	10 mg/mL	Limiting of bacterial biofilm formation	Jasim et al. (2020)

Table 3.2 Classes of antibiotics and their mechanism of action

Antibiotic class				Examples	Effective against	Mechanism of action	References
β-lactam	Penicillins	Penicillinase sensible	Natural penicillins	Penicillin VK and penicillin G	*E. coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *streptococcus pneumonia*, *Haemophilus influenza*, *Bacteroides fragilis*, *Neisseria gonorrhoeae* *Klebsiella* spp. and *Enterobacter* spp.	Inhibition of cell wall synthesis	Graber et al. (1981), White et al. (1979), and Wise and Andrews (1982)
			Aminopenicillins	Ampicillin and amoxicillin			
		Penicillinase-stable penicillins		Methicillin, nafcillin and oxacillin			
		Extended-spectrum penicillins	Ureidopenicillins	Piperacillin, azlocillin, and mezlocillin			
			Carboxypenicillins	Carbenicillin, ticarcillin			
	Cephalosporins	First generation		Cephaloridine, cephacetrile, cephapirin, cefadroxil cephalexin, cephadrine and cephalothin	*Haemophilus influenza*, *Moraxella catarrhalis*, *Streptococcus pneumonia*, *Streptococcus pyogenes*, *MRSA*, *E. coli* and *Klebsiella* spp.		Jones et al. (2007), Morrissey et al. (2009), and Sader et al. (2014, 2015)
		Second generation		Cefotetan, cefaclor, cefoxitin, cefamandole, cefonicid, cefmetazole, cefuroxime and cefuroxime axetil			
		Third generation		Cefoperazone, cefetamet, cefpodoxime, cefixime, cefovecin, cefsulodin, cefotaxime, ceftiofur and ceftriaxone. Cefdinir, ceftizoxime, ceftazidime and ceftizoxime proxetil			
		Fourth generation		Cefepime, cefpirome and cefquinome			
		Fifth generation		Ceftaroline and ceftolozane			
	Carbapenems			Meropenem, doripenem, ertapenem imipenem and tomopenem	*E. coli*, *Pseudomonas aeruginosa*, *MRSA*, *Acinetobacter baumannii*, *Aeromonas* spp. and multiple β-lactamase (TEM, SHV, CTX-M, OXA, CMY types)-producing *E. coli*		Castanheira et al. (2009), Davies et al. (2008), Jones et al. (2005), and Koga et al. (2008)
	Monobactam			Aztreonam	*E. coli*, *Klebsiella pneumonia*, *Serratia marcescens*, *Pseudomonas aeruginosa* and *Enterobacteriaceae*		Shibl (1989) and Van Laethem et al. (1984)
No lactam	Glycopeptides			Oritavancin, vancomycin, telavancin, dalbavancin and teicoplanin	*Clostridium difficile*, *Clostridium innocuum*, *MRSA*, methicillin-resistant *S. epidermidis* (MRSE) and *enterococci*		Bartoloni et al. (1990) and Biavasco et al. (1991)
	Others			Isoniazid, colistin, polymyxin B and daptomycin	Methicillin-resistant *Staphylococcus aureus*, methicillin-resistant *S. epidermidis*, vancomycin-intermediate-susceptible *S. aureus* and vancomycin-resistant *enterococci*		Rybak et al. (2000)

Antibiotic class	Examples	Effective against	Mechanism of action	References
Amino-glycosides	Neomycin, amikacin, kanamycin, gentamicin, tobramycin, dactimicin and plazomicin	Carbapenem-resistant *Enterobacteriaceae*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Acinetobacter* species	Inhibition of protein synthesis	Castanheira et al. (2018)
Tetracyclines	Petracycline, minocycline, demeclocycline, doxycycline, tigecycline, omadacycline and eravacycline	*Moraxella catarrhalis*, *Mycobacterium abscessus*, *Mycobacterium chelonae*, *Mycobacterium fortuitum*, *Mycobacterium mucogenicum*, *Mycobacterium immunogenum*, methicillin-resistant *Staphylococcus aureus*, *Streptococcus pneumonia*, *β-hemolytic streptococci*, *Enterococcus* spp., *Haemophilus influenza*, *Escherichia coli*, *Klebsiella oxytoca*, *Enterobacter cloacae*, vancomycin-resistant *enterococci* (VRE), penicillin-resistant *S. pneumoniae* (PRSPN) and extended-spectrum β-lactamase-producing *E. coli*		Brown-Elliott and Wallace (2021), Carvalhaes (2019), and Sun et al. (2021)
Oxazolidinones	Tedizolid and linezolid	Methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA), *Streptococcus pyogenes*, *Streptococcus anginosus* group, *Streptococcus agalactiae* and *enterococci*		Barber et al. (2016), Keel et al. (2012), and Pfaller et al. (2016)
Streptogramins	Quinupristin-dalfopristin	*Vancomycin*-resistant *S. aureus* (VRSA), *Bacteroides fragilis*, vancomycin-resistant *E. faecium*, methicillin-susceptible or methicillin-resistant *S. aureus*		Betriu et al. (1999), Cha et al. (2003), and Dowzicky et al. (1998)
Chloramphenicol		*Haemophilus influenza*, *S. aureus*, *Streptococcus pneumoniae* and *Neisseria meningitides*		Rahal and Simberkoff (1979)
Macrolides	Erythromycin, roxithromycin, troleandomycin, clarithromycin, telithromycin and maridomycin	*Mycobacterium smegmatis*, *M. avium*, *Streptococcus pneumonia*, MRSA, *Neisseria gonorrhoeae* and *Vibrio cholerae*		Champney and Burdine (1998), Doucet-Populaire (1998), Farrell et al. (2004), and Kondo et al. (1973)
Lincosamides	Lincomycin, clindamycin and pirlimycin	*Porphyromonas gingivalis*, *Prevotella intermedia*, *Clostridium perfringens*, *Peptostreptococcus magnus*, *Bacteroides fragilis*, *B. vulgatus*, *B. oralis* and *Eubacterium lentum*		Kondo et al. (1973) and Miyake et al. (1995)

Table 3.1 (continued)

Antibiotic class	Examples	Effective against	Mechanism of action	References
Fluoroquinolones	Norfloxacin, ciprofloxacin, levofloxacin, sparfloxacin, clinafloxacin, moxifloxacin, trovafloxacin, besifloxacin, sarafloxacin, enrofloxacin and temafloxacin	*Ciprofloxacin-resistant MRSA*, *Mycobacterium tuberculosis*, *Pseudomonas aeruginosa*, *S. aureus*, *S. epidermidis*, *Streptococcus pneumonia*, *Haemophilus influenza*, *Aeromonas salmonicida*, *E. coli*, *Mycobacterium avium* complex, *Streptococcus pyogenes*, *Legionella pneumophila* and *Listeria monocytogenes*	Inhibition of DNA topoisomerase	Abdi-Ali et al. (2006), BARNES et al. (1991), Cherubin and Stratton (1994), Cremades et al. (2011), Haas et al. (2010), Jacobs (1991), Jones et al. (1999), Mizunaga et al. (2005), and Stein et al. (2008)
Quinolones	Nalidixic acid			
Sulfonamides	Sulfisoxazole, sulfasalazine, sulfamethoxazole and Ag sulfadiazine	Carbapenem-resistant *Acinetobacter baumannii* (CRAB), *Neisseria meningitides* and *Haemophilus influenza*	Inhibition of folic acid synthesis	McGowan et al. (1976), Nepka et al. (2016), and Neuharold and Feldman (1973)
DHFR inhibitors	Pyrimethamine, trimethoprim			
Nitroimidazoles	Tinidazole and metronidazole	*Obligate anaerobic bacteria* and *Bacteroides fragilis*, *Clostridium difficile* and *Peptostreptococcus anaerobius*	DNA damage	Jokipii and Jokipii (1977) and Jokipii and Jokipii (1987)
Rifampicin		*Mycobacterium tuberculosis*, *Neisseria meningitides*, *Neisseria gonorrhoeae*, *Haemophilus influenzae* and *Listeria monocytogenes*	Inhibition of mRNA synthesis	Hu et al. (2015), Simmons et al. (2000), and Thornsberry et al. (1983)

Component A interferes with polypeptide elongation by preventing binding of aminoacyl-tRNA to ribosome whereas component B destabilizes the peptidyl-tRNA resulting in enhanced bactericidal activity (Lee 2006).

Macrolides are a different group of compounds that has a lactone ring (14–16 atoms) bonded to one or more deoxy sugar and classified according to the number of carbon atoms in the lactone ring; 14 membered includes erythromycin, roxithromycin, troleandomycin, clarithromycin and dirithromycin, whereas 15 membered includes azithromycin and 16 membered includes spiramycin, josamycin, midecamycin and spiramycin (Kuruvilla 2018). Macrolide antibiotics inhibit protein synthesis by targeting bacterial ribosomes, further binding at nascent peptide exit tunnel and partially occluding it. Thus macrolides are viewed as 'tunnel plugs' that stop protein synthesis (Vázquez-Laslop and Mankin 2018). Another class of antimicrobial agents, the lincosamides, are derived from *Streptomyces* spp. Lincosamide structure consists of three components: an amino acid (L-proline substituted by a 4′-alkyl chain), a sugar (lincosamide) and an amide bond connecting these two moieties (Kwon 2017). Lincosamides inhibit protein synthesis by binding to 50S subunit at a site that overlaps both P and A sites on the bacterial ribosome, preventing charged tRNA docking and their movement through the peptidyl transferase centre (Sauberan and Bradley 2018). Lincomycin, clindamycin and pirlimycin are three antibiotics present in the lincosamide group.

Besides macrolides, antibiotics and lincosamides, the quinolones are another family of synthetic antimicrobial drugs that have been reported to be effective against various bacterial infections. The first quinolone reported, nalidixic acid, was introduced in 1964, and its further chemical manipulation and advancements resulted in the development of fluorinated quinolones (fluoroquinolones) that includes danofloxacin, difloxacin, marbofloxacin, orbifloxacin, enrofloxacin, ciprofloxacin, moxifloxacin and levofloxacin. The major mechanism involved in the inhibition of topoisomerase II (DNA gyrase and topoisomerase IV), regulates under-winding and over-winding of DNA. The binding of quinolones to enzyme-DNA complex results in the conformational changes of enzyme further inhibiting relegation of broken DNA strands leading to the bactericidal effect. Besides quinolones, sulfonamides are one of the oldest groups of antibacterial agents introduced into medical practice even before the discovery of penicillin and have broad-spectrum use concerning both Gram-negative and Gram-positive microorganisms. Sulfonamide drugs are the structural analogs of para-aminobenzoic acid (PABA), an essential component in the folic acid pathway. Sulfonamides inhibit the bacterial dihydropteroate synthetase (DPS) enzyme of the folic acid pathway, blocking bacterial nucleic acid synthesis. Sulfonamides also contribute in preventing the conversion of PABA to dihydrofolic acid by substituting competitively for PABA. Combinations with trimethoprim have also shown an excellent bactericidal effect. Trimethoprim inhibits dihydrofolic acid reductase thereby preventing the subsequent conversion of dihydrofolic acid to tetrahydrofolic acid thus blocking two successive steps in the folic acid pathway and exhibiting enhanced bactericidal effect (Ahern and Richardson 2012). Metronidazole and tinidazole are the main representatives of nitroimidazoles. Metronidazole is active against some anaerobic

bacteria (e.g. *Clostridium difficile*), protozoan infections and microaerophilic bacteria (*Gardenia vaginalis* and *helicobacter pylori*). Metronidazole first diffuses across the membrane and then gets reduced by intracellular protein under anaerobic conditions, hence exerting its effect through cytotoxic intermediate and free radical's formation that provoke DNA damage (Bury-Moné 2014).

Apart from the wide-spectrum activity and fast-action advantages of antibiotics, they face some disadvantages such as side effects, hypersensitivity, drug interaction and toxicity and negative effect on commensal microflora (Weledji et al. 2017). In addition to injudicious usage of conventional and commonly available antibiotics in human health, veterinary agriculture further adds to the evolution, persistence and spread of AMR with emergence of new drug-resistant bacterial strains at a frightening rate resulting in the inefficacy of existing drugs with very few or no solutions in sight. Therefore, to successfully combat the escalating problem of AMR, novel and effective antimicrobial agents are recommended such as phytochemicals, metal, metal-based complexes, metallic nanoparticles and AMPs.

3.4.2 Antimicrobial Peptides (AMPs)

AMPs are broadly defined as 'naturally occurring polypeptide sequences of 12–15 residues comprising cationic and hydrophobic amino acid with direct antibacterial activity' (Li et al. 2021). AMPs are produced by all organisms ranging from bacteria, plants, invertebrates and vertebrates and have a wide range of inhibitory effects against fungi, bacteria, viruses and parasites (Kumar et al. 2018a). AMPs have several advantages over conventional antibiotics showing the multifunctional mechanism of antibacterial action altering cell membrane and also attacking specific targets that take part in the development of different intracellular processes such as bacterial cell wall formation, transcription and translation that has antimicrobial activity against multidrug-resistant pathogens (León-Buitimea et al. 2020).

AMPs are found to be highly effective against Gram-negative bacteria which are more challenging to treat than their Gram-positive counterparts because of the outer membrane composition in the earlier that makes them impermeable to most of the conventional antibiotic drugs. AMPs are often introduced in literature as a 'promising alternative to antibiotics' and 'potential to address the growing problem of antibiotic resistance' and 'hold promise to be developed as novel antibiotics' (Li et al. 2021) because of a non-specific mechanism involving membrane target, oxidative damage, damage to intracellular molecules, potent microbicidal activity in the micromolar range and rapid drug action increasing difficulty in resistance development because of limited time for extensive mutation and growth (Koo and Seo 2019). In addition, AMPs are also known as host defence peptides (HDPs) as they can also enhance immune response highlighting the clinical potential of AMPs to stimulate innate immunity (Li et al. 2021). AMPs such as HPA3P (*Helicobacter pylori*-derived AMP) loaded onto a gold nanoparticle-DNA aptamer (AuNP-Apt) conjugate (AuNP-Apt-HPA3P^{His}) when utilized against *Vibrio vulnificus* resulted in

HPA3P^{His}-induced bacterial cell death via disruption of membrane integrity and 100% survival rate in *Vibrio vulnificus*-infected mice resulting in complete inhibition of *Vibrio vulnificus* colonization, hence displaying effective drug delivery of AMPs (Lee et al. 2017).

AMPs are commonly known for non-receptor-mediated membrane-lytic bactericidal activity. Membrane-targeting mechanisms of AMPs can be described through pole and carpet models, barrel-stave models and toroidal pore models (Fig. 3.2). In the toroidal-pore model, the initial binding of the peptide to the membrane is followed by cascade aggregation of incoming monomer units, causing the lipid moieties of inner and outer membranes to fold inward, forming continuous channels lined by multiple peptide units and thus tightly associating lipid head groups of membrane phospholipids with peptides. A typical example of this model includes magainin 2, lacticin Q, arenicin and melittin (Huan et al. 2020). However, the barrel-stave model differs from the toroidal pore model by the peptide monomers inserted into the membrane arranged parallelly to phospholipid molecules of the membrane. Besides membrane penetration and pore formation, AMPs have another mechanism of action which includes inhibition of protein synthesis by affecting transcription, translation, protein folding and assembly of newly synthesized proteins. For example, PR-39, a proline, and arginine-rich AMP isolated from pigs' small intestine were found primarily to penetrate rapidly into *E. coli* outer membrane that led to protein synthesis inhibition and degradation of the protein (Boman et al. 1993). Following penetration, inhibition of nucleic acid biosynthesis occurs by affecting the key enzymes of DNA synthesis or inducing degradation of the nucleic acid molecule. By inhibiting the DNA replication, DNA damage response (SOS response), causing chromosomal separation failure blocking cell cycle, and inhibiting cell division is the process of AMPs. Cruz et al. (2020) identified 40-amino acid residue MciZ as an effective inhibitor of bacterial cell division, Z-ring formation and localization. Histatin, eNAP-2 and indolicidin were also found to have strong protease inhibition mechanisms (Huan et al. 2020). Similarly, investigations on NP-6 from Sichuan pepper seeds showed inhibition of beta-galactosidase activity in *E. coli* (Hou et al. 2019). These multifunctional mechanisms of antibacterial action thus highlight the AMPs as a promising alternative to antibiotics.

3.4.3 Phytochemicals

Plants produce a wide array of phytochemicals that have been utilized for centuries in ethnomedicine or folk medicines. Phytochemicals are compounds that occur naturally in plants as secondary metabolites (Bai et al. 2011) and can be classified into many major classes depending upon the chemical structure (alkaloids, polyphenols(flavonoids and non-flavonoids), terpenoids, sulphur-containing phytochemicals), biosynthetic pathways, biological pathways and botanical origins (Górniak et al. 2019; Belščak-Cvitanović et al. 2018). Two major sub-classes of phenolic acid include hydroxybenzoic acid (e.g. gallic acid, vanillic acid,

protocatechuic acid, salicylic acid, syringe) and hydroxycinnamic acid (e.g. chlorogenic acid, coumaric acid, caffeic acid, ferulic acid curcumin, caftaric acid, cinnamic acid) (Flamini and De Rosso 2018). Similar to phenolic acids, tannins are a group of structurally complex polyphenols comprising condensed (proanthocyanidins) and hydrolyzable tannins that can form complexes with proteins by non-specific interactions. Therefore, displaying antimicrobial activity may be associated with their potential to denature microbial transport protein, adhesins and microbial enzymes preventing microbial growth through deprivation of metal ions and substrates (Gupta and Pandey 2019). Bacterial cells can be affected by phytochemicals in several ways due to the greater diversity displayed by phytochemicals. The major mechanism of phytochemicals action includes membrane permeabilization, cell membrane disruption, EP inhibition, inhibition of biofilm formation and quorum sensing, targeting resistant plasmid, inhibition of cell division and DNA and protein synthesis (Table 3.1) (Navarro-Martínez et al. 2005; Gradišar et al. 2007; Domadia et al. 2008; Wu et al. 2008; Boulet et al. 2018). For instance, studies have shown enhanced bactericidal activity of thymol against *S. aureus* and *E. coli* by encapsulating thymol in hollow mesoporous silica sphere with cell membrane disruption as an inhibitory mechanism of action, thus highlighting enhanced resistance reversal potential antimicrobial agent when combined with nanocarriers (Liu et al. 2021a) that could speed up the successful application of antimicrobial agents in clinical settings.

Similarly, essential oils are known for their broad-spectrum antimicrobial potentials mainly attributable to their abilities of targeting major determinants of drug resistance, pathogenicity and spread, which include EPs, cell membrane, quorum sensing, resistant plasmids and biofilms. Recent reports confirm that essential oils show both direct killing (bactericidal) or re-sensitizing (or resistance-reversal) potentials providing effective solutions for tackling AMR and the potential to rejuvenate or replace otherwise fading antibiotic arsenal (Yu et al. 2020). Recent years have witnessed the use of nanomaterials as synergistic agents with essential oils as well as their carriers. Montmorillonite nanosheet-based (MMT-based) drug nanoplatform involving antibacterial metal copper ions, quaternized chitosan (QCS) and antibiotic 5-fluorocytosine (5-FC) [QCS/MMT/5-FCCu] strongly inhibited *S. aureus*, *E. coli* and *Candida albicans* with high drug-loading capacity, excellent wound healing and good biocompatibility in a mouse model infected with wound demonstrating enhanced killing effect against both bacteria (Sun et al. 2019). Similarly, cinnamaldehyde-loaded liposomes decorated with chitosan also showed strong antibacterial efficacy against *S. aureus* by damaging cell membrane integrity, causing cell death by leakage of intracellular components (Wang et al. 2021).

3.4.4 Metals, Metal-Based Complexes and Metallic Nanoparticles

Since ancient times, antimicrobial activities of metals such as silver (Ag), gold (Au), copper (Cu) titanium (Ti), mercury (Hg) and tellurium (Te) consisting of different properties defining the spectrum of activity and potencies are known that are used as antimicrobial agents because of their microbiocidal activity at extremely low concentration. Previous reports on *E. coli* and *S. aureus* treated with $AgNO_3$ resulted in losing their replication ability and protein inactivation resulting in strong antibacterial activity of metals (Woo et al. 2008). The major mechanism of antibacterial action of metals includes production of ROS, impairing membrane function, interfering with nutrient assimilation, inducing genotoxicity, protein dysfunction and loss of enzyme activity (Lemire et al. 2013). For example, tellurite (TeO_3^{2-}) toxicity in *E. coli* by treatment of K_2TeO_3 in *E. coli* leads to superoxide formation (Pérez et al. 2007). Similar results with loosening of cell walls, cytoplasmic aggregation and cell wall rupture were observed when *Erwinia carotovora* subsp. *atroseptica* was treated with aluminium chloride resulting in increased mortality (Yaganza et al. 2004). Further, as there is a chemical similarity between iron (Fe) and gallium (Ga), Ga can substitute Fe in a different biological system and inhibits Fe-dependent processes, for example, inhibition of growth, biofilm formation and death of *P. aeruginosa* by Ga-induced reduced uptake of Fe and reduced expression of genes involved in Fe uptake suggesting the importance of Ga in interference of nutrient assimilation. In addition, since Ga is FDA approved for *intravenous* (*IV*) administration suggesting Ga as potentially promising therapeutics in the dearth of new antibiotic development (Kaneko et al. 2007).

Treatment of *E. coli* (lacking copper homeotic system) with copper metal resulted in rapid inactivation of isopropyl malate dehydratase (an iron-sulphur cluster enzyme in the pathway of branched-chain amino acid synthesis) damaging essential enzymes of biosynthetic pathways (Macomber and Imlay 2009). In addition to this, metals when used in nanoformulations or complexed with other antimicrobial agents such as phytochemicals, antibiotics and synthetic metal complex show greater inhibitory effects against bacteria compared to their free ligand, exhibiting potent broad-spectrum antimicrobial activity, with low toxicity (Lemire et al. 2013). For example, when a metal complex of Ga and flavonoid quercetin (metal complex 1) and H2bbppd and Cu(II) (metal complex 2) were evaluated against *Staphylococcus aureus* (ATCC SP 25923), *Escherichia coli* (ATCC SP 11229), *Enterococcus faecalis* (ATCC SP 19433) and *Pseudomonas fluorescens* (ATCC SP 13525), both metal complex showed greater inhibitory effects as compared to their ligand with lower MIC $\leq$250 μg/ml, confirming broad-spectrum strong antibacterial activities.

3.5 Nanomaterial-Based Antimicrobial Delivery Targeting Drug-Resistant Determinants

3.5.1 Bacterial Cell Membrane

The first line of defence in bacteria is the cell membrane that maintains the necessary osmotic balance between the outer environment and the cytoplasm (Yeh et al. 2020). Various nanomaterials have been found interacting with the bacterial cell membrane to increase the membrane permeability via the generation of ROS and production of radicals [singlet oxygen ($^1O_2$), electrons (e^-), hydroxyl radicals (●OH) and superoxide radicals ($O_2^{\bullet-}$)] (Wang et al. 2017). As an alternative to traditional antibiotics, photothermally active nanomaterials have emerged as a potential drug delivery system to target bacterial drug-resistant determinants (Borzenkov et al. 2020; Kaur et al. 2021). Multifunctional drug delivery nanoparticle (MDD-NP) and crystalline ruthenium polypyridine nanoparticles (Sph-Ru-MMT@PZ) consisting of adhesive and surface-anchoring properties, under 670 nm red irradiation therapy (R-IT), resulted in bacterial destruction and cell lysis of *E. coli* via ROS production (Yin et al. 2021). Further in vivo studies in mice revealed synergistic anti-infective effects of nanoparticles, hence promoting wound healing. Vancomycin-encapsulated, pH-responsive, surface charge-switching poly(D,l-lactic-co-glycolic acid)-*b*-poly(l-histidine)-*b*-poly(ethylene glycol) (PLGA-PLH-PEG) nanocarriers demonstrated pH-sensitive NP binding to bacteria (pH 6.0) and drug delivery to bacterial cell membrane of *S. aureus* causing cystic fibrosis with an 1.3-fold increase in MIC (Radovic-Moreno et al. 2012). A study on controlled release of drug at the injection site was conducted with kanamycin-loaded TiO_2 nanotubes (NTs) under NIR irradiation via disrupting the bacterial cell membrane integrity by damaging bacterial cell wall and radical-induced inflammation and cytotoxicity resulting in ≥99.9% reduction in *E. coli* (Xu et al. 2021). Similar results were observed in eco-friendly chitosan-based nanoantibiotic system (LD@CN/DA) for potential delivery of linezolid (LD) with 3,5-dinitrosalicylic acid (DA) as antimicrobial agents with 98.4% drug release efficiency against MRSA, *E. coli* and *E. faecalis* resulting in the formation of ROS and enhancing pathogen-specific activity (Teaima et al. 2020).

3.5.2 Biofilms

Human infections can be caused by bacteria that are in the form of biofilms, planktonic cultures and intracellular residence depending on their surroundings and growth parameters (Yeh et al. 2020). Biofilms are well-organized community of bacteria that adhere to the host cells to protect themselves from the harsh environmental, physiological conditions and action of antibiotics (Sharma et al. 2019). Recent reports on worldwide human infections caused by biofilms have crossed

60% making them the primary cause of various treatment failures in medicine (Huang et al. 2021). Therefore, biofilms have emerged as one of the major resistance mechanisms and spreading AMR. Recent years have witnessed the successful applications of nanomaterials in eradicating biofilms as well as in carrying effective anti-biofilm agents.

Endophthalmitis is defined as the bacterial infections caused by various microorganisms inside the eye vitreous and aqueous humour (Durand 2013). Chen et al. (2019) studied the eradication of *E. coli*, *S. aureus* and MRSA biofilms causing endophthalmitis using ammonium methylbenzene blue-loaded pH-responsive zeolitic imidazolate framework-8-polyacrylic acid (ZIF-8-PAA) modified with $AgNO_3$ and secondary modification of vancomycin/NH_2-polyethylene glycol (Van/NH_2-PEG) composite nanomaterial (ZIF-8-PAA-MB@AgNPs@Van-PEG). Further in vitro retinal pigment epithelium cellular experiments and in vivo mice endophthalmitis models resulted in effective drug release, biocompatibility and antibacterial efficiency of composite nanomaterial against biofilm-causing bacteria (Chen et al. 2019). *Pseudomonas aeruginosa*, another pathogen found in adult patients infected with cystic fibrosis (CF), is the major biofilm-forming bacteria (Davies 2002). The development of novel aerosolized ciprofloxacin-loaded poly(lactic-co-glycolic (PLGA) acid) nanocarriers onto the in vitro model of *Pseudomonas aeruginosa* biofilm-infected human bronchial epithelial cells resulted in the eradication of planktonic bacteria and reduced biofilm fraction by log 6 revealing their potential avenues in preclinical studies (Juntke et al. 2021).

Nitric oxide has emerged as a promising agent for disrupting biofilms and promoting wound healing (Englande and Friedman 2010). Hasan et al. (2019) developed polyethyleneimine/diazeniumdiolate (PEI/NONOate)-doped PLGA nanoparticles (PLGA-PEI/NO NPs) against MRSA biofilm of diabetic wounds resulting in binding of NPs to biofilm matrix facilitating NO delivery and enhanced anti-biofilm activity. Further in vivo studies in MRSA biofilm-infected wounds in diabetic mice accelerated healing via biofilm binding NO release from NPs (Hasan et al. 2019). Amikacin and ciprofloxacin drugs encapsulated in liposomes have shown their effective penetration abilities in *P. aeruginosa* biofilms (Zhang et al. 2018; Chalmers et al. 2021). Besides liposomes, AMP-based nanocarriers have greatly enhanced their medicinal benefits by improving stability, solubility and in vivo half-life in various pulmonary, gastrointestinal and wound infections (Song et al. 2021) (Table 3.3).

3.5.3 Efflux Pumps (EPs)

Extrusion of therapeutically relevant antimicrobial agents/drugs from inside cells to the extracellular environment via EPs has been frequently involved in microbial antibiotic resistance and spreading AMR (Alav et al. 2018). Investigations have identified several EP genes in chromosomes and plasmids of different bacterial species that mediate drug resistance (Li and Nikaido 2009). EPs are also found to play

Table 3.3 List of nanocarriers conjugated with antimicrobial agents as delivery systems in microbes

Antimicrobial agent/drug	Nanocarrier	Drug resistance determinant target	Observation/action mechanism	Name of microorganism	Experiment model	References
Vancomycin	Vancomycin-loaded solid lipid nanoparticles (VM-ATS-SLN)	Biofilm formation	VM release in response to acidified pH and lipase enzyme with biofilm growth inhibition	MRSA	Skin-infected mice model	Ibrahim et al. (2021)
Microcin J25 (MccJ25)	Chitosan nanoparticles (CNs)-antimicrobial peptide microcin J25 (MccJ25) conjugates (CNMs)	Cell membrane	Binding to outer membrane protein A (OmpA) and lipopolysaccharide (LPS) of bacteria causing membrane damage	*E. coli* K88 and MRSA	Human embryonic kidney cells (HEK293T)	Yu et al. (2021a)
Folic acid	Nanoscale metal-organic frameworks (NMOFs) encapsulating the antibacterial ligand (lysine carbon dots, Lys-CDs) and targeted drug (folic acid, FA)	Biofilm formation	Sharp increase of reactive oxygen species (ROS) inside the bacterial cells by FA functionalizing NMOFs	*S. aureus* and *E. coli*	In vitro NIH-3 T3 mouse fibroblast cell	Yu et al. (2021b)
Piper betle aqueous extract	Piper betle-based silver nanoparticles (PbAgNPs)	Quorum sensing	Inhibition of QS-mediated virulence factors such as prodigiosin, protease, biofilm formation, exopolysaccharides and hydrophobicity productions with downregulation of fimA, fimC, flhD and bsmB genes	*Proteus mirabilis* and *Serratia marcescens*	*Caenorhabditis elegans*	Ho et al. (2020)

Antimicrobial agent/drug	Nanocarrier	Drug resistance determinant target	Observation/action mechanism	Name of microorganism	Experiment model	References
Quercetrin and afzelin	Quercetrin and afzelin capped silver nanoparticles (AgNPs)	Biofilm formation	Reduced cell surface hydrophobicity, decreased surface adherence loss of membrane potential	*Salmonella typhimurium*	Zebrafish infection model	Lotha et al. (2018)
Gentamicin	Silica-gentamicin nanohybrids (SiO2-G)	Biofilm formation	Holistic ultrastructural deformation of the *E. coli* biofilms in the form of an utter deterioration of cell shapes and apt damage and wrinkling of their cell walls	MRSA and *E. coli*	Zebrafish embryos	Mosselhy et al. (2018)
Cinnamaldehyde (CA)	CA-loaded mesoporous silica-coated gold nanorod (CA@AuMN-HA)	Cell membrane	On-demand release of CA under NIR irradiation with enhanced antibacterial activity	MRSA and drug-resistant *E. coli*	MRSA-infected mice wound model and *C. elegans*	Sun et al. (2018b)
In-house-designed potent USAMP named RBRBR	RBRBR encapsulated chitosan-based nanoparticles (CS-NPs)	Cell membrane and biofilm formation	98% (5-log reduction) biofilm bacterial reduction at 10 mg/mL formulation	MRSA	Mammalian Vero cell line	Almaaytah et al. (2017)

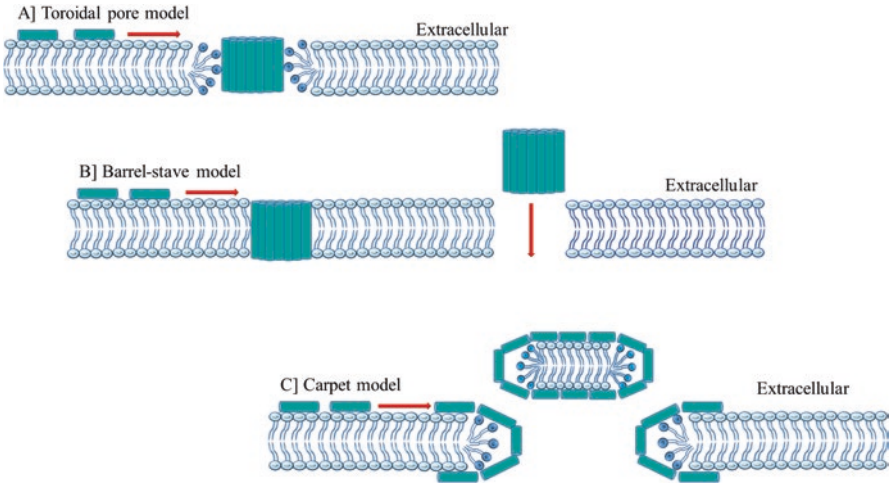

Fig. 3.2 Membrane-targeting mechanism of antimicrobial peptides (AMPs)

key roles in biofilm formation by extruding quorum sensing molecules and quorum quenchers that mediate the formation of biofilm matrix, thus promoting surface adhesion (Ugwuanyi et al. 2021). EPs have been characterized as one of the major drug-resistant determinants. Numerous nanomaterials for delivering antimicrobials to EP target sites have been investigated using in vivo and in vitro models as a potential tool for treating bacterial infection (Prasher et al. 2021). A recent study has reported on the synergistic effects of ciprofloxacin with embelin-loaded chitosan-gold nanoparticles against environmental MDR *P. aeruginosa* and *E. coli* strains by inhibiting EPs by interacting with PA-r (MexA, MexB and OprM) and EC-r (AcrA, AcrB and TolC) active sites (Khare et al. 2021). Further advancements in the microfluidic assembly of pomegranate-like hierarchical microspheres and meropenem-loaded porous silica (MCM-48), for efflux regulation in oral drug delivery against *S. aureus* and *P. aeruginosa*, demonstrated reduced efflux of MER back into the gastrointestinal lumen (Raza et al. 2021). One of the recent innovative strategies includes the application of combinations of different antibiotics on nanomaterials to combat MDR bacteria. Khameneh et al. (2015) investigated the antibacterial activity of co-loaded piperine and gentamicin nanoliposomes in MRSA resulting in EP inhibition with MIC of 32 and 100 μg/mL, respectively. Similarly, liposome-encapsulated phenylalanine-arginine β-naphthylamide (PAβN), an EP inhibitor (EPI), has been proven a cost-effective and worthwhile delivery system against MDR *P. aeruginosa* in lung infections (Ray et al. 2021). However, deeper studies are much further required in this field.

3.5.4 *Quorum Sensing*

The communication mechanism between the bacteria cells with each other that entails the synthesis, detection and autoinducer extracellular signalling molecules is defined as quorum sensing (QS) (Rutherford and Bassler 2012). Molecular mechanisms involving acyl-homoserine lactones, peptide autoinducers and autoinducer 2 are the major QS systems present in bacteria involved in intercellular signalling during human bacterial infections (Irie and Parsek 2008). As a result, there is an increasing demand for viable, non-toxic/anti-QS agents exhibiting dual actin modes addressing both biofilm formation and QS in bacterial infections. In recent years, nanomaterials as antimicrobial agents/drug delivery systems have been reported as an effective tool for QS elimination and treating microbial infections. Bueloni et al. (2020) developed vanadium-nalidixic acid complex (V-NA) nanoencapsulated into myristyl myristate nanostructured lipid carriers (NLCs), and polymeric nanoparticles of Eudragit NE 30D (EuNPs) with enhanced antibacterial and anti-quorum sensing properties against *P. aeruginosa* and *Chromobacterium violaceum* resulted in controlled release of V-NA (30–40% for 3 days) with 59.3 and 129.9 μM MIC values, respectively. Similar results were observed in chitosan-gum acacia gold nanocomposite (CS-GA-AuNC) against MDR *P. aeruginosa* with a greater reduction in Las-R gene expression levels majorly involved as a virulence factor in biofilm formation and QS (Raja Namasivayam et al. 2020). Further in vivo studies on murine macrophage cell line revealed their excellent biocompatibility, an excellent property for drug delivery systems. Recently, the formulations of AMP dendrimers and QSIs (anti-MvfR compounds) for treating burn wound infections caused by *P. aeruginosa* were developed that inhibited the MvfR virulence pathway in the QS system of the bacteria (Jafari et al. 2021). Similar results in tobramycin antibiotic and alkylquinolone quorum sensing inhibitor (QSI)-loaded squalenyl hydrogen sulphate nanoparticles (SqNPs) in in vitro models of pulmonary *P. aeruginosa* infections were observed with improved biofilm penetration and enhanced antimicrobial efficiency (Ho et al. 2020).

3.6 Conclusion and Future Perspectives

Biocompatibility, cost-effectiveness, controlled drug release, deep penetration, target specificity and sustainability properties of nanocarriers make them ideal drug carriers, for delivering wide-ranging antimicrobial agents. However, despite the seemingly large corpus of research and development of a nanomaterial-based delivery system of antimicrobial agents, numerous hurdles need to be overcome before nanomaterial-based approaches for the optimum treatment of drug-resistant bacterial infections may be successfully translated to clinical settings. Silver-oxide and zinc-oxide nanomaterials being approved by the FDA have increased the likelihood of clinical settings among the current leads. Antimicrobial agents such as phytochemicals, AMPs, antibiotics and metallic complexes comprising great

biocompatibility and enhanced antimicrobial activity in conjugation with nanocarriers such as liposomes, nanoparticles, nanocomposites and dendrimers are the emerging promising tools for prolonged and regulated release of drugs/antimicrobial agents against microbial infections. These nanomaterial-based drug delivery systems are proven to be targeting key drug-resistant determinants (cell membrane, EPs, biofilm formation, QS) in pathogenic and threatening bacteria. Nanoliposomes are been already employed in clinical settings for delivering antimicrobials to biofilm-forming bacterial infections. PLGA NPs and GO-NPs have the broadest drug delivery range including AMPs that are found to target biofilms and QS systems. However, deeper research is still required in the field of nanomaterial-based delivery of antimicrobials targeting specific EPs, drug release kinetics, biodegradation, pharmacokinetics and their clearance. For their development, research necessitates multidisciplinary clinical and industrial collaborations for fighting these human microbial infections and making them available from bench to bedside.

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Chapter 4
Nanoparticle Functionalization: Approaches and Applications

Uttara Oak and Tushar Khare

Abstract The field of nanotechnology which presents the nanoparticles (NPs) with the dimensions of just some nanometers and unique physicochemical and optical properties is currently a highly promising field due to the wide range of applications of NPs. However, the applications of the NPs can be found to be even greatly improved if the NPs are accompanied by different molecules (of biological or non-biological origin). The concept of functionalization involves the different approaches aiming the modification of NP surface which help in improving the target specificity along with the reduction in possible nano-toxicity. This attachment of the molecules is achieved by different ways including covalent or non-covalent conjugation as well as via the linker molecules. The functionalization of the NPs can be accomplished by means of attaching various kinds of entities either singly or in combination. These include attachments of drugs, natural products, antibodies, polymers, peptides, fluorescent probes, aptamers, etc. Based on the superior characters of the molecules used for functionalization, the nanomaterial can be used for several applications. One of the most explored areas in this context includes efficient and targeted drug delivery. The functionalized nanomaterials have successfully demonstrated their use in tissue engineering and development of different types of scaffolds. Also, the functionalized NPs can be effectively used for bio-imaging during detection and treatment of different diseases, more prominently in cancer localization. Interestingly the functionalized nanomaterial has also displayed the promising activity against the drug-resistant bacterial pathogens. Moreover, all the efficient properties of these functionalized NPs made them a prime candidate for development of various kinds of biosensors. The present chapter hence describes the functionalization of the NPs and different approaches used to achieve the functionalization.

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The chapter also provides the prominent applications of the functionalized NPs in the field of biological and/or medical sciences.

Keywords Nanoparticles · Functionalization · Covalent/non-covalent conjugation · Linkers · Bioimaging · Tissue engineering · Biosensors

4.1 Introduction

Owing to the nanoscale size, nanoparticles (NPs) show unique properties that are distinct from the bulk material. These innate optical properties, shape, and high surface area to volume ratio allow them to interact with the biological systems at molecular levels that offers a good scope for their applications in the living world (Thiruppathi et al. 2017). However, the NP applications can be limited due to the low target specificity, inability to cross biological hurdles such as blood-brain barrier, and high toxicity resulting from the molecular cascade event triggered on NPs-cell interaction (Carreño-Fuentes et al. 2014; Marano et al. 2016).

Development of functionalized NPs to overcome the abovementioned limitations can be the focus of advancement in the nanosciences and is believed to be a promising technique in widening the applications (Subbiah et al. 2010). NP functionalization refers to surface modification and may impart target specificity, reduction in the toxicity, and anti-agglomeration property to the NPs. Functionalization enables tuning in the properties that one is interested in including in the NPs. This enables the application of NPs in diverse fields: to list a few, field of imaging, drug delivery, therapeutics, tissue engineering, diagnostics, and sensing.

The biomolecules interacting with the NPs possess functional groups on their surface. These entities can bind covalently or non-covalently to the NPs through amine-aldehyde or sulfhydryl interactions (Miyamura et al. 2015; Ma et al. 2019). The non-covalent interactions include ionic, electrostatic, and hydrophobic interactions. The molecules that do not possess either of the functional groups mentioned above or those that cannot interact covalently can still be functionalized through linker molecules (Fig. 4.1). The linker molecules can link the NP and the desired ligand molecule and thereby achieve the expected functionality (Conde et al. 2014). Biotin-avidin is a strong interaction seen in the biological system which has been commonly applied for linking biotinylated NP to ligand attached to avidin group. This interaction is applied by many researchers for effective functionalization (Jin et al. 2020; Bage and Kar 2021). Many other biomolecules that possess functional groups are used as linker molecules. These molecules interact with the ligand, and the NPs or are coated on the NP surface. They may be multifunctional as they serve the linking function along with acting as smart delivery vehicle, enhancing uptake or reducing toxicity or agglomeration (Tam and Lo 2015; Wang et al. 2021). Apart from the biotin-avidin interaction, polyethylene glycol (PEG), chitosan, DNA, and peptides can be used as linker molecules.

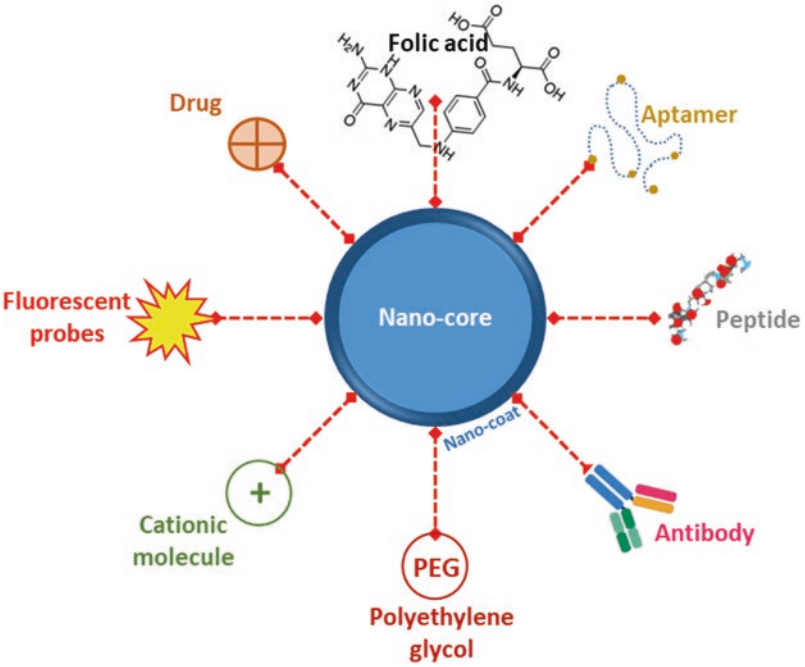

Fig. 4.1 Schematic representation of the functionalized nanoparticles with various targeting ligand agents for specific application

The ligand molecules which connect the functionalized NPs and are eventually involved in the target activity include the DNA and oligonucleotides in gene delivery; peptides, proteins, enzymes, and antibody for metabolic, catalytic, and immunological activity; and drugs for the smart and targeted delivery to the infected or cancer cells (Fig. 4.2).

The current chapter is focused on highlighting the benefits of NP functionalization and understanding the interactions that functionalize the NPs and discusses their applications of functionalized NPs in the varied walks of biomedical sciences.

4.2 Nanoparticle Functionalization

Nanobiotechnology is being successfully used for applications in development of biological assays, study of systems, therapeutics, drug delivery, diagnostics, and sensing. The attributes of the nano-systems such as size, shape, properties, and functionality can be designed and tuned as per the requirements (Nagamune 2017). The biomolecular engineering branch goes hand in hand with the nanotechnology for further enhancing the applications of the nanomaterials. These upcoming biomedical applications of NP are very often based on the binding of the NP probe to

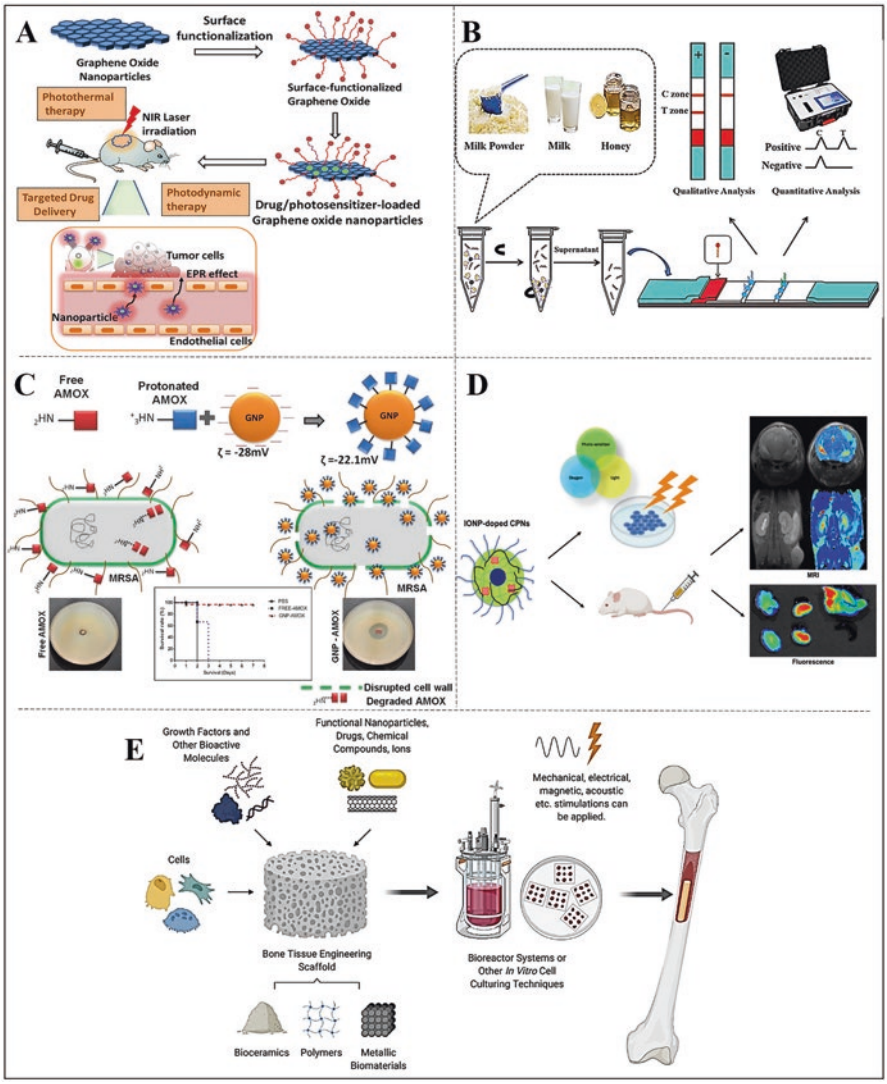

Fig. 4.2 Examples of the application of the functionalized nanomaterials in (**a**) targeted drug delivery. (Reproduced under the terms of creative commons from Sharma and Mondal (2020), Copyright 2020, MDPI), (**b**) biosensing (Reproduced with permission from Ou et al. (2019), Copyright 2019, Elsevier), (**c**) combating drug-resistant microbes. (Reproduced with permission from Kalita et al. (2016), Copyright 2016, Elsevier), (**d**) bioimaging. (Reproduced under the terms of creative commons from Arias-Ramos et al. (2021), Copyright 2021, MDPI), and (**e**) tissue engineering and scaffold generation. (Reproduced with permission from Jodati et al. (2020), Copyright 2020, Elsevier)

a particular substrate. The substrates used are often biomolecules that bind either in covalent/non-covalent interactions or through linker molecules (Atay et al. 2009). Many polymeric NPs are used as drug carriers, or some, for instance, chitosan themselves show antimicrobial properties, and thus improved biocompatibility is attained. On conjugation with polyethylene glycol (PEG), it was reported that silver (Ag) NPs and magnetic NPs showed reduced toxicity in vivo. In addition to this, the NPs did not interfere with normal histology and blood parameters in mice; varied cellular uptake and ROS formation and cell morphology (Yu et al. 2012; Pinzaru et al. 2018). Thus, NP functionalization has proved to widen NP application, bring in reduction in toxicity to the host system where it is applied, and enhance its intracellular uptake and biocompatibility.

4.3 Approaches to Functionalization

NPs show unique optical and plasmonic properties that are altered by the size and composition modifications. Such reorganization of NPs can be made by integration of a NP with another NP or small molecules/polymers which renders them diverse functionalities. Some of the conjugation strategies adopted for NP functionalization are discussed in the subsequent section.

4.3.1 Non-covalent Conjugation

Weak interactions such as adsorption, hydrogen and ionic bonding and affinity, and van der Waal and hydrophobic interaction are involved in non-covalent conjugation (Bohara et al. 2016; Cooper et al. 2017; Saallah and Lenggoro 2018; Sanità et al. 2020). These interactions offer an ease of surface functionalization without change in the interacting NP structure. The non-covalent interactions are reversible and hence can be employed to release the molecules such as drugs or RNAi at a target site (Bao et al. 2013; Conde et al. 2014).

Non-covalent conjugates are easily formed between oppositely charged biological/polymeric molecules and NPs or that between two oppositely charged NPs (Rana et al. 2010; Lu et al. 2021). Proteins adsorb on NPs in an ionic interaction especially rendering them specificity for tumor cells and enhanced cellular uptake and also prevented aggregation of NPs. Various studies reported use of such conjugation strategies. Assembly of amphiphilic molecules such as helices and liposomes are based on the hydrophobic interactions and are self-assembled in the aqueous environments. The hydrophilic core will often hold the molecules to be delivered such as proteins, DNA, or RNA for biomedical applications.

Avidin-biotin is one of the strong non-covalent interactions found in biological systems. It offers ease in functionalization of NPs without losing their biological property and function. This system has been extensively applied in the field of

nanotechnology. The tobacco etch virus protease and human rhinovirus protease were immobilized on iron oxide NPs. The proteases were fused to streptavidin and then added to biotinylated iron oxide NPs and effectively used as a tool for protein purification (Norris et al. 2020). Hauser et al. (2010) and Huang et al. (2015) have reported use of this system for nucleic acids, which was instrumental for successful delivery and uptake by the target cells.

Kim et al. (2011) studied mesoporous silica NP adsorbed with DNA molecules for treatment of tumors. This non-covalent interaction of the NP with a biomolecule was found to be successful in gene delivery for the treatment. In a similar study, proteins were attached to aldehyde-functionalized mesoporous silica NP as a therapy for cancer (Tian et al. 2017).

In different studies, Fan et al. (2016), Liu et al. (2016), and Masoudipour et al. (2017) reported graphene oxide/reduced graphene oxide functionalized non-covalently with phosphorylcholine oligomer-grafted perylene, adipic acid dihydrazide (that introduced an amide group), and N-hydroxysuccinimide (introduction of imide group), respectively. These NPs were used as effective delivery system for anticancer drugs.

4.3.2 Covalent Conjugation

The required bifunctionality of diagnostics, delivery, and therapy can be rendered to NPs when they are linked to one of the following molecules such as nucleic acids, oligonucleotides, peptides, enzymes, antibodies and other proteins, fluorescent dyes, polymers, drugs, and tumor markers. These ligand molecules are often coupled with NPs using covalent linkages directly to NP surface or through a surface-bound linker molecule (Conde et al. 2014; Ye et al. 2018). As compared to the non-covalent linkages, the covalent bonds are strong and stable.

The most common covalent interaction is between amines and the aldehydes, epoxides, or anhydrides. Most proteins contain the amine groups on their surface and can be directly conjugated to NPs. Some NPs may also possess amine groups and coupled with molecules containing aldehydes or epoxides (Miyamura et al. 2015; Esmi et al. 2021). Esmi et al. (2021) and Ashjari et al. (2020) used magnetic NPs functionalized with amine/aldehyde group for immobilization of lipases that were applied for biodiesel production by transesterification of olive oil and waste cooking oil, respectively.

Protein-based subunit nano-vaccines are reported (Lu et al. 2021) wherein different covalent associations of antigens and NPs are discussed. The bioconjugation strategies involved antigen linkage to either inner/outer surface or both and with or without adjuvant. The major chemical reactions that fall in the categories will be discussed in the subsequent sections of this chapter, and they are EDC coupling reactions, Maleimide coupling reactions, and click chemistry reactions (Conde et al. 2014; Bohara et al. 2016; Nevagi et al. 2019).

4.3.3 Use of Linker Molecules

Certain biomolecules may not possess functional groups required for their covalent/non-covalent interactions with NPs. Some oligonucleotides, antibodies, carbohydrates, and some peptides do not include functional groups and need to modify before bioconjugation which may compromise their bioactivity. Use of linker molecules for conjugation of such molecules renders functional groups on molecular surface and thus functionalizing them.

EDC coupling is one such reaction where 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) is used as a crosslinking agent to couple carboxyl or phosphate groups to primary amines. The amine then reacts with the carboxyl group of a biomolecule, forming an amine-reactive O-acylisourea intermediate (Conde et al. 2014; Bohara et al. 2016). Biomolecules functionalized using EDC coupling reactions were found to be effective as they showed reduced toxicity and enhanced functions for use as agent in biomimetics (Bartczak and Kanaras 2011; Keleştemur et al. 2017; Zhang et al. 2017).

Biomolecules that possess reactive thiol or amine groups can be conjugated through maleimide coupling reaction. Maleimide reacts with free sulfhydryl groups, forming stable thioether linkages, at physiological pH. It is useful for bioconjugation of proteins with −SH groups and the coupling of two thiols to form a disulfide linkage. Lee et al. (2020) and Zhu et al. (2012) have demonstrated cellular uptake and other bioactivities of functionalized gold (Au) NPs, whereas Nieves et al. (2014) used monovalent maleimide reaction for coupling fibro-blast growth factor 2 and an oligosaccharide to Au NPs.

Click chemistry is a group of reactions that are fast, simple to use, and easy to purify. These reactions are highly regiospecific and give high product yields. The copper(I)-catalyzed azide-alkyne cycloaddition (CuAAC) is one such click reaction that has been employed in biofunctionalization of NPs. Azide and alkynes are almost inert to the biological entities and also to the intracellular conditions and hence are easy to introduce into organic molecules. Thus, this reaction has been used for coupling Au NPs and quantum dots to biomolecules such as proteins, enzymes, and polymers. These reactions were employed for bioconjugation of Au NPs for targeted intracellular delivery (Yi et al. 2018; Van Der Meer et al. 2019).

Variety of polymeric compounds and biomolecules have been employed as linker molecules. Polyethylene glycol (PEG) renders hydrophilicity to NPs and allows them to escape the immune system. However, the PEG layer is lost on binding of target ligand to the NPs. As reported by Badkas et al. (2018), a PEG NP conjugated to a commercial antibody (herceptin) to HER-2-positive breast cancer cells lost the immune evasion property on binding of the antibody to the cells. This evasion was restored when an additional PEG linker molecule was attached to the antibody, thus restoring the biofunctionality.

DNA-linked NPs have found applications in diverse field as the NPs are rendered functionality. Hayes et al. (2018) constructed a novel 3D lattice structure with application in colloidal crystallization by linking two protein molecules by interprotein

DNA bond. In another study, highly stable Au NPs were developed that were synthesized using DNA linkers, and the structure was applied in development of assays as an enhancing agent (Hinman et al. 2017).

4.4 Hybrid Nanoparticles

4.4.1 Metal Organic Framework

The metal organic frameworks (MOFs) are nanoporous materials with metal centers linked to various organic linkers to form three-dimensional porous structures with varied pore volumes, internal and external surface areas, and properties rendering them functionality as building blocks for colloidal crystal engineering, biological probes, sensors, membrane separation materials, catalysis, and drug delivery systems (Horcajada et al. 2010; Bae et al. 2010; Li et al. 2016). The MOFs are functionalized post-synthesis wherein the preexisting amine, carboxylic acids, and azide groups react with the carbonyl, amine, and alkyne groups, respectively, possessed by the ligand molecules. For such functionalization covalent conjugation methodologies (discussed in above section) are employed.

Horcajada et al. (2010) have reported use of such MOF as successful drug carriers and imaging agents for anticancer drugs. Several other reports have reviewed applications of MOF for anticancer drug delivery (Cai et al. 2015; Liu et al. 2019; Ding et al. 2020) and others as antibacterial agents that could reverse antimicrobial resistance to drugs (Arenas-Vivo et al. 2019; Liu et al. 2021). Zn-MOF (Restrepo et al. 2017) and Ag-MOF (Arenas-Vivo et al. 2019; Yang et al. 2020) have shown promising antibacterial effects.

4.4.2 Mesoporous Silica Nanoparticles

Mesoporous silica NPs (MSNs) display uniform particle size and good dispersity enabling them to use in variety of functions such as catalysis, drug delivery, optical devices, and bioimaging (Wu and Lin 2013). Juneja et al. (2020) reported conjugation of MSN to nucleic acid NPs wherein the two species had strong electrostatic attraction. The complex proved to be immunostimulatory in nature and offered a novel approach to treat diseases including cancer by targeting specific metabolic pathways. Doxorubicin was delivered to gonadotropin-releasing hormone (GnRH) – overexpressing cancer cells using MSNs functionalized with PEG and GnRH receptor decapeptide. The functionalized delivery showed better uptake of the drug and increased cytotoxicity to the target cancer cells as compared to the bare MSNs as delivery vehicle (Tambe et al. 2018).

4.4.3 Conjugation of NPs with Organic Molecules

Organic-inorganic hybrid NPs show improved properties due to the structural diversity and enhanced activity due to synergism between the two components in the system. Several magnetic NPs are reported to be conjugated with polymer molecules that proved to be better candidates for drug delivery.

In different studies, Biswas et al. (2014) and Chatterjee et al. (2005) reported capping of NiCu NPs with PEG and applied for use in hyperthermia and drug delivery. Au NPs functionalized with PEG proved to protect drugs from degradation, leakage in the physiological environment, and its clearance by the immune cells (Suk et al. 2016; Li et al. 2019).

Another such polymer, chitosan, is widely applied in bioconjugation. Silvestri et al. (2018) functionalized Au NPs with poly hydroxybutyrate-chitosan polymer conjugate which enabled the NP to circumvent the tissue toxicity while maintaining the catalytic function. Chitosan-carboxymethyl-5-fluorouracil-folate conjugate was synthesized and along with microwave treatment facilitated skin drug retention and intracellular drug delivery (Nawaz and Wong 2018). In another study folic acid-conjugated temozolomide-loaded chitosan NPs showed increased affinity for the target lung cancer cells both in vitro and in vivo (Li et al. 2017a).

The Au NPs were employed to detect infectious disease or biomarkers in body fluids with limited sensitivity. Kang et al. (2019) developed a single-stranded DNA binding protein (RPA70A) and enabled signal intensity enhancement, thereby improving sensitivity. Curcumin and resveratrol are polyphenols with strong antioxidant properties and therefore have nutraceutical implications. A protein-phenolic conjugate used for co-delivery also showed increased stability and bio-accessibility (Liu et al. 2018a).

Antimicrobial peptides show broad spectrum antibacterial activity. However, they have low penetration ability in the cells and are instable. Lee et al. (2017) demonstrated that an antimicrobial peptide, HPA3PH when combined to Au NPs-DNA aptamer (AuNP-Apt), the conjugate (AuNP-Apt-HPA3PHis) is an effective therapeutic tool against drug resistant *Vibrio vulnificus*. The *V. vulnificus*-infected HeLa cells showed 90% reduction in the microbial load and complete elimination of the bacteria in vivo in mice when treated with the AMP conjugated with AuNP-Apt. In addition, this treatment showed enhanced intracellular uptake with reduction in toxicity proving it to be a successful mode of treatment to combat antimicrobial resistance. In the other studies, NP-DNA conjugates have been employed in photodymanic therapy, bioimaging, and as chemical and biological sensors (Jia et al. 2018; Liu et al. 2018d).

Like the biomolecules discussed above, lipids are also an important class of biomolecules/NPs that are employed as delivery vehicles, for instance, the liposomes that are instrumental in protecting and delivering the mRNA to cells in the currently used COVID-19 mRNA vaccines (Tenchov et al. 2021). Lipid nanocarriers that are complex in design but have robust stability can encapsulate and deliver therapeutic agents to specific target, thus proving to be a helpful platform for treatment. These

lipid NPs include solid lipid NPs, cationic lipid-nucleic acid complexes, and nanostructured lipid carriers (Du et al. 2018; Alavi and Hamidi 2019; Tenchov et al. 2021). In different studies, solid lipid NPs are successfully reported as anti-oral cancer drug carriers (Ding et al. 2018; Nasirizadeh and Malaekeh-Nikouei 2020).

4.5 Applications of the Functionalized Nanomaterials

The functionalization of the NPs with various compounds allowed the use of nanomaterials in miscellaneous areas, where properties of both the NPs and the linked compound can be fully explored to achieve the desired goals (Table 4.1). Following are the broad areas where functionalized nanomaterials have displayed their great potential.

4.5.1 Drug Delivery

Owing to the superior ability of functionalized nanomaterials to mimic the actual drugs along with the target-specific (tailored) features, the functionalized nanomaterial is considered as one of the promising platforms for the efficient drug delivery. The functionalized nanomaterials, especially with the biological entities, can be applied as the long-circulating drug carriers, carriers in photodynamic therapies, sustained drug delivery applications, as well as targeted drug delivery (Bose et al. 2016). Various nanomaterials have been explored for this task, which includes drug molecules, core material (metallic/nonmetallic) in nano-form, and/or some prominent polymers such as albumin, chitosan, gelatin, PEG, ovomucin, etc. (Sur et al. 2019). The drug delivery applications of these nanomaterials are diverse, as such nanomaterials have been successfully explored for the treatments of various kinds of diseases. For instance, the camptothecin-loaded graphene oxide NPs loaded with PEG and folic acid (GO-PEG-FA-CPT) were successfully demonstrated for the anticancer drug delivery. This developed system displayed pH-dependent release of the drug, and the enhanced anticancer activity of this developed material was demonstrated against the MCF-7 breast cancer cell lines (Deb and Vimala 2018). The alginate-chitosan NP system was used for encapsulation of the anticancerous drug amygdalin. The amygdalin-loaded alginate-chitosan NPs displayed greater yet sustainable inhibitory effects on the H1299 cell lines in dose-dependent manner (Sohail and Abbas 2020). The starch-encapsulated copper oxide NPs (synthesized using *Helianthus tuberosus* extracts) functionalized with folic acid (FA-ST-HtCuONPs) facilitated and targeted release in MDA-MB-231 cells. This anti-breast cancer potential was linked to the generation of reactive oxygen species (ROS), damage to the nucleus, reduced mitochondrial membrane potential, and activation of apoptosis pathways (Mariadoss et al. 2020). Apart from the cancer-related drug delivery, the functionalized NPs have also displayed the potential to facilitate the drug transit

Table 4.1 Summary of the applications of the functionalized nanoparticles

Application	Functionalized nanomaterial system	Description	References
Drug delivery	Camptothecin-loaded GO NPs loaded with PEG and FA	Displayed pH-dependent release of drug and enhanced anticancer activity against the MCF-7 breast cancer cell lines	Deb and Vimala (2018)
	Amygdalin-loaded alginate-chitosan NPs	Dose-dependent and sustainable inhibitory effect on H1299 cell lines	Sohail and Abbas (2020)
	Starch-encapsulated CO NPs functionalized with FA	Facilitated and targeted release of drug in MDA-MB-231 cells with ROS generation, nuclear damage, reduced mitochondrial membrane potential, and activation of apoptosis pathways	Mariadoss et al. (2020)
	Apolipoprotein E-functionalized polymeric NPs	Transport of drugs (doxorubicin, loperamide, dalargin) and nerve growth factor across the blood-brain barrier via LDL receptor-mediated transcytosis	Hartl et al. (2021)
	Cpl-1-loaded chitosan NPs	Biocompatible system for efficient delivery of Cpl-1 to improve treatment efficiency against *S. pneumoniae*	Gondil et al. (2020)

(continued)

Table 4.1 (continued)

Application	Functionalized nanomaterial system	Description	References
Tissue engineering	Chitosan/biphasic calcium phosphate scaffolds functionalized with Arg-Gly-Asp and BMP-2-loaded NPs	Developed scaffold was equivalent to the bone extracellular matrix and proved to be providing an optimal micro-environment for bone formation	Gan et al. (2018)
	Nanotopographical polyurethane-based bioactive scaffolds harboring uniformly dispersed functionalized multi-walled CNTs and ZnO NPs	The scaffold was able to stimulate the nucleation of the calcium phosphate and displayed great potential in promoting osteogenic differentiation of pre-osteoblasts	Shrestha et al. (2017)
	Randomly oriented and aligned electro-active (fibrous) scaffolds of poly-L-lactic acid with ferroelectric ceramic NPs	Scaffolds stimulated the early osteogenic differentiation and polygonal spreading in mesenchymal stem cells and promoted the cellular elongation to enhance osteogenic differentiation	Li et al. (2017c)
	Peptide-functionalized system for the delivery of the β-catenin agonist, a GSK-3β inhibitor	Displayed significant uptake of the regenerative mesenchymal stem cells and osteoblasts at the fractured bone, with improved formation of the bones and micro-architecture, higher torsional rigidity, volume of regenerated bone	Wang et al. (2017)
	Spermine-acetalated dextran-based NPs functionalized with the PEG and atrial natriuretic peptide	The pH-triggered transport of small molecules, CHIR99021 and SB431542, which improved the reprogramming efficacy of the fibroblasts to the cardiomyocytes	Ferreira et al. (2018)
	Magnetic NPs functionalized with chitosan	A promising system to treat peripheral nerve injuries	Pop et al. (2021)

(continued)

Table 4.1 (continued)

Application	Functionalized nanomaterial system	Description	References
Antimicrobial resistance	Au NPs functionalized with the amoxicillin	System helped in subversion of antibiotic resistance via targeting the high levels of β lactamase in methicillin-resistant *Staphylococcus aureus* (MRSA)	Kalita et al. (2016)
	Au NPs functionalized with carbapenems (meropenem and imipenem)	Nanoparticles reduced the carbapenem resistance in drug-resistant isolates of *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Acinetobacter baumannii*	Shaker and Shaaban (2017)
	DNase-I-functionalized chitosan NPs loaded with ciprofloxacin	Significant inhibitory activities, biofilm prevention, and biofilm dispersal abilities which proved effective against the *Pseudomonas aeruginosa* possessing the biofilm-mediated resistance	Patel et al. (2020)
	Mannose-functionalized chitosan NP	Improved activity against the resistant biofilms in resistant *P. aeruginosa* and *S. aureus*	Ejaz et al. (2020)
	ZnO NPs conjugated to TS and functionalized with glutamic acid and ciprofloxacin	Decreased efflux pump activities with noticeable reduction in the expression levels of the genes coding for the efflux pumps (namely, norA, norB, and tet38) of resistant *S. aureus*	Nejabatdoust et al. (2019)
	Embelin-loaded chitosan Au NP	Inhibition of the efflux pump activities in drug-resistant environmental isolates of *E. coli* and *P. aeruginosa*	Khare et al. (2021)

(continued)

Table 4.1 (continued)

Application	Functionalized nanomaterial system	Description	References
Molecular imaging	Hyaluronic acid-functionalized bismuth oxide NPs	System with improved biocompatibility, water solubility, and uptake by the CD44 receptors, which proved to be favorable for active-targeting CT imaging with enhanced radio-sensitivity for tumor inhibition	Du et al. (2017)
	Peptide-functionalized drug-loaded phase transformation NPs	Nanoconjugates with ability to pass through the vascular endothelial gaps of tumor cells which helped in the low-intensity focused ultrasound imaging of the tumor and precise therapy	Zhu et al. (2018)
	FA-functionalized polyethylenimine superparamagnetic iron oxide NPs	A delivery system for the PDL1 siRNA which act as contrast agent (T_2-weighted) to enhance the cellular magnetic resonance imaging during cancer treatment	Luo et al. (2017)
	Rare earth NPs coated with carbon shells and Au NPs functionalized with peptide	Enhance the red up-conversion luminescence and near-infrared II imaging intensity for diagnosis and therapy of tongue squamous cell carcinoma	Lin et al. (2021)
	Tryptophan-functionalized Ag NPs	The NPs interacted with the *E. coli* cell membrane via tryptophan insertion in lipid bilayer which was trackable after the synchrotron excitation deep ultraviolet (DUV) fluorescence imaging	Dojčilović et al. (2016)
Biosensors	Luminol-functionalized Ag NP-decorated GO	The visual electrochemiluminescence biosensing of the aflatoxin M1	Khoshfetrat et al. (2018)
	Kanamycin-specific aptamer-modified Au NPs (probe) and oligonucleotide DNA1-functionalized Ag NPs (signal amplification)	Lateral flow strips biosensor for rapid, accurate, cost-effective, and on-site recognition of kanamycin in various food samples	Liu et al. (2018c)
	Anti-*E. coli* O157:H7-functionalized CNTs	Ability to detect the bacteria with the concentration as low as 1 CFU mL^{-1}	Li et al. (2017b)

NP nanoparticles, *GO* graphene oxide, *PEG* polyethylene glycol, *FA* folic acid, *CuO* copper oxide, *ROS* reactive oxygen species, *LDL* low-density lipoprotein, *Cpl-1* endolysin from Cp-1 phage, *BMP-2* bone morphogenetic protein-2, *CNT* carbon nanotubes, *ZnO* zinc oxide, *GSK-3β* glycogen synthase kinase 3 beta, *Au* gold, *TSC* thiosemicarbazide, *PDL1* programmed death ligand-1

through blood-brain barrier. The apolipoprotein E-functionalized polymeric NPs are able to transport drugs such as doxorubicin, loperamide, and dalargin, and nerve growth factor across the blood-brain barrier via low density lipoprotein (LDL) receptor-mediated transcytosis (Hartl et al. 2021). Similarly, the Cpl-1 (an endolysin from Cp-1 phage, effective against pneumococcal infections)-loaded chitosan NPs were found to be a promising biocompatible system for efficient delivery of Cpl-1 to improve the efficiency of the treatment for *S. pneumoniae* infection (Gondil et al. 2020).

4.5.2 Tissue Engineering

The field of tissue engineering and regenerative medicine research aims to develope the approaches for stimulation of natural regeneration and/or repair of the tissue. The fundamental components of these approaches are cells, scaffolds of the biomaterial, and signalling molecules. A key to the successful tissue regeneration is fine-tuning of the growth factor concentrations at the biomaterial scaffolds (Monteiro et al. 2015). In this context, the potential of the nanomaterials for efficient delivery of the growth factors (controlled release of the growth factors) has been explored. Tissue engineering strategies were developed in recent years with combination of the NPs functionalized with the bioactive agents and scaffolds (which can be used as reservoir of the bioactive agents as well) (Quaglia 2008; Kulkarni et al. 2010). For instance, Gan et al. (2018) developed chitosan/biphasic calcium phosphate scaffolds which were functionalized with Arg-Gly-Asp and bone morphogenetic protein-2 (BMP-2, a protein with high osteo-inductive activity)-loaded NPs. In terms of the structure and the composition, the developed scaffold was equivalent to the bone extracellular matrix, and the developed scaffold was proved to be providing an optimal micro-environment for bone formation (Gan et al. 2018). Nanotopographical polyurethane-based bioactive scaffolds were prepared by Shrestha and group (Shrestha et al. 2017) which harbored uniformly dispersed functionalized multi-walled carbon nanotubes and zinc oxide NPs. The scaffold displayed good antibacterial potential, was able to stimulate the nucleation of the calcium phosphate, and displayed great potential in promoting osteogenic differentiation of pre-osteoblasts (Shrestha et al. 2017). The randomly oriented and aligned electro-active (fibrous) scaffolds of poly-L-lactic acid with ferroelectric ceramic NPs were fabricated and investigated for the osteogenic response in mesenchymal stem cells from bone marrow (Li et al. 2017c). Scaffolds with random orientation markedly stimulated the early osteogenic differentiation and polygonal spreading in mesenchymal stem cells, whereas the aligned scaffolds promoted the cellular elongation, proving the potential of this system in enhancing osteogenic differentiation (Li et al. 2017c). Bone targeting system for the delivery of the β-catenin agonist, 3-amino-6-(4-((4-methylpiperazin-1-yl)sulfonyl)phenyl)-N-(pyridin-3-yl)pyrazine-2-carboxamide, a glycogen synthase kinase 3 beta (GSK-3β) inhibitor aiming for fracture healing was developed by Wang et al. (2017). This peptide-functionalized system displayed

significant uptake of the regenerative mesenchymal stem cells and osteoblasts at the fractured bone. The improved formation of the bones and micro-architecture was observed in the mice after being treated with the developed nano-system with higher torsional rigidity and volume of regenerated bone referring to the expedite fracture healing (Wang et al. 2017). Apart from the bone regeneration/healing-specific application, the functionalization of the nanomaterials was also attempted to treat other health conditions. For instance, the spermine-acetalated dextran-based functional NPs were formulated by Ferreira et al. (2018) for pH-triggered transport of small molecules, CHIR99021 and SB431542, to improve the reprogramming efficacy of the fibroblasts to the cardiomyocytes. To improve the biocompatibility, the system was functionalized with the PEG and atrial natriuretic peptide. The results of the study highlighted the potential of this nano-functionalized system in the cardiac regeneration therapy (Ferreira et al. 2018). The magnetic NPs functionalized with chitosan were successfully demonstrated as promising candidates to treat peripheral nerve injuries using rat model system (Pop et al. 2021).

4.5.3 Antimicrobial Resistance

Antimicrobial resistance refers to the development of the resilience in the bacterial pathogens due to the over-application of the antimicrobial agents (mainly antibiotics), which is proving to be a major hurdle in the drug discovery process and eventually making the treatments difficult to cure and costly (Shriram et al. 2018; Yu et al. 2020). The progression, persistence, and spread of the antimicrobial resistance are usually correlated to the injudicious antibiotic usage in health(care) products, animal husbandry, agricultural applications, food industries, and related sectors (Venter et al. 2017; Buckner et al. 2018; Tiwari et al. 2021). The nanotechnology has been used to address this problem where the NPs are being developed to specifically target the molecular determinants of the antimicrobial resistance. The biocompatible Au NPs were synthesized using the extracts prepared from the aerial parts of pteridophyte *Adiantum philippense* and were surface functionalized with the amoxicillin (broad-spectrum β lactam antibiotic) by Kalita et al. (2016). The amoxicillin-functionalized NPs were proved to subvert the antibiotic resistance by tackling the effects of high levels of β lactamase (enzymes responsible to deactivate the β lactam antibiotics) in methicillin-resistant *Staphylococcus aureus* (MRSA) (Kalita et al. 2016). Another study revealed the successful surface conjugation of two carbapenems (meropenem and imipenem) with Au NPs, which were responsible for destroying the carbapenem resistance in drug-resistant isolates of *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Acinetobacter baumannii* (Shaker and Shaaban 2017). A study by Patel et al. (2020) demonstrated the successful fabrication of the DNase-I-functionalized chitosan NPs loaded with ciprofloxacin. This nano-conjugate displayed significant inhibitory activities, biofilm prevention, and excellent biofilm dispersal abilities. This system proved to be effective against the *Pseudomonas aeruginosa* pathogens possessing the biofilm-mediated resistance

(Patel et al. 2020). Similarly, mannose-functionalized chitosan NP system was developed, which displayed improved activity against the resistant biofilms in resistant pathogens including *P. aeruginosa* and *S. aureus* (Ejaz et al. 2020). The zinc oxide NPs conjugated to thiosemicarbazide (TSC) and functionalized with glutamic acid (ZnO@Glu-TSC) as well as ciprofloxacin (CIP) were synthesized by Nejabatdoust et al. (2019) to target the efflux pumps (a major mechanism in the resistant pathogens responsible for the expulsion of the drug molecules out of the cells) of the multi-drug-resistant *S. aureus*. The results of the study suggested the reduced efflux pump activities in the pathogen with marked reduction in the expression levels of the genes coding for the efflux pumps (namely, norA, norB, and tet38) (Nejabatdoust et al. 2019). On the similar line, the potential of the embelin was explored using the embelin-loaded chitosan Au NP system, which displayed inhibition of the efflux pump activities in drug-resistant environmental isolates of *E. coli* and *P. aeruginosa* (Khare et al. 2021).

4.5.4 Molecular Imaging

The functionalization of the NPs also aids a specific imaging modality approach. This helps not only in getting best possible quality image but also improves applicability and functionality of imaging modality. This functionalization may include the incorporation of dyes or other compounds with fluorescent properties or other/more than one nanomaterial as well (Thiruppathi et al. 2017). Aiming for the accurate localization of the tumors, the hyaluronic acid-functionalized bismuth oxide NPs were prepared by Du et al. (2017). The functionalization process improved the biocompatibility, water solubility, and the uptake by the CD44 receptors of the cancerous cells. These properties were further proved to be favorable for active-targeting CT imaging with enhanced radio-sensitivity for tumor inhibition (Du et al. 2017). The study by Zhu et al. (2018) presented the novel tumor penetration peptide-functionalized drug-loaded phase transformation NPs with ability to pass through the vascular endothelial gaps of tumor cells. The application of this system helped in the low-intensity focused ultrasound imaging of the tumor and precise therapy (Zhu et al. 2018). The folic acid-functionalized polyethylenimine superparamagnetic iron oxide NPs were reported by Luo et al. (2017) to improve the treatment course of gastric cancer. The system works as delivery agent for the programmed death ligand-1 (PD-L1) siRNA. The study showed that the developed system can act as contrast agent (T_2-weighted) which enhances the cellular magnetic resonance imaging during cancer treatment (Luo et al. 2017). The rare earth NPs functionalized with peptide were designed for the dual mode optical imaging of the tongue squamous cell carcinoma (Lin et al. 2021). Rare earth NPs coated with carbon shells and Au NPs after functionalized with peptide proved to enhance the red upconversion luminescence and near-infrared II imaging intensity, demonstrating its potential in diagnosis and therapy of tongue squamous cell carcinoma (Lin et al. 2021). The NP-based imaging has been used for detection/tracking of bacterial pathogens

as well. For example, the human serum albumin-protected Au nanoclusters can be used as fluorescent probes due to their photoluminescent properties for detection of the MRSA (Chan and Chen 2012). In an investigation by Dojčilović et al. (2016), the amino acid tryptophan was used as an environmentally sensitive fluorescent probe for functionalization of Ag NPs. The study revealed that the NPs interact with the *E. coli* cell membrane through insertion of tryptophan into lipid bilayer and enter the cells. This was trackable after the synchrotron excitation deep ultraviolet (DUV) fluorescence imaging (Dojčilović et al. 2016).

4.5.5 Biosensors

The nanomaterials divulge diverse and astonishing properties which make them very appealing as well as reasonable candidates to be exploited as a part of electrochemistry as well as development and/or improvement of the biosensors. Nanomaterials based on the carbon or Au are broadly explored for biosensor development due to their favorable properties (detection, distinguishing, and imaging) (Alim et al. 2018). Such biosensors can be used to sense very diverse types of analytes including toxins, amino acids, proteins, nucleic acids, pollutants, antigens, drugs, as well as bacteria and viruses (Alim et al. 2018). To name few examples, the luminol-functionalized Ag NP-decorated graphene oxide was reported for the visual electrochemiluminescence biosensing of the aflatoxin M1. The developed aptasensor demonstrated satisfactory performance when used for real samples and displayed promising results during smartphone-based detection of aflatoxin M1 (Khoshfetrat et al. 2018). A strip biosensor for rapid, accurate, cost-effective, and on-site recognition of kanamycin was developed by Liu et al. (2018c) using kanamycin-specific aptamer-modified Au NPs as a probe and oligonucleotide DNA1-functionalized Ag NPs for signal amplification. These lateral flow strips were found to have very high stability and specificity and were able to detect the kanamycin in various food samples (Liu et al. 2018c). The anti-*E. coli* O157:H7-functionalized carbon nanotube-based multilayer biosensor was successfully designed by Li et al. (2017b). This biosensor was able to demonstrate the capability of the system to detect the bacteria with the concentration as low as 1 CFU mL^{-1} (Li et al. 2017b).

With respect to the progressions in the field of nanotechnology, now we can observe pairing of a variety of compounds with various kinds on nanomaterials, which also enabled the application of such nanomaterials in numerous fields. These diverse applications include applications in energy devices including solar energy conversion (photovoltaic and photoelectrochemical devices, artificial photosynthesis), electrochemical energy (lithium batteries, supercapacitors, fuel cells), as well as environmental applications which may include detection and removal of pollutants (heavy metals), detection and removal of organic substances, sensing of gases as well as organic/inorganic vapors, as well as sensing of pathogens in the environment (reviewed by Chang and Wu 2013).

4.6 Conclusion

The progression in the field of nanotechnology has enabled the extensive and efficient use of the NPs in diverse areas. The efficacy of the NPs' application is even more enriched via various functionalization-based approaches. The diversity of the molecular entities which can be used for functionalization of the NPs is vast; therefore, the successive functionalized nanomaterials gain enormous applicability. The use of functionalized NPs has evidenced as an effective approach for proficient and targeted drug delivery, tissue engineering, combating the resistant pathogens, and bioimaging and as a biosensor. The functionalization approach holds a great potential in diagnosis as well as treatments of various health conditions as well as other detection-oriented applications. This potential still can be explored further which may enable even more applicability of the different kinds of nanomaterials.

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Chapter 5
Nano-adjuvants as Effective Next-Generation Antimicrobial Agents

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Abstract Adaptive immunity is a feature peculiar to vertebrates which is responsible for defence against invading pathogens. It is a system that recognizes pathogenic antigens from a previous encounter and builds a robust immune response that neutralizes the pathogen. Vaccines are a perfect application of exploiting our adaptive immunity where pathogenic antigens are introduced into our body resulting in the generation of long-lived immune cells known as memory cells. These cells recognize pathogenic signatures upon infection and initiate the adaptive immune response. An essential component of a vaccine is an adjuvant which enhances the efficiency of an antigen to produce a robust immune response. The exact mechanisms behind adjuvant enhancement of immune response are not fully understood; thus, they have been referred to as 'the immunologists' dirty little secret' – Janeway. Recent studies have shown that nano-adjuvants are very efficient immune regulators and double Toll-like receptor (TLR) stimulation might be the reason for their effi-

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cacy. Adjuvant development is emerging as an important trend in vaccine delivery, since a wide range of antigens that are not immunogenic need to be enhanced. This chapter aims to highlight the potential role of nanoparticles as nano-adjuvants and the recent developments made in this evolving field.

Keywords Adjuvant · Vaccine · Nanoparticle

5.1 Introduction

The development of vaccines has become increasingly important due to the emergence of deadly pathogens that are difficult to control. Microbial diseases cause millions of deaths each year. In recent years, antimicrobial resistance has become a major health concern, and many studies have been conducted to improve antimicrobial methods. According to evidence, more than 70% of bacteria responsible for poisoning and infection are resistant to one or more antimicrobial agents that are normally used to eradicate infection and treat poisoning. There appears to be a great need for new and effective antimicrobial agents. Currently, we need improved vaccines, for example, subunit vaccines, to boost the currently available vaccine or to serve as stand-alone treatments for immunocompromised patients. Subunit vaccines can be effectively used with adjuvants to enhance the duration and magnitude of adaptive immunity. Subunit vaccines are generally easier to administer than whole pathogen vaccines, but are generally less immunogenic than whole pathogen vaccines. Several alternative approaches have been proposed to overcome these issues, including the use of adjuvants to stimulate the immune system when adding adjuvants to vaccine formulations. Adjuvants are substances, compounds or even strategies that enhance or modulate a humoral or cellular response to an antigen (Cox and Coulter 1997; McGeary et al. 2003; Sarkar et al. 2019). The adjuvants in vaccines are used since almost a century as components of inactivated antimicrobial vaccines. Le Moinic first reported the immune-boosting effects of a Vaseline-water emulsion in 1916 when vaccinating mice with inactivated *Salmonella typhimurium* (Le Moinic 1916). Vaccine adjuvants based on aluminium and emulsion are still commonly used by themselves as well as in complex combinations. The adjuvant properties of aluminium-based formulations were discovered by Glenny and colleagues in 1926 (Glenny 1926). Trial and error have often been used to develop and create vaccines and adjuvants, and for many existing vaccines, it is unclear exactly how immunity is induced. Some consider adjuvant research to be a form of alchemy, despite increasing knowledge of immune system function.

In general, adjuvants are classified according to their chemical properties, origin or physical-chemical properties; however, they often consist of related compounds (Hazen 2000). Normally adjuvants are classified based on their ability to stimulate Th1 (T-helper-1) or Th2 (T-helper-2) immunity. Their chemical composition can also be used to subclassify them. PAMPs (pathogen-associated molecular patterns), CpG-DNA and LPS (lipopolysaccharides) were among the adjuvants associated

with innate immunity, which bind to Toll-like receptors (TLRs) and pattern recognition receptors (PRRs) and trigger the activation of nuclear factor-kB which further activates the production of inflammatory cytokines and B7 ligand production (Marciani 2003), Press et al. 2000). A variety of natural and synthetic adjuvants were used to promote adaptive immunity, including cytokines, quillaja saponins and tucaresol (Cibulski et al. 2021; Rivera-Patron et al. 2021; Belz et al. 2020). Co-stimulatory signals stimulate T cells through these adjuvants, activating them to instigate cytokine-regulated gene expression (Bendelac and Medzhitov 2002). An adjuvant's main characteristics are its potency in stimulating the immune system (Fig. 5.1), thereby reducing the number of antigens and the number of booster vaccinations needed to produce long-lasting immunity, but it also has to be stable and safe.

The mechanisms governing how adjuvants work in vaccines are not fully understood, however. Researchers are slowly unravelling the mystery of adjuvant activity based on studies from the past decade (Schijns and Lavelle 2011). A combination of mechanisms can contribute to the action of adjuvants, such as formation of the

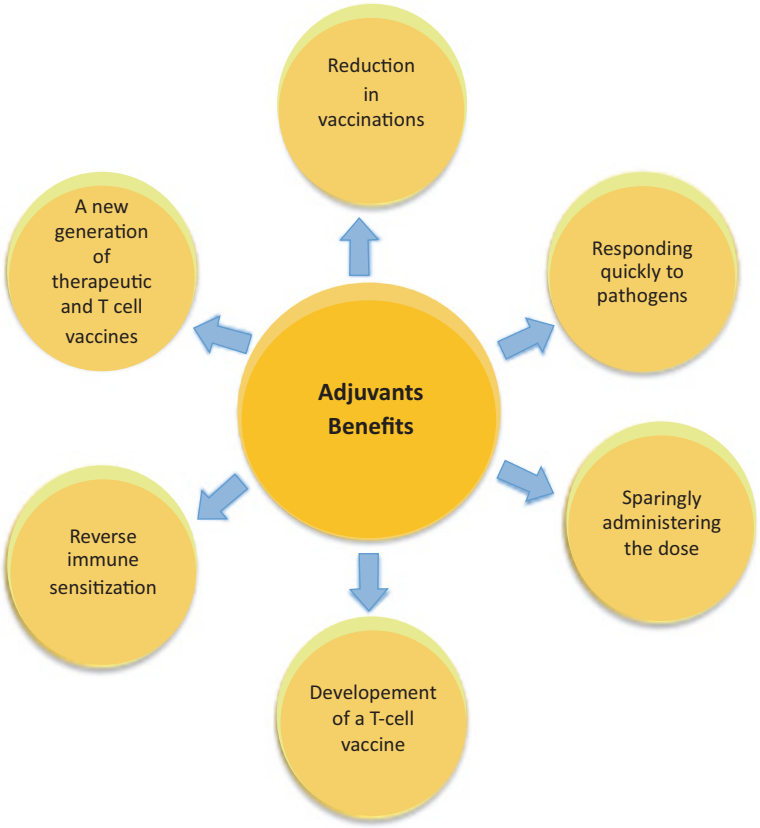

Fig. 5.1 Benefits of adjuvants

depot, activation of cytokines and chemokines, recruitment of immune cells and enhancement of antigen presentation and transport. A local immunocompetent environment at the injection site appears to be created by adjuvants activating innate immune responses. In addition to optimizing innate immune responses, adjuvants can also modify adaptive immune responses based on the type of innate responses stimulated. A better understanding of adjuvant mechanisms will show how innate immunity influences the development of adaptive immunity, can contribute to the rational design of vaccines against various diseases and will improve adjuvant safety. A major challenge for future research will be a thorough understanding of how adjuvants play a role in intracellular signalling and intercellular communication.

New solutions are needed in order to combat microorganisms and biofilms on wounds. In general, antimicrobial treatments are considered the gold standard for treating overt infections. Its unique characteristics have made nanotechnology a major topic of interest. Nanoparticles do indeed exhibit many of these characteristics (Percival et al. 2011). The small size of these molecules, combined with their high surface-to-volume ratio, allows them to pass through fenestrated capillaries' interendothelium gaps. Other mechanisms than antibiotics can be employed to inhibit microbial growth and promote cell death through direct interaction between nanoparticles and cell membrane/wall proteins. Interestingly, the molecular interaction properties of nanoparticles can be manipulated to improve their action and promote these interactions (Blecher et al. 2011; Friedman et al. 2013).

5.2 Nano-adjuvants Against Microbes

A modern subunit vaccine delivers its antigen component through an adjuvant and/or a delivery system. This helps boost immunity. Adjuvants mimic PAMPs that can be associated with infection. Table 5.1 lists the most potent nano-adjuvants as antimicrobials.

Table 5.1 List of nano-adjuvants activated against microbes

Adjuvant name	Type of immune response/stimulated	Activated against	Reference
AuSNs	Dendritic cells, T cells	Foot-and-mouth disease virus	Teng et al. (2018)
Chitosan	Dendritic cells, B and T lymphocytes	*Staphylococcus aureus*, *Candida albicans*	Banche et al. (2015)
Mannan	Dendritic cell maturation	HIV	Cordeiro et al. (2015); Pifferi et al. (2021)
Saponins	Th1, Th2, T cells	H7N1 influenza infection.	Tong et al. (2018); Zhao et al. (2017)
ZOTEN	T cells	HSV-2 genital infection	Agelidis et al. (2019); Antoine et al. (2016)

5.2.1 Carbohydrate-Based Adjuvants

Carbon-containing nanoparticles possessing a moderating effect on immune system activity and particulate carriers capable of delivering antigens effectively stimulate the humoral and cellular immune systems simultaneously. In the soluble form, carbohydrate-containing nanoparticles may enhance cellular uptake of vaccine adjuvants and enhance their activity (Cordeiro et al. 2015). A carbohydrate-containing nanoparticle is similar to a pathogen particle in some ways (Marradi et al. 2011). In addition to their size, nanoparticles contain carbohydrate patterns that bind to pattern recognition receptors on immune cells (Zhao et al. 2014). The carbohydrate component of a vaccine increases its immunogenicity by binding to specific glycan-binding receptors on the surface of APCs (antigen-presenting cells) (Zhao et al. 2014). Through phagocytosis and endocytosis, carbohydrate and associated antigen are absorbed (Mosaiab et al. 2019).

Every year, chronic wounds such as ulcers, bedsores, burns and diabetes-associated vasculopathies cost developed countries millions of dollars. Diabetics and immunocompromised patients tend to have chronic wounds caused by *Candida albicans* and methicillin-resistant *Staphylococcus aureus* (MRSA). In vitro T cell proliferation and maturation can be enhanced by chitosan and nanoparticles as they act as intrinsic adjuvants as well as mucoadhesive materials. It has been reported that chitosan and chitin possess antimicrobial properties. Furthermore, chitosan is also thought to possess antimicrobial properties, especially in relation to bacteria, yeasts and filamentous fungi. Chitosan is an antibacterial polymer, because of its hydrophilic and polycationic nature.

Ionic gelation is most commonly used to prepare chitosan nanoparticles (Lugade et al. 2013). This can be achieved by using chitosan with a molecular weight of 200–400 kDa. Through the gelation of ionic compounds, such as tripolyphosphate, chitosan powder is dissolved in acetic acid (1–2%) and then added to the solution. Chitosan particles might stimulate macrophages, DCs and B and T lymphocytes. Chitosan nanoparticles can serve as immunological adjuvants to stimulate humoral and cellular immunity (Banche et al. 2015). Banche et al. (2015) explored nanodroplets' antimicrobial properties against MRSA and *C. albicans*, as well as their toxicity to human keratinocytes (HaCaT) and ultrasound-triggered transdermal delivery. In particular, US-activated chitosan-shelled OLNs (oxygen-loaded nanodroplets) are promising, innovative and nonconventional tools for treating infected chronic wounds.

Joshi et al. (2019) report for the first time the detection of plant-fungal pathogenic chitin in cell walls using a polyclonal serum raised against trimethyl chitosan nanoparticles in mice. In addition to producing antibodies, their method also employs the adjuvant properties of trimethylchitosan and its nanoparticles (Joshi et al. 2019). Chitosan's adjuvanticity is affected by its quaternization degree. H5N1 (highly pathogenic Asian avian influenza a) adjuvants containing moderate degrees of quaternization induced higher immune responses (Wang et al. 2016).

As vaccine adjuvants, mannan nanoparticles can enhance immune responses, especially in vaccines against the human immunodeficiency virus (HIV). A glycosidic bond of 1,4 hydroxyl groups is linked to D-mannose in mannan to form a molecule abundant in plants and microbial cells. Inflammasome activation by mannan may stimulate DC maturation under TLR4 stimulation (Cordeiro et al. 2015; Pifferi et al. 2021). Mannosylated surfaces closely mimic the native structure of virion surfaces and as such are a good candidate for inclusion in HIV vaccines as adjuvants. Mannan-coated liposomes have been tested in an anti-HIV vaccine, according to Toda et al. (1997). A major advantage of mannan-modified nanoparticles in HIV vaccines is their potent adjuvanticity. Mannan-modified nanoparticles would be appropriate vehicles to deliver antigen. Nanoparticles modified with mannan promote further immune response stimulation when combined with antigens.

Saponins have been widely known for their adjuvanticity for decades. Saponin-containing nanoparticles exhibit strong adjuvanticity in vaccines. The saponins of terrestrial higher plants were previously recognized as steroidal or triterpene aglycones connected to hydrophilic chains of carbohydrate. Bark of the tree *Quillaja saponaria* Molina, a source of triterpene saponins, has been found to provide powerful adjuvant effects. One of the most widely studied extracts from this source, Quil A, contains heterogeneous mixtures of saponins with structurally related properties. A saponin fraction, QS-21, was purified from Quil A and had the best adjuvanticity among all the fractions. To reduce the haemolytic activity of Quil A saponins, formulation optimization efforts were undertaken (Kensil et al. 1995). Different licensed or test vaccines are using nanoparticulate adjuvants with QS-21 or other Quil A fractions that demonstrate good safety profiles. Kazakhstani plants produced three saponins that are immunostimulating and have low toxicity. A nanocomplex of this saponin, lipid and antigen of the H7N1 influenza virus was prepared via extensive dialysis. Nanocomplexes containing these three saponins stimulated robust Th1 and Th2 responses upon subcutaneous immunization. The result of these treatments was elevated antibody titres (both IgG1 and IgG2a) and enhanced cytokine production in Th1 and Th2. In addition, mice that had been preimmunized with these nanocomplexes displayed 100% protection against H7N1 influenza infection. Moreover, plant saponins have been reported to have adjuvanticity (Tong et al. 2018; Zhao et al. 2017).

5.2.2 Gold-Based Nanoparticle Adjuvant

The inherent ability of gold nanoparticles (AuNPs) to tune and regulate immune responses has made them a promising tool for vaccine development. As both delivery systems and adjuvants, AuNPs can be highly efficacious. Most commonly, AuNPs are synthesized via the reductive reaction of chloroauric acid using Turkevich-Schriffins procedures. They are then stabilized using surface modification and stabilization (Compostella et al. 2017). The functionalization of 2 nm AuNPs with four components led to development of a candidate against different

serotypes of *Streptococcus pneumoniae*, a respiratory pathogen responsible for over two million deaths each year (Masomian et al. 2020; Vetro et al. 2017). Mouse IgG antibodies specific to influenza matrix 2 protein (M2e) were induced by intranasal injection of 12 nm AuNPs containing a highly conserved extracellular region. AuNPs and CpG induced cross-protection against diverse influenza viral strains when administered together as an adjuvant (Tao et al. 2014). More recent approaches utilize AuNPs of 18 nm coupled with hemagglutinin (HA), Aichi/2/68 (H3N2) and flagellin, the TLR5 agonist (Wang et al. 2017). Using intranasal administration of the conjugated AuNPs in vivo and in vitro, the conjugated AuNPs were able to stimulate a strong immune response by activating DCs and T cells. There is evidence that gold nanostars have shape-dependent adjuvant effects on macrophages and induce immunity against foot-and-mouth disease using virus-like particle nanoformulations containing gold nanostars (Teng et al. 2018).

5.2.3 Zinc-Based Nanoparticle Adjuvant

The efficacy of ZOTEN (zinc oxide tetrapod nanoparticles) was comparable to that of alum, and it exhibited strong adjuvant-like properties against HSV-2 female genital herpes infections. ZOTEN exhibits new and multifunctional antiviral properties with both therapeutic and prophylactic potential due to its targeting of viral particles and manipulation of host immune systems (Agelidis et al. 2019). ZOTEN provides the platform for capturing and presenting neutralized viruses to mucosal APCs for the purpose of triggering and boosting adaptive immunity (Antoine et al. 2016). Similarly, ZOTEN also showed suppression in Coleman and Shukla (2013) study. According to the study, there were fewer clinical signs of vaginal infection and fewer deaths in the animals (Coleman and Shukla 2013). ZOTEN is also shown to reduce the likelihood of infection using any live viral vaccine when given alongside. In mice with HSV-2 infection, ZOTEN decreased CD45+, Gr-1+ and F4/80+ cell infiltration, which was correlated with vaginal inflammation (Yadavalli and Shukla 2016). It is important to note that sexually transmitted infections by herpes simplex virus type-2 (HSV-2) pose a serious health threat. Human immunodeficiency virus (HIV) susceptibility can also be significantly increased by HSV-2 infections. ZOTEN treatment has greater effects on developing adaptive immunity and memory to resist pathogens. Even if more studies are required, ZOTEN's live virus vaccine platform may contribute to the development of new and more effective vaccine strategies.

5.3 Conclusion

In recent decades, vaccine adjuvants have been developed. Adjuvants for this vaccine have gone through a long process from the bench to the clinic. There are several factors to consider when manufacturing a product, including compatibility, stability, effectiveness and complexity. Vaccines based on nanomaterials are an emerging and promising research field. In spite of nanotechnology's limitations, it provides hope to develop new antimicrobial agents that will stand the test of time and help us combat emerging and existing infectious diseases. There are several types of metallic and organic nanoparticles each with their own advantages and disadvantages. Through the use of novel nanomaterials or their combinations, researchers are striving to enhance the efficacy of these antimicrobial agents and reduce or eliminate their associated side effects. Whenever nano-adjuvants are approved for use as antimicrobial agents, they should also be evaluated for their impact on the environment when released.

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Chapter 6
Limiting Antibiotic-Resistant Bacteria Using Multifunctional Nanomaterials

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Abstract In the current scenario, antibiotic-resistant bacteria become a global threat to human health, and it has been predicted that by the year 2050, death caused due to bacterial infection will surpass the cancer-related death. Existing antibiotic therapy experiences several limitations like side effects, poor stability, and solubility which leads to its inefficiency in antimicrobial therapy. To overcome these limitations, research has been focused on alternative strategies like use of nanomaterials in the formulation of antimicrobial agents due to advantages like drug-targeting ability, biodistribution, enhanced uptake, and favored physicochemical properties. Nanomaterials interact with the cellular component of microbes, and their antimicrobial behavior depends majorly on surface chemistry, size, shape, and core material. This chapter elaborates on the drug-resistant mechanism of microbes as well as the role of nanomaterials (nitric oxide-releasing, chitosan-based, and metallic) in combating drug resistance. Various bacterial-based diseases in animals are also liable to be transferred in humans and cause serious illness. The potential of nanomaterials in the prevention and treatment of diseases in animal models is also the highlight in the present article. Finally, we also discussed the clinical approaches of nanoformulation in combating drug-resistant microbes.

Keywords Multidrug resistance · Nanomaterials · Antimicrobial · Biofilm · Animal disease · Clinical trials · Antimicrobial peptides · Antitoxins

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6.1 Introduction

Multidrug-resistant (MDR) bacteria remain a key challenge for the treatment of bacteria-driven life-threatening diseases. According to an estimate, by the year 2050, the deaths caused by bacteria-driven diseases may surpass the mortality caused by cancer. Currently, several bacterial species are reported to be resistant to the essential drugs like methicillin, carbapenem, and vancomycin, thus producing methicillin-resistant *Staphylococcus aureus* (MRSA), carbapenem-resistant *Enterobacteriaceae*, and vancomycin-resistant *Enterococcus*, respectively (Ssekatawa et al. 2020; Willyard 2017). Some of the common reasons for drug resistance are irrational and overuse of antibiotics, bacterial adaptation to biofilms formation, and prolonged use of antibiotics for the treatment of bacterial diseases (Munir et al. 2020). Excess use of antibiotics causes selective pressure on microbes which in turn develop the genes encoding antibiotic resistance and thus produce new strains in which the resistance is transferred via horizontal or vertical transmission (Arzanlou et al. 2017a).

Antibiotics inhibit the growth of bacteria by different mechanisms including inhibition of cell wall synthesis and hindering DNA, RNA, protein synthesis, and biofilm formation. MecA genes in bacterial cells are reported to impart resistance against antibiotics such as penicillin and methicillin (Berger-Bachi 1994). Single microbe-like superbugs can acquire MDR by adapting drug-resistant genes from other bacteria. The enzyme, such as New Delhi metallo-b-lactamase-1 (NDM-1), can degrade the β-lactam ring, thus making a range of antibiotics ineffective against the bacterial strain (Rolain et al. 2010). *Mycobacterium tuberculosis* and *S. aureus* are other common examples of drug-resistant bacteria causing serious concern and threat to the global healthcare community (Munir et al. 2020). Thus, the origin of antibiotic-resistant pathogenic bacterial strains requires immediate attention, and some novel approaches are required to inhibit their growth and transmission. In this context, nanotechnology-based novel therapeutic strategies have shown promising results in controlling the growth of MDR microbes.

To overcome the antibiotic-resistant complications, it is important to understand the mechanism by which microbes escape the traditional antibiotic therapy. There are two types of bacterial growth: (i) planktonic growth, described as unicellular, free-swimming microbes not attached to a surface, and (ii) biofilm growth phase, characterized as multicellular sessile state which forms communities (Berlanga and Guerrero 2016).

Biofilm formation is an advanced method that allows bacteria to survive in harsh circumstances by developing permanent colonies with great ability to dissociate and form new colonies (Rizzato et al. 2019; Majumdar and Pal 2017). Bacterial biofilms are made up of a dense and hydrated clump of bacteria that are attached to a surface and are encased in a dense external matrix of exopolysaccharides, extracellular deoxyribonucleic acid (DNA), and amino acids (Blair et al. 2008). While biofilm formation in the common bacteria like *Staphylococcus epidermis* and *Pseudomonas aeruginosa* is well-known to protect them from various antibiotics, diverse other

biofilm-forming microbes also exist which confer resistance against wide range of antibiotics. For example, yeast *candida albicans* and obligate anaerobe *Porphyromonas gingivalis*, when grown in the biofilm, have been reported to be less susceptible to antibiotics in comparison to free-floating cells (Stewart 2002). Human lung, urethra, colon, ear infections, infective endocarditis, gum infection, and wound-related infections are linked to biofilms formation (Valappil 2018). In comparison to planktonic bacterial growth, biofilms are thought to be ~1000 times more resistant to antibiotics (Rossi-Fedele, Roberts 2007). Biofilm bacteria are subjected to cell density-dependent control from their extracellular polymeric substances (EPS) matrix, and thus as a result of high density, they are discharged into the surroundings as free-floating bacteria. Furthermore, both biofilms and host immune responses enhance the transformation of normal nonpathogenic commensal bacteria into virulent forms in the human body (Marsich et al. 2012). The evolution of survival mechanisms has been aided by the increased genetic mutation rates within biofilms. The expression of certain efflux pumps and upregulation of various proteins could cause diffusion across the biofilm. In this context, deletion of genes encoding the biofilm-specific efflux pump, PA1874–1877, confers the *P. aeruginosa* sensitivity to antibiotics like gentamicin and ciprofloxacin. These genes are not found to be overexpressed in planktonic cells proving their importance in biofilm resistance. Furthermore, increased production of toxin-antitoxin modules inhibits important cell operations like translation (Zhang and Mah 2008; Eleraky et al. 2020). Thus, due to the diversity and anonymity of biofilm-resistant processes, innovative nanosystems are envisaged to effectively inhibit the spread of resistant bacterial strains.

Nanoparticles (NPs) offer multifunctional aspects of eradicating MDR microbes because of their ability to act as transporters for common antibiotics as well as natural antibacterial substances (Wang et al. 2017c). The most widely used aspect of nanomaterials (NMs)-based drug delivery system is its ability to introduce a diverse array of therapies being linked to or confined inside their huge surface area and controlled rate of targeted delivery to infected site (Gholipourmalekabadi et al. 2017; Baptista et al. 2018). NMs-mediated delivery can improve the therapeutic index and pharmacokinetic profile of encapsulated drugs in comparison to free drugs which leads to decrease in the required dose to achieve an equivalent clinical effect. This will reduce the adverse toxic side effect caused due to high and frequent dose administration (Gao et al. 2018). Various NPs are reported to be used as efficient drug delivery agents, i.e., liposome, polymeric NPs, inorganic NPs, dendrimers, etc. Rinaldi et al. demonstrated the rifampicin-loaded liposome (Rif-Lipo) for the treatment of pulmonary infection caused due to *Mycobacterium abscessus* (Rinaldi et al. 2021). Synthesized nanoformulation was found to be stable at room temperature and 4 °C for 90 days. The authors showed that 18 h exposure of 96 μM Rif-Lipo nanoformulation inhibits the *M. abscessus* infection with a similar effect of 192 μM rifampicin alone. Targeted delivery of drugs and antibiotics can also be achieved efficiently by using various NPs. In this context, Güncüm et al. demonstrated the antibacterial activity of polymeric (poly(vinyl alcohol)/sodium alginate) NPs containing amoxicillin (poly-AmoNPs) against *Escherichia coli (E. coli)* and

S. aureus (Güncüm et al. 2018). The result showed that with a decrease in pH, the release of amoxicillin also decreases which induces the controlled release of drug at infectious site. This formulation has a similar effect as a free drug against *E. coli* and *S. aureus*. Further, Wang et al. synthesized gold-silver nanocage coated with the pattern recognition receptors (PRRs) (found in macrophage membrane) (Shi et al. 2018). Macrophages were treated with *S. aureus* and *E. coli* to confirm the expression of pathogen-related receptors on their membrane surface. This formulation promotes the adherence of NPs to specific bacteria for targeted therapy and delivery of the drug. Also, the gold-silver nanocages can convert NIR (laser light) into heat to destroy the bacteria by using laser irradiation treatment. The hollow structure of nanocage can be well utilized for the encapsulation of drugs for the targeted therapy.

6.2 Mechanism Underlying Antibiotic Resistance in Microbes

Existing antibiotics are reported to inhibit the growth of microbes via affecting three different machineries: DNA replication, translation process and cell wall synthesis. Interestingly, bacterial cells evolve various strategies to counter the inhibitory functions of antibiotics by developing resistance mediated by mutations in chromosomes. Non-resistant bacteria get eradicated by antibiotics, whereas resistant species survive the exposure and eventually transfer the resistance mechanism to next generation through horizontal or vertical transfer (Arzanlou et al. 2017b; Ruddaraju et al. 2020). Various mechanisms of antibiotic resistance in microbes are discussed below in detail and summarized in Fig. 6.1.

6.2.1 Competition to Antibiotics and Resistance to Persister

Bacterial cells can produce molecules displaying competitive inhibition for each antibiotic to acquire resistance against the antibiotics. In this context, sulfonamide-resistant bacterial cells produce a high amount of pare-aminobenzoic acid (PABA) to confer resistance. Sulfonamides hinder bacterial nucleic acid synthesis by inhibiting the bacterial enzyme dihydropteroate synthetase (DHPs) in the folic acid pathway. In *Neisseria meningitidis* and *S. aureus*, PABA competes with the sulfonamides for enzyme DHPs and leads to resistance in bacterial cells (Ponce et al. 2017).

Inert persisters occur in the infected bacterial community causing a repetition of infection even after the treatment due to acquired resistance against antibacterial drugs. Persister cells decreased their metabolic rate by gene shift to achieve resistance against antibiotics. Bacterial community exposed to antibiotics showed that some of the populations are sensitive to drugs, while other remains unaffected

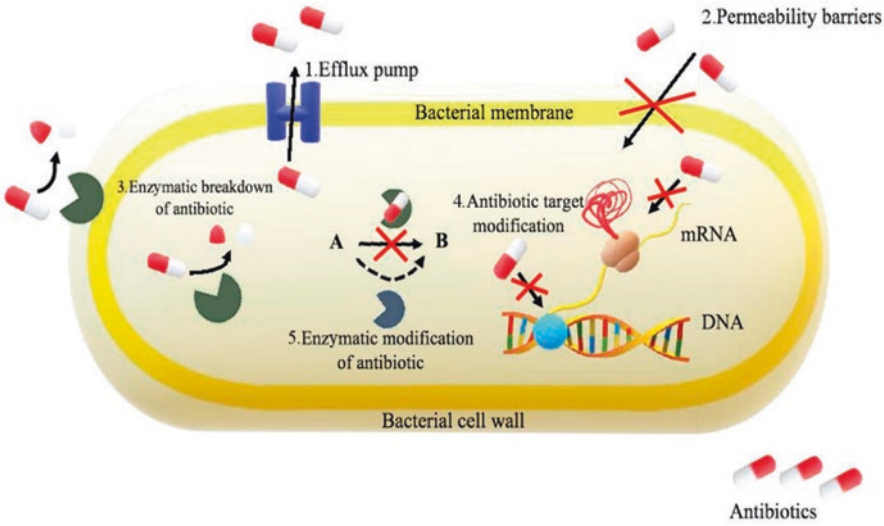

Fig. 6.1 Scheme showing mechanistic action of antimicrobial resistance (Pauter et al. 2020)

indicating the treatment completion for certain infection. In some cases, persisters shift to an active metabolic state again to cause reinfection.

6.2.2 Low Drug Uptake and High Efflux

Reduced rate of drug uptake and high efflux rate are two major mechanisms that simultaneously regulate antibiotic resistance. For example, *P. aeruginosa* low sensitivity for a drug could be due to the presence of inner membrane protein (H^+/drug antiporter protein) in the periplasmic space attached to a linker protein. Regulatory protein suppresses the gene encoding efflux protein; thus, mutation in regulating protein leads to overexpression of efflux protein and high MDR of *P. aeruginosa* (Nikaido 2009). In addition, energy driven by transmembrane protein could also be utilized by nine efflux pumps expressed in *E. coli* that facilitate the development of resistance in bacteria by expelling many antibiotics (Du et al. 2018).

Several genes are reported in both gram-negative and gram-positive bacteria that encode efflux pumps such as tetracycline efflux pump encoded by TetB, TetK, and TetA. Transfer of these genes to bacterial cells may be attributed to transposons and horizontal gene transfer on the plasmid (Blair et al. 2015). Most of the gram-negative bacteria are reported to be resistant against chloramphenicol and fluoroquinolones primarily due to the efflux effect. *Enterococcus faecalis* develop resistance against dalfopristin and quinupristin antibiotics by using the same efflux mechanism. Further, decreased uptake of antimicrobial drugs in gram-negative bacteria causes

resistance against aminoglycosides, whereas resistance to vancomycin may be attributed to the increased thickness of cell walls in microbes (Blair et al. 2015).

6.2.3 Biofilm Formation

Resistance in bacteria from several antibiotics may be attributed to the biofilm formation, which causes chronic infections. Biofilm-producing bacteria exhibit ~1000 times more resistance against antibiotics than bacterial species that do not form biofilms (Arciola et al. 2018). At the initial stage of biofilm formation, antibiotic treatment is found to be more effective because the microbial population is not completely adapted into the biofilm community (Munoz-Egea et al. 2016). During biofilm formation, EPS assembles to facilitate the localization of bacterial community; however, it acts as a diffusion barrier for antibiotics. Various mechanisms are suggested regarding EPS mediating antibiotic inhibition. Here, at the initial stage, the pore size of the matrix is small enough to hinder the entry of antibiotics. The negatively charged matrix further inhibits the effect of antibiotics on bacterial cells, and the enzymes located in EPS induce covalent modification in antibiotics that lead to the inhibition of their antimicrobial action (Ferreira et al. 2010). Additionally, EPS also acts as a barrier to nutrient and oxygen supply, thus promoting indirect resistance to bacteria against antibiotics. Bacterial cells located deep inside the biofilm exhibit lower metabolic rates due to less supply of nutrients and thus are also less susceptible to antibiotics.

The multicellular nature of biofilm is one of the key factors responsible for antibiotic resistance. EPS accumulates the bacterial cells together and develops the multicellular consortia, which forms a heterogeneous environment inside the biofilm and establishes a multicellular system. If the steps of multicellular structure formation of biofilm can be disrupted, antibiotic efficacy as well as host defense system could be improved. Another mechanism regarding the resistance of the biofilm community is the internalization of resistance genes by horizontal gene transfer via conjugation method (Mah 2012). Biofilm provides a compatible environment for gene transfer such as high genetic competence, accumulation of genetic elements, and high cell density (Fux et al. 2005). Several studies have reported that the conjugation process is more efficient in biofilms compared to planktonic cells (Van Meervenne et al. 2014; Sharma et al. 2019).

6.2.4 Antibiotic Modification

Several microorganisms express the drug-resistant genes, which encode the enzymes responsible for covalent modification of antibiotics such as aminoglycosides, tetracycline, quinolones and β-lactams (Laxminarayan et al. 2013). β-Ring of β-lactam has been reported to be hydrolyzed by β-lactamase enzyme leading to resistance in

β-lactamase-sensitive microbes. Horizontal transfer of β-lactamase gene on bacterial plasmids or decreased activity of a repressor protein that inhibits β-lactamase gene transcription is the major cause of resistance development in microbes (Moyá et al. 2012; Munir et al. 2020).

Recently, New Delhi metallo-β-lactamase 1 (NDM-1)-producing carbapenem-resistant microbes have been discovered. Several NDM-1-expressing bacteria are resistant against a majority of antibacterial drugs used to treat serious infections. Research on samples collected from various regions reports that they are tolerant to monobactam aztreonam, aminoglycosides, quinolones, tetracycline and β-lactam antibiotics (Kumarasamy et al. 2010). Moreover, the aminoglycoside resistance gene encodes enzymes causing a covalent modification of the OH and NH_2 group of aminoglycosides which leads to the decreased affinity with the 30S ribosomal subunit, thus inhibiting the antibacterial activity. Robicsek et al. reported the reduced susceptibility of clinical bacterial isolates against ciprofloxacin due to the expression of gene encoding aminoglycoside acetyltransferase. This enzyme inhibits ciprofloxacin's activity by N-acetylating the amino nitrogen on the piperazinyl group (Robicsek et al. 2006).

6.2.5 Swarming

Swarming represents social motility enabling differentiated bacterial cells to migrate. Swarming is similar to the biofilm community and characterized by a high level of resistance to antimicrobial treatments (Lai et al. 2009). Several swarming bacterial species like *P. aeruginosa*, *Salmonella enterica*, and *B. subtilis* are reported to exhibit multiple antibiotic resistance. Swarming bacteria exhibit three different strategies against antibiotics including high cell density, lower exposure to antibiotics due to circulation within the multilayer structure, and death of directly exposed individuals (Butler et al. 2010). Reports showed that even in the absence of swarming, high cell density promotes bacterial survival; however, movement ability, as well as the speed of movement, offers an extra advantage to swarm as an effective strategy against antimicrobials agents (Butler et al. 2010).

6.3 Nanotechnology-Mediated Strategies to Overcome MDR in Microbes

The emergence of new drug-resistant microbial species and the limited production of antimicrobial drugs have created a serious concern for human health. Synthesis of new antibiotics is a complex process and takes around 10–15 years for approval and also has a very high production cost (Eleraky et al. 2020). Therefore, as an alternative, people have looked at using NMs as an effective antimicrobial. NPs are

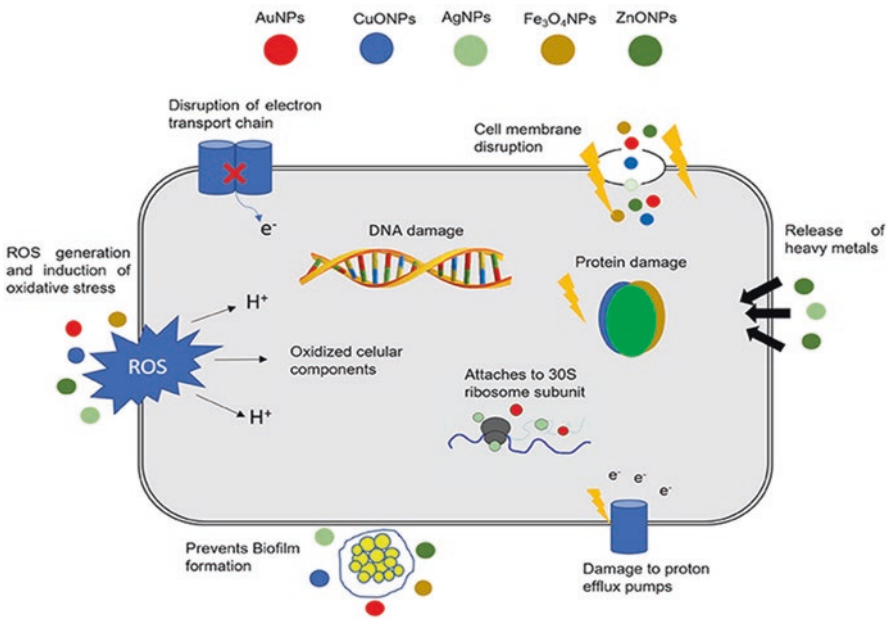

Fig. 6.2 NPs act on bacterial cells through different mechanisms (Baptista et al. 2018)

also reported to enhance the physicochemical property and stability of existing antibiotics, prolong their release, and facilitate targeted delivery to the infection site, with reduced side effects (Patra et al. 2018). NMs possess various mechanisms to overcome microbial drug resistance (Fig. 6.2), which can be governed by their physicochemical properties like size, surface charge, and solubility. Nitric oxide-releasing NMs, metal-based NMs, and chitosan-derived NMs are reported to prevent microbe resistance (Pelgrift and Friedman 2013). Encapsulation of antibiotics in NMs, inhibition of biofilm, increasing drug influx, and decreasing efflux are some of the mechanisms underlying their antimicrobial efficacy (Kaur et al. 2019; Tang and Zheng 2018). Table 6.1 shows the list of various NPs with their inhibitory mechanism against MDR microbes.

6.3.1 Nitric Oxide-Releasing Nanomaterials

In recent years, gas-releasing agents especially nitric oxide-releasing NMs (NO-NMs) have found applications in combating multidrug resistance in microbes (Rong et al. 2019). NO released from NMs reacts with superoxide ($O_2^{\cdot -}$) to produce reactive nitrogen intermediates (RNOS) causing bacterial cell death. NO (>1 mM) shows toxicity against microbes by various mechanisms (Wang et al. 2017c; Nguyen et al. 2016) including (i) interaction of RNOS with prosthetic groups of proteins, (ii)

inducing nitrosative damage to DNA, (iii) reacting with bacterial protein residues, (iv) lipid peroxidation, and (v) interference with zinc metalloproteins to hinder cellular respiration. Additionally, NO can also trigger the immune response in humans and animals (Dolansky et al. 2018).

Kafshgari et al. reported the antibacterial efficacy of NO-releasing porous silicon NPs (Hasanzadeh Kafshgari et al. 2016). In the presence of ascorbic acid, NO inhibited *E. coli* and *S. aureus* growth within 2 h of exposure. However, after 24 h of exposure, bacterial growth decreased to 1 log than untreated cells. Gehring et al. have also demonstrated the antibacterial activity from NO-releasing mesoporous organosilica (Gehring et al. 2016). The antibacterial effect of NO alone and along with gentamicin in polymeric NMs was also investigated (Nguyen et al. 2016). It was found that both agents were simultaneously released and displayed a synergistic effect causing a decrease in the planktonic cell viability and biofilm formation by 95% and 90%, respectively. NO-NMs are also reported to cause interference in adhesion of MR *S. aureus* and also prevent the biofilm formation when tested in rat central venous catheter model of infection (Mihu et al. 2017). NO-releasing silver NPs (AgNPs) are also reported to show antibacterial activity. The presence of NO on the particle surface enhances the antibacterial effect due to the synergistic effect of AgNPs and NO (Seabra et al. 2017). So far, there is no report on the development of resistance against NO, which could be due to the no increase in minimum inhibitory concentration (MIC) of exposure (Privett et al. 2012). However, some bacteria express enzymes (flavohemoglobin, DNA repair enzymes, lactate dehydrogenase) that protect them from the nitrosative effect of NO at a physiological quantity of NO, but at an adequate concentration of NO, these enzymes also become ineffective (Hall et al. 2020). At a concentration of 1.25–5 mM, NO-NMs are reported to completely eradicate the bacterial cells and also lower the bacterial burden when applied on lesions and intramuscular and dermal abscesses (Schairer et al. 2012).

NO-NMs are also reported to be effective against fungal infections. Bio-screen C analysis and time-lapse microscopy showed NO-NMs induced inhibition of fungal colonies by decreasing the cell division, filament, and bud formation (Rosen et al. 2016). NO-NMs are effective against the *Candida albicans* biofilm formation activity (Hetrick et al. 2009). NO-NMs treatment was found to decrease fungal load and accelerate wound closure in mice. This is also supported by the tissue histology showing a lack of fungal hyphae structures within the dermis and decreased inflammation with increased fibrin and collagen deposition.

6.3.2 Metal-Based Nanoparticles

Different metallic NMs, i.e., gold (Au), silver (Ag), zinc (Zn), magnesium (Mg), and titanium (Ti), prevent drug resistance in microbes by employing a different mechanism to hinder the growth (Wyszogrodzka et al. 2016). Polyclonal antibody-decorated bismuth NPs are reported to enhance the effect of X-ray irradiation to eradicate the MDR bacterial species. The result showed a significant antibacterial effect due to the combined action of bismuth and X-ray, thus killing ~90% of

bacteria, whereas only ~6% death was observed when only X-ray was exposed (Luo et al. 2013). On the other hand, no significant toxicity was observed when human cells were exposed to same concentration. Some of the common metallic NPs that are reported to exhibit antimicrobial effects are discussed below.

6.3.2.1 Titanium Dioxide Nanoparticles

Titanium dioxide NPs (TiO_2 NPs) exert antimicrobial action possibly by the following two mechanisms: (i) production of reactive oxygen species (ROS) when irradiated in the near-UV region leading to the damage of bacterial cell membrane (Ranjan and Ramalingam 2016) and (ii) TiO_2 NPs itself inhibit the growth of microbes by an unknown mechanism (Venkatasubbu et al. 2016). In this context, Liu et al. demonstrated the antibacterial activity of TiO_2 nanocrystals with [101] [001] surface heterojunction promoting electron-hole spatial separation at [101] and [001] facets leading to ROS generation and thus antimicrobial effect (Liu et al. 2017). SEM images revealed that the surface of both *E. coli* and *S. aureus* has been altered by the TiO_2 nanocrystals and the antibacterial effect was due to the depletion of glutathione, membrane lipid peroxidation, and intracellular oxidative stress. Further, Arora et al. reported the antibacterial activity due to the use of a combination of TiO_2 NPs, ceftazidime and cefotaxime in MDR *P. aeruginosa* isolated from sputum, pus, endotracheal tract, and bronchoalveolar lavage (Arora et al. 2015). NPs showed toxicity at 350 μg/mL concentration in presence of UV light for an hour.

6.3.2.2 Zinc Oxide Nanoparticles

Zinc oxide NPs (ZnO NPs) are reported to exhibit antibacterial activity regulated by different mechanisms and also reduce the likelihood of resistance development (Sirelkhatim et al. 2015). Polyvinyl alcohol-coated ZnO NPs showed rapid internalization into the cell cytoplasm by increasing the permeability of the bacterial membrane and inducing oxidative stress within the cytoplasm. In the cytoplasm, ZnO NPs bind to bacterial membrane and destroy the lipid and membrane protein to release the cytoplasmic content, thus resulting in cell death. Additionally, ZnO NPs also produce Zn^{2+} ions, which may rupture the bacterial membrane and promote intracellular ROS generation (Sirelkhatim et al. 2015; Siddiqi et al. 2018). In a study by Patra et al., ciprofloxacin-functionalized ZnO NPs were developed to demonstrate the antibacterial activity against MDR *E. coli*, *S. aureus*, and *Klebsiella* sp. Results showed that this nanoconjugate has a lower MIC than only drug (Patra et al. 2014). Further, the two antibiotics (ciprofloxacin and ceftazidime) were conjugated with ZnO NMs and tested against MDR *Acinetobacter baumannii* (Ghasemi and Jalal 2016). Results showed that there was an increase in the internalization of antibiotics in bacterial cells that supported the change in shape of cells from rods to cocci form. ZnO NPs conjugated with clinically approved drugs (ceftriaxone,

amphotericin B, quercetin, naringin, and ampicillin) have also been evaluated for their antibacterial effect against gram-positive and gram-negative bacteria. Ceftriaxone- and ampicillin-conjugated ZnO-NMs exhibited high antibacterial activity but were found nontoxic to human cells (Akbar et al. 2021).

6.3.2.3 Silver Nanoparticles

Antimicrobial property of AgNMs is mainly considered due to the slow release of silver ions (Ag^+) in an aqueous solution (Ramalingam et al. 2016). Ag^+ react with the bacterial cell membrane and rupture them to release cytoplasmic content leading to cell death. Comparatively, gram-positive bacteria are less sensitive than gram-negative bacteria to Ag^+ exposure, which could be due to the thin cell wall in the latter case (Dakal et al. 2016; Li et al. 2019b). Ag^+ are also reported to be less likely to penetrate the gram-negative bacterial cells due to its strong binding to negatively charged lipopolysaccharide (LPS) of gram-negative bacteria (Acharya et al. 2018). Further, some other mechanisms suggested in the favor of Ag^+ ions exerting antimicrobial effect are (i) damage to genetic material and prevention of p DNA duplication, thus arresting cell division, (ii) binding with cytochrome to interfere electron transport chain, and (iii) inhibition of cell wall formation in gram-positive bacteria (Brown et al. 2012; Munir et al. 2020).

AgNPs are effective against a wide range of pathogens, including drug-resistant fungus, bacteria, and viruses. AgNMs bactericidal effects have been reported against ampicillin-resistant *E. coli* and *S. pyogenes* and MDR *P. aeruginosa*. Combining AgNMs with several drugs (amoxicillin, penicillin G, vancomycin, clindamycin, and erythromycin) is reported to exhibit significant antimicrobial activity (Kaur et al. 2019; Li et al. 2019b). Wang et al. reported the synergistic antibacterial effect of levofloxacin decorated on Ag core-embedded silica nanoplatform (Ag@MSNs@LEVO) against drug-resistant bacteria (Fig. 6.3) (Wang et al. 2016). Results showed that upon treatment with the Ag@MSNs@LEVO to in vivo acute peritonitis model, *E. coli* infection in the peritoneal cavity of the mice reduced to three-fold and pathological effects from spleen and peritoneum were also found to be vanished without exerting any toxic side effect on mice. Thus, this data strongly suggests that Ag@MSNs@LEVO has the potential to be a safe therapeutic option for clinical drug-resistant infections. Mottais et al. synthesized N-heterocyclic carbene-coated silver complexes (Ag-NHCs) featuring a lipid chain and investigated their antibacterial potency (Mottais et al. 2019). It was found that the aqueous formulation of Ag-NHCs showed a better antibacterial effect against some strains of *S. aureus* and *P. aeruginosa*. Additionally, when combined with cationic lipid and DNA, it can also be used to deliver therapeutic genes to infected lungs via aerosolization. Taken together, the data presented herein suggest the use of n-alkyl chain Ag-NHC as a promising alternative to traditional antibiotics in the treatment of respiratory infections and to fight against the rise of MDR bacteria.

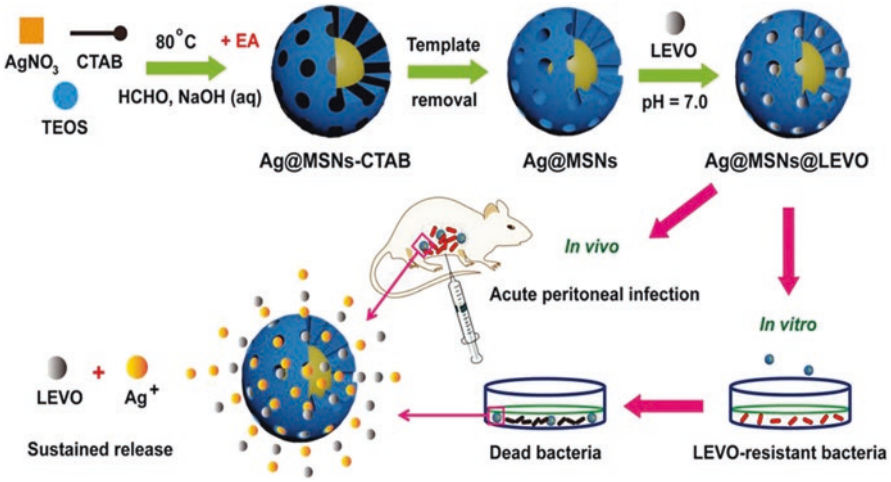

Fig. 6.3 Schematic illustration showing the fabrication of Ag@MSNs@LEVO nanoplatform and its synergistic application over drug-resistant infections in vitro and in vivo. (Reprinted with permission from *Biomaterials,* Copyright 2021, Elsevier (Wang et al. 2016))

6.3.2.4 Copper Oxide Nanoparticles

Copper oxide NPs (CuONPs) are reported as weak but wide spectrum antimicrobial agents, mostly reported effective against *Listeria monocytogenes*, *E. coli*, *S. aureus*, and *S. cerevisiae*. CuONPs utilize two different mechanisms for antimicrobial action: (i) excess amount of Cu ions cause generation of ROS to prevent both DNA replication and amino acid synthesis; and (ii) Cu ions react with amino and carboxyl groups presented on the bacterial surface (Ananth et al. 2015). Agarwal et al. demonstrated the activity of CuONPs against MDR biofilm-forming bacteria (Agarwala et al. 2014). The result showed that CuONPs exposure displayed a zone of inhibition against MR *S. aureus* (22 ± 1 nm) followed by *E. coli* (18 ± 1 nm). It has been reasoned that Cu ions damage the microorganism's envelope and subsequently bind with DNA leading to multiple damages mediated by OH radicals. However, in some cases, it has been reported that copper-mediated oxidative damage follows the Fenton mechanism (Borkow and Gabbay 2009).

6.3.2.5 Bismuth Nanoparticles

Bismuth NPs (BiNPs) are reported to be a potent antimicrobial agent against drug-resistant microbes (Hernandez-Delgadillo et al. 2012). BiNPs are synthesized in different ways with controlled shape and size required to display maximum antibacterial activity (Wang et al. 2008). Besides the use of visible, topical, and UV radiation, X-rays have a high impact to eradicate the bacterial infection when used with BiNPs. It leads to the reduced requirement of radiation dose needed to eradicate

bacteria, thus making it less harmful to humans. Mechanistically, Bi releases electrons by photoelectric effect and generates free radicals following X-ray irradiation, which gradually destroys the bacterial DNA (Luo et al. 2013). Antibiotic conjugation with BiNMs reduces the average distance between targeted microbes and NPs and thus enhances the bactericidal action (Gao et al. 2014). Further, BiNMs radiation therapy is also reported to be highly effective against MDR *P. aeruginosa* (Luo et al. 2013). The bacterial sample was incubated with polyclonal antibody-modified BiNMs and irradiated with X-rays. The result suggested that ~90% of bacteria were killed upon exposure to 200 μg/mL BiNMs, whereas only 6% were killed when exposed to X-ray alone. Additionally, no significant toxicity was observed on human cells, thus establishing the possibility of future clinical applications.

6.3.2.6 Graphene-Based Nanomaterials

Graphene is a single-layer carbon sheet that has been emerging as a potent antimicrobial agent along with other applications. It acts both by physical and chemical methods, and its sharp edges can disrupt the bacterial membrane leading to cell death. Graphene-based NMs (GNMs) are also utilized as dispersing and stabilizing agents for other NMs resulting in high antibacterial competence due to the synergistic effect (Xia et al. 2019). In this context, Aunkor et al. reported the antibacterial activity of graphene oxide (GO) nanosheets against MDR superbugs (*E. coli*, *Klebsiella pneumoniae*, *P. aeruginosa*, *P. mirabilis*, *S. marcescens*, and *S. aureus*) obtained from hospitals (Aunkor et al. 2020). Antibacterial activity of GO nanosheets was compared with commonly used antibiotics (azithromycin, cotrimoxazole, ciprofloxacin, amoxicillin, ceftriaxone, imipenem, gentamycin, and cefixime). The result suggested that GO nanosheets may act as "Nano knives" due to the sharp edges and thus rupture the bacterial cell wall. Secondly, bacterial cells may be entrapped in GO nanosheets and detached from the external environment restricting them to access nutrient supply leading to cell death. Entrapment activity depends on the size of nanosheets, and larger-sized nanosheets showed better entrapment (Liu et al. 2012). Further, Wu et al. reported antimicrobial activity of GO in three different MDR bacteria, i.e., *K. pneumoniae*, *E. coli*, and *P. aeruginosa* (Wu et al. 2017). Result demonstrated that upon GO exposure, *K. pneumoniae* colony is eradicated from the agar dish, thus protecting the alveolar macrophage from infection in culture. GO can inhibit the growth and spread of *K. pneumoniae* both in vitro and in vivo which leads to increased cell survival rate, suppressed inflammatory response, less tissue injury, and prolonged mice survival. Further, Pan et al. demonstrated the antibacterial effect of a nanocomposite system based on reduced GO-iron oxide NPs (rGO-IONPs) against MR *S. aureus* (Pan et al. 2016). Mechanism of rGO-IONPs antibacterial effect was suggested due to the heat and OH radical generation causing bacterial cell death both in vitro and in vivo (Fig. 6.4). These findings suggest that GO may be used as promising NMs for efficiently combating MDR infections.

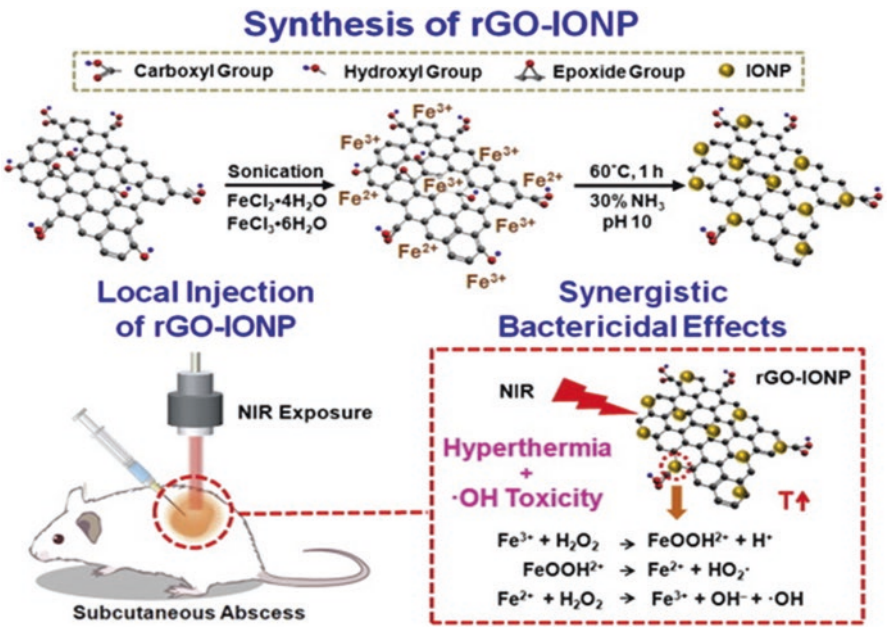

Fig. 6.4 Schematic demonstrating rGO–IONP synthesis and their mechanism of action to inactivate MRSA in subcutaneous abscesses created in a mouse model. (Reprinted with permission from *Nanomedicine: Nanotechnology, Biology and Medicine,* Copyright 2021, Elsevier (Pan et al. 2016))

6.3.2.7 Bimetallic Nanomaterials

Bimetallic NPs composed of AgNPs and AuNPs have been extensively investigated for their antibacterial activity (Singh et al. 2016). AgNPs are a well-known antimicrobial agent. Since the functionalization of AgNPs with biomolecules and drugs remains challenging, therefore, use of bimetallic/alloy NPs has been synthesized to realize its efficient antibacterial property. AuNPs, being biocompatible, are reported as an ideal vector for the delivery of pharmacological compounds. Bimetallic NMs display superior electrical, optical, and catalytic characteristics than their monometallic counterparts (Latif ur et al. 2015). AuNPs and AgNPs bimetallic NMs comprise the properties of both individual NMs, i.e., antimicrobial activity of silver with stability and easy surface functionalization provided by gold (dos Santos et al. 2012). In this context, Fakhri et al. demonstrated the synthesis and functionalization of bimetallic AgAuNPs with tetracycline. The result showed that in combination with bimetallic NPs, antibiotics show a synergistic effect and produce high bactericidal results than their free forms (Fakhri et al. 2017). Recently, Baker et al. synthesized AgAuNPs from the cell-free supernatant of *Pseudomonas veronii* strain AS41G inhabiting *Annona squamosa L.* and demonstrated their antimicrobial efficacy against bacitracin-resistant strain of *B. subtilis*, *E. coli*, and *K. pneumoniae*. Result showed that the synergistic antibacterial effect with antibiotics, bacitracin, kanamycin, gentamicin, streptomycin, erythromycin, and chloramphenicol resulted

in 87.5, 18.5, 11.15, 10, 9.7, and 9.4% fold increase in the activity, respectively (Baker et al. 2017). Further, bimetallic NPs of Au and platinum (AuPtNPs) have also shown enhanced antibacterial activity against sensitive and drug-resistant bacteria (Zhao et al. 2014). Mechanism of action revealed the elevation of adenosine triphosphate (ATP) level and dissipation of bacterial membrane potential.

6.3.2.8 Silica Nanoparticles and Their Derivatives

Silica NPs (SiNPs) offer a variety of functional properties that make them a useful candidate to fight against bacterial infections and are reported to inhibit biofilms from wearable medical implants (Selvarajan et al. 2020). In this context, Kanugala et al. demonstrated the antibacterial activity of phenazine-1-carboxamide (PCN)-loaded SiNPs (PCN-SiNPs) against planktonic *C. albicans* and biofilms of *C. albicans-S. aureus* (Kanugala et al. 2019). Results showed that the antimicrobial activity of PCN-SiNPs was enhanced significantly than PCN and SiNPs alone on silicone urethral catheters. The mechanistic study revealed that released PCN induces ROS production in all microbes, thus resulting in disrupted homeostasis, reduced ergosterol content, altered membrane permeability, and leakage of Na^+, K^+, and Ca^{2+}. Similarly, Wang et al. proposed that silica-gentamycin NPs incorporated in gelatin matrix cross-linked on microarc-oxidized titanium could be used for coating percutaneous implants (Wang et al. 2017b). The antibacterial titanium coating was found to be biocompatible and capable of inhibiting the growth of *S. aureus*. It was also found that the nano-delivery system is biocompatible and thus can be utilized to prevent infection around percutaneous implants.

In various cases, loaded drug amount is insufficient because the physically absorbed drugs in the mesopores suffer from quick release during encapsulation. To overcome this limitation, Kankala et al. developed metal-doped SiNPs where metal embedded in the siliceous frameworks acts as an anchor for drug molecules by establishing the coordination interaction (Kankala et al. 2020). Host-guest interaction among metal and ligands facilitates the high loading capacity compared to the naked SiNPs to allow targeted delivery in the acidic environment at the bacterial infection site. Thus, the synthesized nanocomposite consists of cu-doped SiNPs and holds a pH-responsive coordination interaction with the molecule tetracycline. Further, the nanocomposite was coated with ultrasmall AgNPs. The released Ag^+ can sensitize the resistant strain due to interaction with the membrane and damage the cytoplasmic components, by free radical via Fenton-like reaction. This formulation showed no significant toxicity to mammalian fibroblast cells; therefore, it can be concluded that this trihybrid nanocomposite having a synergistic effect and pH-responsive delivery of antibiotics could play a significant role in combating MDR bacterial species.

Efflux pump system-mediated antibiotic discharge is one of the major causes of MDR in bacteria. To overcome this challenge, novel nanocarriers are designed that can significantly inhibit the growth of MDR bacteria by increasing the retention time of antibiotics (Chen et al. 2018). Based on this, Chen et al. demonstrated the

pH-responsive SiNPs nanocarrier coated with folic acid and calcium phosphate via electrostatic interaction and biomineralization, respectively (Chen et al. 2018). Further, the nanocarrier was loaded with ampicillin with increased uptake and reduced efflux effect in *E. coli* and *S. aureus* via folic acid targeting. The mechanistic study revealed that nanoformulation reduced the protein content and also inhibited the protein activity in MDR bacteria, which leads to the destruction of the bacterial membrane and finally cells' death.

6.3.2.9 Iron Oxide Nanoparticles

Reports suggest that antimicrobial activity of iron oxide NPs (IONPs) is mainly due to ROS generation leading to DNA damage, lipid peroxidation, cellular integrity disruption, and release of metal ions. All these events significantly alter cellular homeostasis and biomolecule coordination (Arias et al. 2018). Antibacterial activity of IONPs has been investigated in bacterial species present in the planktonic free state as well as in biofilms. Reports showed that MIC of IONPs-conjugated amoxicillin nanosystem is approximately three to four times lower than the antibiotic alone when tested in *E. coli* and *S. aureus* (Grumezescu et al. 2014). It reduces the bacterial cell adhesion to polystyrene surface at the initial stage of biofilm formation. In addition, IONPs-chitosan (IONPs-CHT)-streptomycin nanosystem exhibited a significant toxic effect on gram-negative bacteria than gram-positive bacteria (El Zowalaty et al. 2015). Wang et al. demonstrated the IONPs-based silver micro flowers conjugated with antibiotic vancomycin and SiO_2 to act synergistically on MR *S. aureus* and *E. coli* (Wang et al. 2017a). Synthesized nanosystem possesses high magnetic response due to the presence of an iron core of ~200 nm and flower-like Ag shell for offering high surface area and thus release of Ag^+. Vancomycin layer increases the permeability of bacterial membrane by binding to the cell wall, thus allowing the easy entry of Ag^+ to induce cell death. The antimicrobial effect of the nanosystem is reported to be more than 90% effective even after five cycles of assays, which proves the stability of the system. Further, Benjamin et al. have synthesized a biocompatible, multi-compartment nanocarrier consisting of hydrophobic IONPs and hydrophilic methicillin for the treatment of infections associated with medical devices (Fig. 6.5) (Geilich et al. 2017). Applying an external magnetic field, the nanocarrier penetrates ~20 μm thick biofilm of *S. epidermis* and eradicates all bacterial population at 40 μg/mL of IONPs carrying 20 μg/mL methicillin. Most importantly, this formulation was effective against biofilm from MR cells but nontoxic to mammalian cells. Thus, evidence suggests that the growth of antibiotic-resistant biofilms can be overcome by manipulating the arrangement of nanocarriers holding two or more therapeutics. Gabrielyan et al. demonstrated the antibacterial effect of IONPs on ampicillin- and kanamycin-resistant *E. coli* strains (Gabrielyan et al. 2019). The result showed that in the presence of ATPase inhibitor, N,N′-dicyclohexylcarbodiimide, IONPs reduces the H^+ flux through the bacterial membrane by two-fold proving the ATP metabolism-dependent antibacterial activity of IONPs.

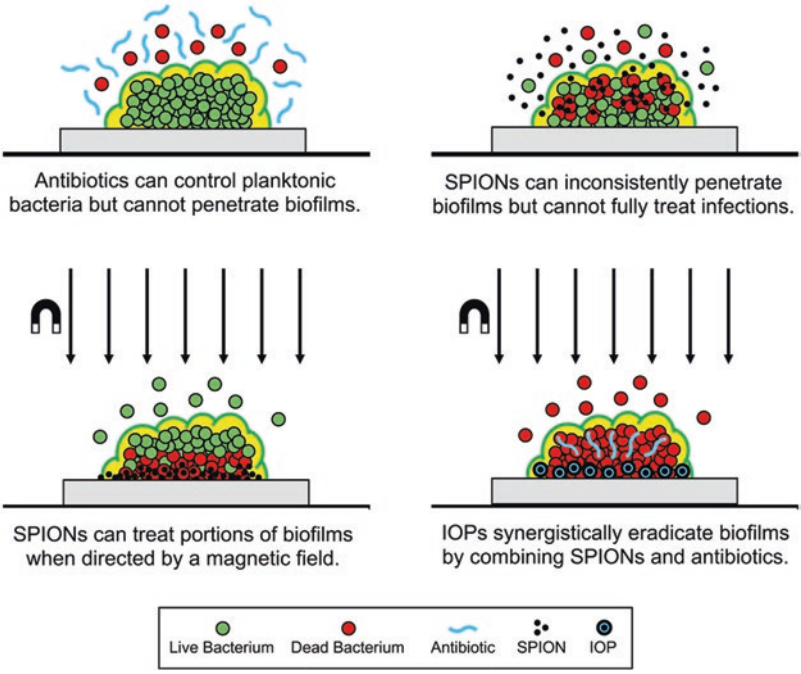

Fig. 6.5 A strategy for biofilm treatment using SPIONs and/or antimicrobials. IOP = iron oxide-encapsulating polymersome. (Reprinted with permission from *Biomaterials,* Copyright 2021, Elsevier (Geilich et al. 2017))

To overcome antibiotic resistance, peroxidase-based therapies have gained tremendous interest because of the knowledge that the peroxidase enzyme is present in blood cells to support innate immunity (Tonoyan et al. 2017). Natural peroxidase enzymes can inhibit microbial infections; however, their application is limited due to the low stability, difficult synthesis and purification process. Nanozymes with peroxidase mimetic activity have shown promising results than natural peroxidase enzymes because the former offers high stability and easy synthesis with tunable properties (Huang et al. 2019). H_2O_2 serves as an initiator in the peroxidase reaction and, therefore, used as a disinfectant in normal practice. However, due to the presence of peroxidase-degrading antioxidant enzymes (SOD and catalase) in bacterial cells, H_2O_2 alone cannot serve the purpose. Thus, a combination of peroxidase mimetic nanozyme with H_2O_2 could be used to kill the bacterial cells by generating $^{\bullet}OH$ radicals (Yin et al. 2016). In this context, Vallabani et al. have demonstrated a synergistic antibacterial mechanism from citrate-coated IONPs in combination with ATP, which facilitates the $^{\bullet}OH$ radical production (Vallabani et al. 2020). The result showed that this strategy exhibits antibacterial activity on both gram-positive and gram-negative bacteria at neutral pH in presence of H_2O_2 and thus can be used as an effective broad-spectrum antibacterial mechanism.

6.3.3 Chitosan-Based Nanomaterials

Chitosan-based NMs (CHT-NMs) exhibit antibacterial effect by various mechanisms including (i) coherence with bacterial and fungal DNA to hinder the transcription and translation processes; (ii) speedy wound recovery as chitosan deposits more collagen III and fibroblasts in addition to inhibition of inflammatory cytokine release; and (iii) removal of the acetyl group from chitosan that causes protonation at pH <6.5 and acquiring a positive charge. Thus, antimicrobial action is due to the osmotic damage caused due to interaction between positively charged molecules with negatively charged microbial cell walls (Ma et al. 2017; Wassel and Khattab 2017).

Encapsulation of chitosan in NMs improves the solubility of chitosan as well as enhanced their antimicrobial activity (Wassel and Khattab 2017). Chitosan promotes surface positive charge and thus facilitates strong interaction between microbes and CHT-NMs. Comparatively, NMs encapsulated in chitosan proved to be more efficient against *E. coli* and *S. aureus* than when used individually. High and low molecular mass chitosan are reported to be more effective against gram-positive and gram-negative bacteria, respectively. In this context, Marangon et al. demonstrated that the combination of chitosan and rhamnolipids (CHT-RL NMs) exhibits enhanced antimicrobial activity against *S. aureus* (Marangon et al. 2020). Rhamnolipids reduce the size and polydispersity index of CHT-NMs and enhance the surface positive charge to improve stability. CHT-NMs alone can only eliminate the bacteria present in the upper layer of biofilm, whereas CHT-RL NMs are more effective against the sessile bacteria and reduce the viable bacterial cells below the detection limit. This may be attributed to increased delivery of chitosan and rhamnolipid to the bacterial cell surface and consequently to their targets in gram-positive bacteria.

6.3.4 Aptamer-Conjugated Nanoparticles

The application of aptamer-conjugated NMs is also proved to be an attractive strategy to significantly enhance the efficiency as a novel class of antibiotics (Gao et al. 2018; Gutiérrez-Santana et al. 2020). Friedman et al. have demonstrated that highly stable 2-fully modified RNA aptamers could be used for targeted delivery of biomaterials. Modification of anti-SpA (*S. aureus* Protein A) aptamer with fGmH (2-F-dG, 2-OMe-dA/dC/dU) provides resistance to aptamers against alkaline hydrolysis and nucleases present in serum. Further, this aptamer was conjugated with AgNPs to show SpA-dependent antimicrobial effect (Friedman et al. 2015). Moreover, the antimicrobial effect of NMs can be further enhanced by conjugation with different aptamers to target the same pathogen. Song et al. demonstrated the TiO_2 NPs conjugated with aptamers specific for *E. coli* surface-specific ssDNA (three different aptamers). Results showed that the TiO_2 conjugated with three different aptamers

eradicated 99.9% of bacteria in 30 min than TiO_2 attached to a single aptamer (60 min) (Song et al. 2016). Due to the proximity between the aptamer and *E. coli*, there is an efficient and fast ROS transfer to the cells causing cell death. The developed nanosystem was found to be specific to *E. coli* even from the mixed culture of *E. coli* and *S. epidermidis*.

Single-wall carbon nanotubes (SWNTs) have been explored for antimicrobial activity. Using a selective aptamer conjugated to ciprofloxacin (Apt-CPX) and SWNTs, ~90% of *P. aeruginosa* biofilm inhibition was achieved (Apt-SWNTs) (Wang et al. 2018). Further, Yeom et al. demonstrated AuNPs-conjugated aptamer for delivery of antimicrobial peptide (AMP) to mammalian cells (Yeom et al. 2016). The result showed that treatment of nanoformulation increased the viability of host cells as well as inhibited the colonization of bacteria and thus promoted a 100% survival rate of infected mice. Therefore, the conjugate served as an efficient and novel agent to eradicate intracellular bacterial infection. Immobilization of vancomycin in the pores of SiNPs and their subsequent conjugation with an anti-*S. aureus* aptamer are an effective strategy for antibiotic delivery to the targeted site and also reduce the MIC and toxicity of pure antibiotics against other related species.

6.4 Use of Nanomaterials in Combating Bacterial Diseases of Animals

Bacterial diseases in animals of industrial importance are becoming a serious global concern. The common animal bacterial diseases are brucellosis (*Brucella melitensis*, *Brucella abortus*, *Brucella canis*), septicemia (*Pasteurella multicoda* and *E. coli*), mastitis (*Staphylococcus aureus*, *Staphylococcus dysgalactiae*, *Staphylococcus agalactiae*), listeriosis (*Listeria monocytogenes*), salmonellosis (*Salmonella enterica*), bovine tuberculosis (*Mycobacterium bovis*), anthrax (*Bacillus anthracis*), etc. Pathogens of these disease are transmitted from animals to humans and cause various disorders (Fig. 6.6). Although there have been several strategies adopted to circumvent the onset of these animal diseases, the use of NMs has shown potential in effective prevention. The following section will comprehend only a few important diseases and provide a summary of the progress of various treatment strategies.

6.4.1 Brucellosis

Brucellosis is a bacterial disease caused by various *Brucella* species such as *Brucella melitensis*, *Brucella abortus*, *Brucella canis*, *Brucella neotomae*, *Brucella ovis*, and *Brucella suis*. These species mainly infect goats, cattle, swine/pig, sheep, etc. *Brucella* can also be transmitted to human upon direct contact with infected animal

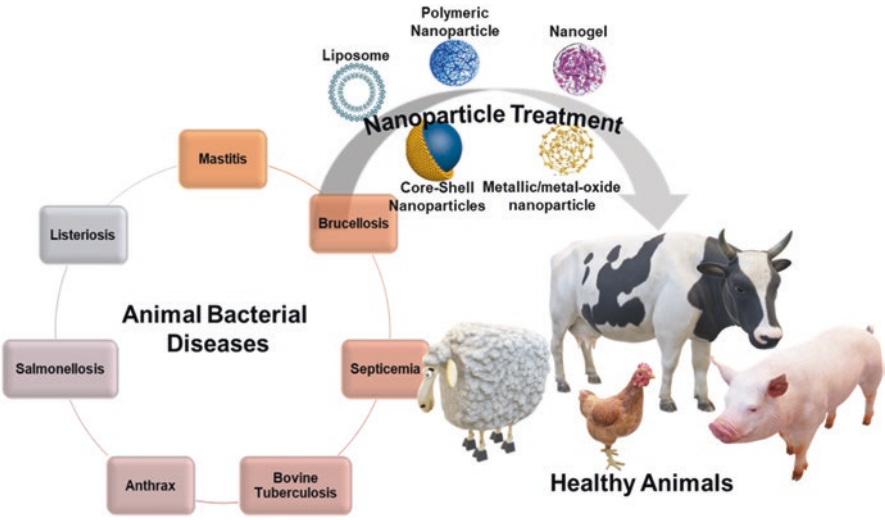

Fig. 6.6 Schematic showing a list of various diseases caused by bacteria in livestock and use of different nanomaterials for the treatment

or due to consumption of contaminated animal products such as unpasteurized milk, raw meat, and other byproducts (Thirumalaivasan et al. 2019). In 2018, a large study from 23 states of India revealed ~12% of cases of brucellosis in cattle and buffalo (Deka et al. 2018) causing an economic loss of ~58.8 million USD per year in the dairy sector (Deka et al. 2018). Brucellosis leads to the reduced milk production, death of young ones, abortion, retained placenta, stillbirths, increased calving intervals, etc. (Holt et al. 2021). Conventional antibiotics are prevalent to be used for the treatment of brucellosis including aminoglycosides, tetracycline, rifampicin, quinolones, doxycycline, streptomycin, and chloramphenicol (Khan, Zahoor 2018). The antibiotics are also delivered as encapsulated in NPs for the treatment of these pathogens. Lueth et al. (Lueth et al. 2019) developed polyanhydride NPs encapsulating doxycycline and rifampicin (individually and in combination) and tested their activity against *Brucella melitensis.* A 1:1 ratio combination of doxycycline and rifampicin in NPs showed the best release performance of drugs under in vivo system (BALB/C mice infected with *Brucella melitensis*). Within 5 days of treatment, the bacterial burden was decreased by 3 $\log_{10}$ times in the liver of mice. A treatment of 21 days led to the bacterial burden in the spleen and liver equal to free drugs (3.5 mg) and nanoformulation (1.5 mg). Thus, the NPs-based delivery improved the drug/s release time and dose sparing without compromising the activity under in vivo system. More examples of the use of NPs to display anti-brucellosis activity are summarized in Table 6.2.

Table 6.1 Nanomaterials frequently used in the treatment of MDR microbes

Nanomaterial type	Size (nm)	Antibiotics used	Targeted microbes	Important observation	References
Conventional liposome (egg yolk lecithin)	75–92.14	Cinnamaldehyde	*S. aureus*	Liposomes improve the stability and durability of cinnamaldehyde and antibacterial efficacy against *S. aureus*	Chen et al. (2019b)
Gold nanoflower	–	Ciprofloxacin	*B. subtilis*, *S. aureus*, *P. aeruginosa*, *and E. coli*	Nanoflower-drug conjugate interacts with the phosphate/amine group present on outer membrane of gram-negative bacteria and exerts its antibacterial activity	Sreedharan and Singh (2019)
Shell-cross-linked knedel-like NPs, AgNPs	22	Minocycline	MDR *P. aeruginosa* and *S. aureus*	Demonstrated enhanced antibacterial effect in comparison to free silver and minocycline at same concentration	Chen et al. (2019a)
Poly lactic-co-glycolic acid (PLGA)	226.00 ± 5.57	Teicoplanin	MR *S. aureus*	~32-fold reduction was observed in the MIC values of *S. aureus* strains when treated with teicoplanin-aptamer-PLGA NPs	Ucak et al. (2020)
Carboxymethyl tamarind polysaccharide capped AgNPs	~20–40	–	MDR *E. coli*	AgNPs inhibit biofilm formation and alter the expression and positioning of bacterial cytoskeletal proteins	Sanyasi et al. (2016)
Vanadium oxide nanodots	3.36 ± 0.23	–	*E. coli* and *S. aureus*	High antibacterial efficiency and good biocompatibility	Ma et al. (2020)
AuRh and AuRu NPs	~5	–	Polymyxin-resistant *E. coli*, *P. aeruginosa*, and *K. pneumoniae*	Destroy cell membrane and increase level of ATP and ROS. AuRh accelerate wound healing caused due to MDR bacteria	Zhao et al. (2020)
CHT-NPs	193 ± 1.9 to 530 ± 13	–	MDR *Neisseria gonorrhoeae*	CHT NPs found to be effective against all tested strains, including multiple antibiotics-resistant strain	Alqahtani et al. (2020)

(continued)

Table 6.1 (continued)

Nanomaterial type	Size (nm)	Antibiotics used	Targeted microbes	Important observation	References
AgNPs	15–20	Amoxicillin, azithromycin, clarithromycin, or linezolid	MR *S. aureus*	Proved to be promising therapy against infections caused due to MR *S. aureus*	Akram et al. (2016)
Oleic acid (OA)-monomethoxy polyethylene glycol (mPEG) nanocarrier	142.9	Vancomycin	MR *S. aureus*	In vivo therapy revealed 1.47-fold greater reduction in bacterial load after treatment with vancomycin-loaded mPEG-OA polymersomes in comparison to bare vancomycin	Omolo et al. (2017)
pH-responsive CHT NPs with new anionic gemini surfactant (AGS)	220.57 ± 5.9	Vancomycin	MR *S. aureus*	At acidic and normal physiological conditions, sustained drug release was reported with enhanced in vivo activity	Kalhapure et al. (2017)
pH-responsive nanostructured lipid carriers (NLC)	225.2 ± 9.1	Vancomycin	MR *S. aureus*	Vancomycin loaded-NLCs had enhanced antibacterial activity and reduced bacterial population 2.5-fold more than the bare drug	Osman et al. (2019)
AgNPs	13.47 ± 12	Alpha-amylase	MDR *K. pneumoniae* and MR *S. aureus*	Demonstrated higher antibacterial activity than AgNPs and α-amylase alone, with significant reduction in the biofilm formation	Abeleda et al. (2020)
CHT-NMs	180 ± 5	Mannose	Resistant *E. coli* and *L. monocytogenes*	Demonstrated enhanced antibacterial activity against biofilm. Inhibit the exponential growth of bacteria by interacting with bacterial membrane	Ejaz et al. (2020)
Graphene/chitosan nanocomposite	–	–	*K. pneumoniae* and *MDR P. aeruginosa*	Inhibited the biofilm-forming strains of *P. aeruginosa* and *K. pneumonia* via membrane damage and disruption of cellular morphology	Muthuchamy et al. (2020)

6.4.2 Septicemia

Septicemia is a type of blood poisoning caused by bacteria through the release of toxins leading to death in livestock. Hemorrhagic septicemia and colisepticemia mainly affect cattle, chickens, and water buffalo. Recovery from this disease is very rare. Hemorrhagic septicemia and colisepticemia are caused by *Pasteurella multicoda* and *E. coli.* According to a study, ~ 792 million USD per year economic loss in India is caused by hemorrhagic septicemia (Singh et al. 2014). Symptoms could be depression, fever, decreased milk production, nasal discharge, swelling on the neck and brisket, dyspnea, abdominal pain, and meningitis. A combination of streptomycin and penicillin, oxytetracycline, trimethoprim, ampicillin, doxycycline, and other antibiotics has shown promising results if the treatment is given at the early stage of the disease (Liu et al. 2018; Zhang et al. 2017). Vaccines could protect animals for 6–12 months. The vaccine is developed using a virulent *Pasteurella multicoda* strain B:3–4, dense bacterin combined with oil or alum as an adjuvant, and formalin-inactivated bacterin (Li et al. 2019a). Awaad et al. (Awaad et al. 2021) studied the clinical and pathological performance of AgNPs on colisepticemia in broiler chickens infected by the exposure of 3×10^8 CFU/mL of *E. coli* (serogroup O78) for 2 days. Chickens fed on 4, 6, and 8 mg/kg of AgNPs revealed that 4 mg/kg dose significantly reduced the bacterial burden, histopathological lesion scores, and virulence of genes. A dose of AgNPs (8 mg/kg) resulted in a severe negative effect on chicken health.

6.4.3 Mastitis

Mastitis is a fatal infection of the mammary gland in cattle, which occurs when bacteria enter a milk duct via a crack in a nipple. Different species of bacteria such as *S. aureus*, *Staphylococcus dysgalactiae*, *Staphylococcus agalactiae*, coagulase-negative *Staphylococcus, Staphylococcus uberis, Enterococci*, coliform bacteria *E. coli*, etc. cause mastitis. The symptoms include reduction in milk production, poor milk quality, swelling and redness in the udder, pus, or clotting in milk (Yashchenok et al. 2012). In India, mastitis is estimated to decrease milk production by 21%, costing about Rs. 575 million USD per annum (Sharun et al. 2021). Common antibiotics such as ampicillin, cloxacillin, tetracycline, penicillin, and streptomycin are used as an ointment and intravenous or intramuscular injection for the treatment of mastitis (Yang et al. 2019). Among NPs-based treatment, Cardozo et al. (Cardozo et al. 2014) synthesized polymeric NPs releasing NO radicals against MDR *S. aureus* and *E. coli.* The polymeric NPs encapsulating mercaptosuccinic acid (MSA) (S-nitroso-MSA particles) worked well as a NO donor. This formulation showed a sustained formation of NO radicals in presence of MDR bacterial strains (MIC 125–250 μg/mL).

6.4.4 Listeriosis

Listeriosis is a food-borne intracellular infectious disease of animals, birds, fishes, and humans. Causative bacteria (*Listeria monocytogenes*) are present in soil, plants, mud, and stream. Goats, sheep, and cattle get sick from eating contaminated corn silage. In India, ~ 7.66% of animals were found to have listeriosis (a survey conducted from 2015 to 2018) (Chaudhari et al. 2021). Common symptoms of listeriosis are inflammation in the brain, loss of balance, dystonia, loss of appetite, fever, etc. Penicillin, sulfonamides, ampicillin, tetracycline, etc. are traditional antibiotics used for the treatment (Dhama et al. 2015). Mohammed and Abdel Aziz (2019) evaluated the biocidal activity of AgNPs alone and in combination with different commercial disinfectants (sodium hypochlorite, H_2O_2, Virkon®S, benzalkonium chloride, and ammonium compound TH^{4+}®) on MDR species of *L. monocytogenes* isolated from 260 samples of animal and human stool. Among the different combination, 2% Virkon®S/AgNPs showed highest antibacterial activity (100%) followed by 5% H_2O_2/AgNPs (90%) and 1% TH^{4+}/AgNPs (90%).

6.4.5 Salmonellosis

Salmonella (*Salmonella enterica*), a gram-negative, rod-shaped bacterium, causes salmonellosis in warm-blooded animals. *Salmonella* is commonly found in contaminated food, the stool of animals, and the intestine of various animals. Different species of *Salmonella* infect different animals causing typhoid-like symptoms such as *Salmonella Gallinarum* in poultry, *Salmonella Abortusovis* in sheep, *Salmonella Choleraesuis* in pigs, *Salmonella Dublin*, and *Salmonella Typhimurium* in cattle. Diarrhea, fever, and abdominal cramps are the major symptoms of salmonellosis (Duraisamy 2016). For the treatment of salmonellosis, a high concentration of antibiotics is found to be effective when delivered at the infected intracellular site (small intestine). Among different NPs-based formulations, Xie et al. (2017) have developed enrofloxacin-encapsulated solid lipid NPs (SLN) for the effective intracellular delivery of the drug. Enrofloxacin-loaded SLN (0.24 and 0.06 μg/mL) showed ~99.97% inhibition in *salmonella CVCC541* (3.80 CFU/mL) growth, whereas same inhibition could be achieved by 0.6 μg/mL of free enrofloxacin (4.15 CFU/mL).

6.4.6 Bovine Tuberculosis

Mycobacterium bovis causes chronic disease, bovine tuberculosis, in pigs, goats, cattle, deer, cats, and dogs. Contaminated food and water are some of the common sources of infection. Bovine tuberculosis is commonly found to affect the lymph glands of the throat and lungs of infected animals. In 2017, 7.3% of the Indian (300

million cows and buffalos) population was found to be infected with bovine tuberculosis (Mydin et al. 2018) causing a ~4% decrease in overall milk production (Rinu et al. 2020). In cattle, mostly respiration-related symptoms (coughing, lymph node enlargement, dyspnea) are realized without any significant clinical signs (Lee et al. 2001). Rifampicin, isoniazid, pyrazinamide, kanamycin, ethambutol, etc. are the traditional antibacterial drugs used for the treatment of *Mycobacterium bovis* infection (Marianelli et al. 2015). Small cationic peptides as antimicrobials are recently reported as one of the best alternatives for the treatment of bovine tuberculosis (AlMatar et al. 2018). Zhou and coworkers (Liang et al. 2020) used PLGA NPs encapsulating small recombinant bovine neutrophil β-defensin-5 (PLGA-B5 NPs) as an antibacterial agent. In in vitro studies (on J774A.1 cells), PLGA-B5 NPs enhanced the expression of IL-1β, tumor necrosis factor, and IL-10. After 4 weeks of PLGA-B5 NPs treatment, *Mycobacterium bovis*-infected BALB/C mice showed a significant decrease in bacterial burden in the lungs, pulmonary area, and spleen. More examples of the use of NPs to display anti-bacterial activity is mentioned in Table 6.2.

6.4.7 Anthrax

Anthrax is a bacterial disease caused by spore-forming *Bacillus anthracis*, commonly infecting livestock animals. According to the study, ~28% of cattle are infected with *Bacillus anthracis* globally (Sushma et al. 2021). The major symptoms of the infection include abrupt fever, convulsions, staggering, depression, cardiac distress, etc. Various antibiotics are reported for the treatment of anthrax such as ofloxacin, gentamicin, doxycycline, imipenem, etc. (Weiss et al. 2011). Sun et al. (2016) synthesized a visible light-responsive carbon-containing TiO_2 NPs (C-TiO_2-NPs), causing a significant increase (~60%) in antibacterial activity than TiO_2 NPs alone. Further, C-TiO_2-NPs with visible light treatment cleared ~90% of anthrax lethal toxins (major virulence factor for anthrax). In another study by Manayani et al. (2007), chimeric virus-like NPs (CV-NPs) were synthesized to work as an antitoxin against an AB-type toxin generated by *Bacillus anthracis*. CV-NPs generated a complex system with protective antigens, which further facilitated the clearance of the toxins and generation of immune response in Sprague-Dawley rats.

6.5 Nanoparticle-Based Antibacterial Strategies in Clinical Studies

A number of nanosystem-based antibacterial strategies are being evaluated in clinical trials (Eleraky et al. 2020). Few strategies are being discussed in the following section.

Table 6.2 Application of NPs for the treatment of animal diseases caused by bacteria

Nanoparticle	Bacterial disease	Bacterial species	Study model	Observations/results	References
Gentamicin-loaded polymeric NPs (GP-NPs)	Brucellosis	*Brucella melitensis*	In vitro: THP-1 macrophage cell line In vivo: BALB/C mice infected with *B. melitensis*	GP-NPs, delivered to liver and spleen, reduced the bacterial infection in THP-1 cell line by 2-$\log_{10}$. Four doses (18 mg/L) of GP-NPs reduced ~50% infection without any adverse effect	Imbuluzqueta et al. (2013)
Curcumin-loaded niosome hydrogel-doxycycline-loaded chitosan-sodium alginate NPs	Brucellosis	*Brucella melitensis*, *Brucella abortus 544*, and *Brucella suis 1330*	Guinea pigs infected with different *Brucella* species	The formulation showed sustained release of curcumin and doxycycline but did not completely remove the artificially created *Brucella* infection. However, bacterial load was significantly lowered (~90%) from spleen and blood	Abo El-Ela et al. (2020)
Cerium oxide NPs (CeNPs)	Septicemia	*E. coli* and *S. aureus*	Sprague-Dawley rats injected with cecal materials to induce sepsis	Exposure of CeNPs to *E. coli* and *S. aureus* led to inhibition of growth of bacteria due to the reduced ROS level and decrease in NF-kB/p65 binding to DNA in rats. Lower levels of IL-6 and blood urea nitrogen were observed suggesting reduced inflammation	Selvaraj et al. (2015)

Melatonin-encapsulated polymeric (poly(ethylene glycol) (PEG) and poly(propylene sulfide) (PPS)) NPs (PEG-PPS-NPs)	Septicemia	*E. coli* serotype O127:B8	C57BL/6 J mice	The formulation released melatonin on demand (ROS-mediated delivery) in in vivo system with highest concentration in the liver, kidney, lung, and pancreas than control groups. This indicates that ROS-mediated delivery of melatonin from the PEG-PPS-NPs works against septicemia	Chen et al. (2017)
2,2,6,6-Tetramethylpiperidine-1-oxyl (TEMPO)-coated polymeric NPs (TEMPO-PNPs)	Listeriosis	*Listeria monocytogenes*	BALB/C mice infected with *Listeria monocytogenes*	TEMPO-PNPs alone did not show significant reduction in bacterial count; however, they protected organs from the oxidative damage due to the infection. A combination of amoxicillin with TEMPO-PNPs resulted in decreased bacterial counts in infected mice	Ikeda et al. (2018)
AgNPs	Listeriosis	*Listeria monocytogenes*	BALB/C mice infected with *Listeria monocytogenes*	AgNPs treatment reduced the bacterial count (10 CFU/mL) in the small intestinal and decreased the oxidative stress and inhibited expression of pro-inflammatory cytokine genes	El-Zamkan et al. (2021)

(continued)

Table 6.2 (continued)

Nanoparticle	Bacterial disease	Bacterial species	Study model	Observations/results	References
AgNPs	Mastitis	*S. aureus* and *Pseudomonas aeruginosa*	Bacterial samples from milk were collected from mastitis-infected goats	AgNPs-treated bacteria showed decreased lactate dehydrogenase activity and adenosine triphosphate levels than control group. Expression of glutathione, SOD, and catalase enzymes was downregulated, but glutathione S-transferase expression was promoted	Yuan et al. (2017)
AgNPs and copper NPs (CuNPs), Ag-Cu complex	Bovine mastitis	*Staphylococcus agalactiae*, *Staphylococcus dysgalactiae*, *Enterococcus faecalis*, *S. aureus*, *Salmonella enteritidis*, *E. coli*, and *Enterobacter cloacae*	–	AgNPs (200 ppm), CuNPs (50 to 100 ppm), and Ag-Cu complex (200 ppm) showed inhibition in biofilm formation by different bacterial species	Lange et al. (2021)
Chitosan-adjuvanted *Salmonella* subunit NPs (CSNPs)	Salmonellosis	*Salmonella*	White Leghorn layer chickens	The mucoadhesive oral drinking water containing CSNPs was used as a nanovaccine that increased the expression of different Toll-like receptors and Th1 and Th2 cytokines in chicken immune cells	Renu et al. (2020)
ZnO NPs	Salmonellosis	*Salmonella typhimurium* and *S. aureus*	–	Results revealed that ZnO NPs (1.33 mM) ruptured the cell wall of the bacteria which resulted in cell death	Akbar et al. (2019)

TiO_2 NPs	Bovine tuberculosis	*Mycobacterium bovis*	–	TiO_2 NPs (10–100 μg/mL) inhibited the biofilm formation. The metabolic activity of *Mycobacterium bovis* was decreased by threefold. Also, this particle was biocompatible to normal lung bronchus cells up to 100 μg/mL concentration	Ramalingam et al. (2019)
PLGA NPs encapsulated with argF antigen	Bovine tuberculosis	*Mycobacterium bovis*	Specific pathogen-free BALB/C female mice	NPs-treated BALB/C mice showed enhanced response of IgA, interferon-λ, and CD^{4+} T cells against *Mycobacterium bovis* infection. Also, inflammatory lesions in lung tissue and bacterial burden were found to be decreased after treatment	Ni et al. (2021)

6.5.1 Nanoparticles Delivering Antibiotics

First clinical use of ciprofloxacin-loaded liposome known as Lipoquin was under phase 1 trial in some healthy volunteers (Bruinenberg et al. 2010), and then it entered in phase 2 trial of 14 days, on 21 adults, to evaluate the initial safety, activity, and pharmacokinetics (one-time inhalation every day). Simultaneously, a double-blind and randomized phase 3 trial (ORBIT-3 and ORBIT-4) was conducted internationally in a similar region to investigate the safety and efficacy of Lipoquin (Haworth et al. 2019). Further, amikacin-loaded liposomes were also studied in clinical trials. In a double-blind phase 2 trial, efficacy, tolerability, and safety of once-daily (QD) dosing of amikacin (590 mg) versus placebo was conducted for 84 days against refractory *nontuberculous mycobacteria* lung infection. Another study also examined the stability and safety of once-daily dosing of 560 mg amikacin-loaded liposome for six cycles over 18 months in the patient suffering from cystic fibrosis and chronic infection by *P. aeruginosa* (Eleraky et al. 2020).

A new study of phase 2 trial of inhaled liposomal amikacin 590 mg, once-daily dosing for 12 months has been evaluated for their safety, tolerability, and efficacy of treatment against *Mycobacterium abscessus* lung disease. Further, a phase 3 trial was also conducted to study the safety and long-term tolerability of inhaled amikacin-loaded liposome (590 mg/day) in the patient suffering from chronic infection of *P. aeruginosa* (Eleraky et al. 2020).

6.5.2 Nanoparticle Delivering Antimicrobial Peptides and Antitoxins

Antimicrobial peptides exhibit less chance of resistance development and produce wide-spectrum antibacterial activity (Molchanova et al. 2017). They mainly target bacterial cell membrane synthesis of protein, nucleic acid, cell wall, enzymatic activity, and ATP efflux process. Some of the antimicrobial peptides are nisin (interfere in cell wall synthesis); indolicidin (interfere in protein synthesis); buforin II (inhibit RNA synthesis); and histatins (alter ATP efflux) (Molchanova et al. 2017). In the treatment of bacterial-driven diseases, targeting the bacterial components which are responsible for their virulence, i.e., toxins, is a major aspect of nanomedicine these days. Bezlotoxumab is a human monoclonal antibody and the first approved antitoxin in 2016 which is designed to target the toxin B of *Clostridium difficile* (Mullard 2016). Several antitoxin agents are still in clinical trials like monoclonal antibodies targeting *S. aureus*' α-toxin and type III toxin secretion moiety of *P. aeruginosa* (Azeredo da Silveira and Perez 2017). Further, a novel empty liposome, CAL02, has been developed which leads to a synergistic effect with drugs and antibiotics and also demonstrated the ability to rescue mice from major infections, such as staphylococci, through the adsorption of toxins (Laterre et al. 2019).

6.5.3 *Limitations of Nanoparticle-Based Antibacterial Agents*

Clinical translation of NPs-based antibacterial agents faces several challenges including biocompatibility, safety, laws and regulations, intellectual property rights (IPRs), and high cost than traditional therapies (Narang et al. 2013; Hua et al. 2018). Important issues which should be considered during clinical translation of nanomedicine are:

(i) *Preclinical evaluation of toxicity*: To avoid the side effects of nanomedicine, they should be initially evaluated in vivo (inappropriate animal models) and analyze their pharmacokinetics and pharmacodynamics properties. Further, biocompatibility and stability of NPs and interaction with the surrounding medium should also be considered (Eleraky et al. 2020).

(ii) *Nanopharmaceuticals design*: In the designing of antimicrobial nanoformulations, factors like biodegradability, administration route, and physical and chemical stability should be considered. Large-scale production of antimicrobial drugs should consider the factors like reproducibility and quality control assay, i.e., polydispersity, the storage stability of final product, charge, morphology, incomplete purification, and consistency of nanomedicine (Tinkle et al. 2014; Teli et al. 2010).

(iii) *Challenges in commercialization*: Launching antimicrobial products in the market is a complex process as it is time- and cost-intensive. Simple techniques should be employed to test the therapeutic efficacy of antimicrobial drugs in the patient. With the use of NMs, biological half-lives of drugs have been greatly enhanced; thus, specialized toxicological tests in animals should be conducted to examine both short- and long-term side effects of antimicrobial nanomedicine (Eleraky et al. 2020). Thus, regulatory guidelines should be developed to examine the nanotoxicological effect and for standard and validated use of NPs in clinical development (Accomasso et al. 2018).

6.6 Conclusion and Expected Future Developments

With the increasing incidence of resistance against antibiotics, it is essential to find alternative methods exhibiting strong antimicrobial activity. In this quest, NPs-based formulations containing essential oils, antimicrobial peptides, and other natural products have been explored. Several studies have confirmed that these NPs-based formulations of certain NPs themselves are better in inhibiting the growth of pathogenic bacterial species than their non-formulated counterparts. Although there are several vaccines available to protect humans from bacterial pathogens, the emergence of new bacterial epidemics and pandemics would require significant efforts for the quick development of new vaccines. Traditional methods of vaccine development take several years; therefore, novel methods must be explored to reduce the development phase in months. Nanotechnology could play an important role to act

as adjuvants to facilitate the delivery of vaccine components. Currently, multifunctional nanocarriers are developed that could be used to deliver multiple vaccines in one shot. These nanocarriers could either encapsulate or carry the vaccine components on their surface and offer high payload delivery to facilitate quick and long-term immune responses.

There has been a lot of effort devoted to develop nanotechnology-based antibacterial agents and vaccines for bacterial diseases in humans; however, limited efforts are made to protect animals from animal diseases caused by microbes. Vaccines for animal diseases (brucella, anthrax, and foot and mouth disease) are developed; however, they have not been improved for decades. These vaccines face several challenges of storage condition, being ineffective in immunocompromised animals, and requiring multiple doses. In the coming years, nanotechnology-based vaccines could be developed to offer long-term stability and better immune response with one-shot treatment. Oral and intranasal vaccines involving the controlled release of vaccine components would be another area of interest. The NPs-based antibacterial agents for animal diseases are so far limited to certain toxic particles such as ZnO and AgNPs, leaving a huge scope of research in developing novel NMs and nanoformulations to combat the drug-resistant bacterial species. NPs conjugated aptamers or another specific biomolecules-based targeting of particular bacterial species would be required to develop. Novel theranostics would also be required for simultaneous detection and treatment of pathogenic and drug-resistant strains of bacteria. Overall, there remains a tremendous amount of research work for developing novel NPs-based antibacterial materials to effectively and selectively cause damage to pathogenic strains barring non-pathogenic strains, humans, and the environment.

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Chapter 7
Microbial Resistance Mechanisms and Potential of Metal-Organic Framework in Mitigation Thereof

Shakil Ahmed Polash, Linda Varadi, and Ravi Shukla

Abstract Though the emergence of drug resistance is a natural phenomenon in the life cycle of a bacterium, the rise of drug-resistant microbial pathogens is a global public health concern. Researchers from all parts of the world are working restlessly to develop an effective and alternative option of existing antibacterial tools. Advanced nanomaterials bring a new hope which kills bacteria in an irreversible mode of action. Metal-organic framework (MOF) is a hybrid nanocarrier system made of an organic ligand connected with inorganic metal ions. The favourable physicochemical properties of MOFs offer the delivery of antibacterial agents. The released metal ions interact strongly with the bacterial membrane and kill the bacteria via cell membrane disruption, reactive active oxygen species (ROS) generation and metabolic interference. In this chapter, we shall summarise the problem of antimicrobial resistance and current tools in controlling drug-resistant bacteria and evaluate the potency of MOFs as a future antimicrobial agent.

Keywords Infectious disease · Antimicrobial resistance · Drug delivery system · Metal-organic framework · Synergistic effect · ROS

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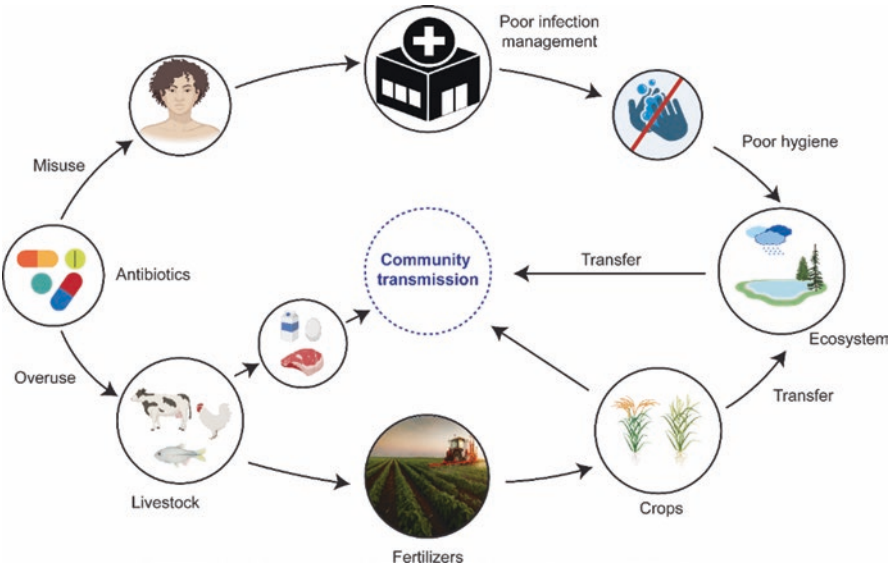
7.1 Introduction

Bacteria and yeasts are the oldest living organisms on earth and evolved as micrometre-sized single-cell organisms with a wide range of species, sizes, shapes, morphologies, functions and habitats. Nearly one trillion species of microbes have been identified on earth including bacteria, fungi, viruses and intracellular parasites. They play an essential role in nutrient recycling by decomposing organic wastes, boosting crop yields by increasing soil fertility and aiding food digestion and the production of essential nutrients of another organism from within (Amin et al. 2021). Although less than 1% of bacteria are responsible for infectious disease development, their impact on human, plant and animal health is greatly burdening.

Antimicrobial resistance (AMR) is one of the most life-threatening health concerns to humans at this moment. The increasing number of AMR species is made worse by the often frequent use of antimicrobial drugs and the lack of new drugs being developed and approved (Michael et al. 2014). Another major contributor to the emergence of AMR is the lack of awareness and compliance with surveillance, especially in low-income and developing countries. For instance, about a 65% increase in global antibiotic consumption was identified in low/middle-income countries (Klein et al. 2018). Other factors like misuse of antibiotics, poor infection management and lack of sanitation accelerate the spread of AMR. As multidrug-resistant (MDR) species are found in both biotic (human, animal) and abiotic (crop, water, soil) sources; humans and animals acquire resistant genes through contaminated water, food and livestock sources (Scheme 7.1). Nowadays, infectious



Scheme 7.1 Transfer of antibiotic resistance genes

pathogens become resistant to existing antibiotics, and we refer to the threat of MDR pathogens as a 'silent pandemic'.

Microbial infections become the top global public health concern, with a stark prediction of over ten million loss of lives globally by the year 2050, if the available and routinely applied surveillance, diagnosis and treatment tools fail to improve (de Kraker et al. 2016). To demonstrate the urgency, more than 2.8 million antibiotic-resistant infection cases are recorded per year on average in the USA only (CDC 2019). The most common disease-causing resistant pathogens include *Enterococcus*, *Staphylococcus*, *Klebsiella*, *Acinetobacter*, *Pseudomonas* and Enterobacter spp. (collectively called as a group of 'ESKAPE' pathogens). These bacterial species are responsible for high healthcare costs and severe infections and even death (Founou et al. 2017). There are an increasing number of global efforts and non-profit organisations joining at the forefront of the battle against AMR. Some of the examples include but are not limited to CARB-X (https://carb-x.org/), Longitude Prize (https://longitudeprize.org/), Bill and Melinda Gates Foundation (https://www.gatesfoundation.org/), Co-ADD (https://www.co-add.org/), Global Antibiotic R&D Partnership (GARDP) (http://gardp.org/), Save Antibiotics (https://www.nrdc.org/save-antibiotics) and British Society for Antimicrobial Chemotherapy (BSAC) (https://bsac.org.uk/). International campaign like World Antimicrobial Awareness Week (WAAW) helps to spread awareness on antimicrobial resistance, globally.

Since AMR occurs over time, the burden of AMR infections on the healthcare system is approximated as 100 trillion dollars per annum (Tauman et al. 2009). Moreover, failure in evidence-based and effective antibiotic treatment leads to further development of MDR species and mistreatment of the patients. Hence, new antibacterial agents are urgently required to control them. These facts also emphasise the need for novel ways of reactivating/salvaging existing and approved antibacterial agents. One such way is to develop drug delivery systems that enable intracellular delivery of existing small molecular or biomolecular drugs that may have been rendered inactive by certain resistance mechanisms.

Beyond classical small molecular drugs, nanomaterials also could play a significant role in AMR control. Different kinds of nanomaterials (e.g. heavy metals and various oxides, lipid/peptide nanoparticles, carbon nanotubes) have been studied so far as potential antimicrobial agents acting via their material properties or acting as targeted delivery systems with the possibility for controlled release (Majhi et al. 2020; Yasuyuki et al. 2010; Ng et al. 2013; Liu et al. 2009; Mocan et al. 2017). An emerging class of nanomaterial used as drug carrier vehicles with a favourable biological activity is called metal-organic framework (MOF). MOF was first discovered and described in the 1990s, and since then the field saw exponential growth to date mostly due to the broad applicability and relatively easy-to-engineer properties. MOF is a promising material for multiple applications including catalysis, carbon capture, energy storage, biomedicine and biosensing (Dhakshinamoorthy et al. 2019; Aniruddha et al. 2020; Baumann et al. 2019; Velásquez-Hernández et al. 2020; Du et al. 2021). In their most hierarchical crystalline form, they were initially mostly used in sensing and gas separation-based applications, but now the exceptional properties of MOFs resulted in their exploration in biological and biomedical

research. MOF is made of a polymeric network where the positively charged metal node connects with electron-rich/negatively charged organic linker molecule via coordination linkage. These are available both in crystalline (Liu et al. 2021) and non-crystalline (i.e. amorphous, liquids and glasses) form (Fonseca et al. 2021). The overall physicochemical properties of MOF rely on the type and relative amount of metal ions and organic linkers and the synthetic methods used, defining the coordination between the building blocks. Moreover, fine-tuning the ligand-linker ratio and solvents used during synthesis offers ways to achieve versatile properties and extends possible applications (Lu et al. 2014a). Most relevantly to this chapter, MOF is valuable as carrier of a diverse group of biologically active compounds: therapeutic proteins, enzymes, fatty acids, nucleic acid and vaccines (Velásquez-Hernández et al. 2020).

In this chapter, we give a brief overview of the currently available small molecular antibiotics and their resistance mechanisms in bacteria, followed by an account on the utility of MOFs in irreversible damage to microorganisms, and/or as drug delivery systems to 'reactivate'/salvage known antibiotics that may have depleted activity by AMR.

7.2 Antimicrobial Agents

Antimicrobials are natural or synthetic agents able to have a debilitating effect on microbes either by killing them (bactericidal) or inhibiting their proliferation and growth (bacteriostatic). In 1928, 'Penicillin', a game-changer in the control of infectious diseases, was discovered by Scottish microbiologist Alexander Fleming resulting in the significant decline of their impact. Antimicrobials can be categorised based on their chemical structures that mostly define their mechanism of action too. Antimicrobial agents include a group of drugs capable to inhibit microbial growth and prevent infections. The main classes of antimicrobials and their target microorganisms are shown in Table 7.1. Antimicrobial drugs can target many different cellular metabolic pathways to achieve growth inhibition or cell death. They can interrupt essential bacterial enzymes or metabolic pathways, trigger oxidative stress, corrupt DNA replication, introduce abnormalities during various stages of protein synthesis (e.g. transcription, translation, post-translational modification, protein folding), or result in the incorporation of dysfunctional building blocks/faults into the cell wall (Kohanski et al. 2010; Anderson et al. 2012) (Fig. 7.1). This eventually induces microbial growth inhibition or death (Cho et al. 2014; Murima et al. 2014).

Despite there are plethora of antimicrobial drugs available, microorganisms have been evolving sophisticated ways to escape the effect of antimicrobials by developing various drug resistance mechanisms. These resistant strains of microbes can survive and reproduce even after exposure to the antimicrobial drug. Repetitive pressure exerted by the antimicrobials on bacteria can induce genetic alterations allowing them to render the drugs inactive. Multiple drug-resistant species were

Table 7.1 Different classes of antibiotics and their mode of action

Antibiotic class	Active against	Common feature(s)	FDA-approved drug(s)	Adverse effect(s)	Reference
A. Targeting DNA					
Fluoroquinolones	Mostly Gram-negative	Fusion of aromatic rings and carboxylic acid groups, exhibit concentration-dependent bactericidal activity, inhibit the activity of DNA gyrase and topoisomerase	Levofloxacin, ciprofloxacin, moxifloxacin, ofloxacin, delafloxacin	Disturbances in the central nervous system, upper gastrointestinal, peripheral neuropathy, tendinopathy	Giguère and Dowling (2013)
Rifamycin	Broad-spectrum	Suppress bacterial RNA synthesis	Rifabutin, rifampin, rifapentine, rifaximin	Hepatitis, myelosuppression, disturbances in the gastrointestinal tract and central nervous system	Koo and DuPont (2010)
Nitroimidazole	Obligate anaerobic	Inhibit DNA synthesis	Metronidazole, tinidazole	Gastrointestinal disturbance, central nervous system effects, peripheral neuropathy	Brook et al. (2013)
B. Targeting metabolic processes					
Sulphonamide	Broad-spectrum	Contain a sulphonamide group, inhibit conversion of p-aminobenzoic acid to dihydropteroate	Mafenide, sulphacetamide, sulphadiazine, sulphadoxine, sulphamethizole, sulphanilamide, sulphisoxazole	Hypersensitivity reactions, crystalluria, hematologic reactions, photosensitivity, insomnia, headache	Sköld (2000)
Trimethoprim	Aerobic bacteria	Block sequential steps in bacterial folate metabolism, prevent dihydrofolate reduction		Folate deficiency, hyperkalaemia, urinary tract infection	Brogden et al. (1982)
Aminoglycoside	Most Gram-negative aerobic and facultative anaerobic bacilli	Have amino sugar substructure, bind to the 30S ribosome	Streptomycin, kanamycin, gentamicin	Renal toxicity, vestibular toxicity, inner ear toxicity	DeMars et al. (2016)

(continued)

Table 7.1 (continued)

Antibiotic class	Active against	Common feature(s)	FDA-approved drug(s)	Adverse effect(s)	Reference
Macrolide	Aerobic and anaerobic Gram-positive cocci	Contain macrocyclic lactone ring, bind to the 50S subunit of the ribosome	Azithromycin, clarithromycin, erythromycin	Gastrointestinal disturbance, inhibition of hepatic metabolism, nausea	Brook et al. (2013)
Tetracycline	Aerobic and anaerobic bacteria	Four adjacent cyclic hydrocarbon rings bind to the 30S subunit of the ribosome	Tetracycline, doxycycline, lymecycline, minocycline	Gastrointestinal disturbance, candidiasis, photosensitivity, fatty liver, nausea, vomiting, rash, loss of appetite	Chopra and Roberts (2001)
Chloramphenicol	Gram-positive and Gram-negative cocci and bacilli	Bind to the 50S subunit of the ribosome	Pentamycetin, chloromycetin	Bone marrow depression, nausea, vomiting, diarrhoea	Sood (2016)
Oxazolidinone	Gram-positive bacteria	Contain 2-oxazolidone, bind to P site at the ribosomal 50S subunit	Linezolid, posizolid, tedizolid	Gastrointestinal disturbance, myelosuppression, neuropathy	Bozdogan and Appelbaum (2004)
C. Targeting cell wall synthesis					
β-Lactam	Broad-spectrum	Has a β-lactam ring in nucleus, inhibits the synthesis of the peptidoglycan layer of bacterial cell walls	Cephalosporins, clavams, carbapenems, monobactams, penicillins	Diarrhoea, nausea, rash, fever	Smet et al. (2010)
Glycopeptide	Gram-positive cocci	Carbohydrate-linked peptides, inhibit peptidoglycan synthesis	Vancomycin, teicoplanin, telavancin, ramoplanin	Kidney failure, neutropenia, deafness	Matsuda et al. (2017)
Daptomycin	Gram-positive bacteria	Cyclic lipopeptide causes rapid depolarisation of the membrane due to potassium efflux; aggregation of daptomycin alters the curvature of the membrane and causes ion leakage via holes		Eosinophilic pneumonia, myopathy, insomnia, rash, fever	Rybak (2006)

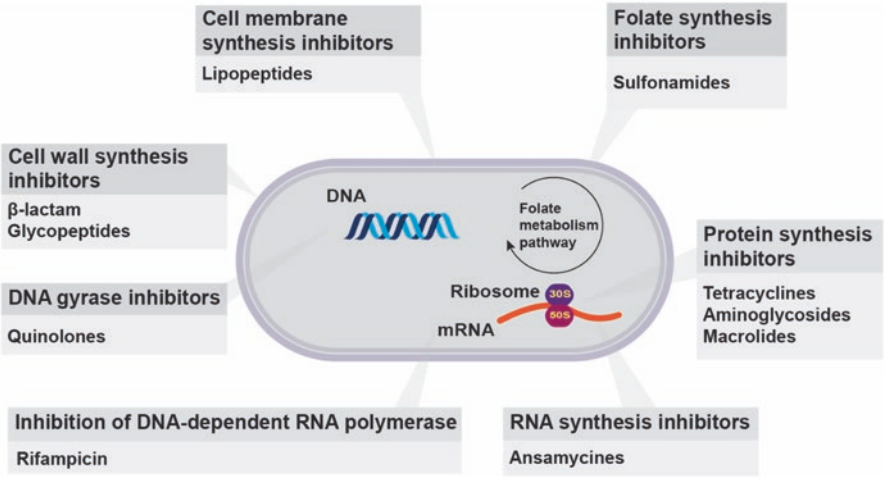


Fig. 7.1 The general mode of action of antibiotics in the microbe

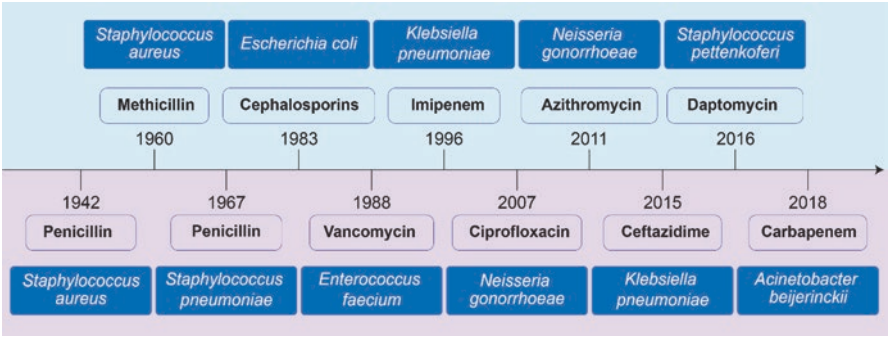


Fig. 7.2 Timeline of major antibiotic resistance event

identified soon after the discovery of penicillin. Soon after that different resistant species were also identified, and this list is still increasing (Fig. 7.2). This increase in MDR pathogens encourages finding an alternative solution to tackle the lethal effect of that species.

7.3 Resistance Mechanisms

Bacteria develop resistance mechanisms naturally through evolutionary processes. Most often, resistant genes can develop via mutation, and this process is often accelerated by continuous exposure to antimicrobials (Fig. 7.3). However, resistant genes can also be carried by extracellular DNA fragments – also called mobile genetic

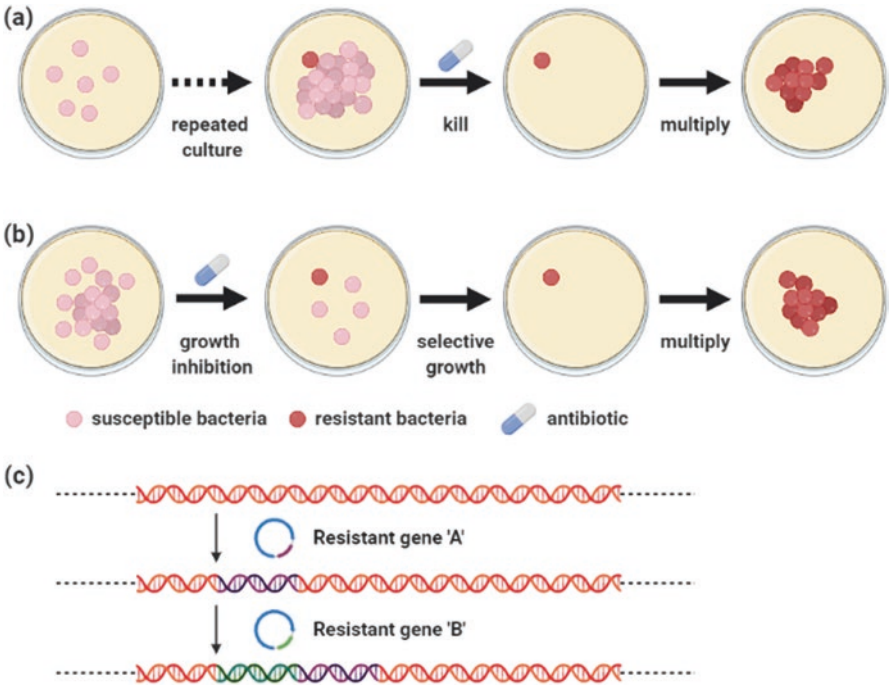


Fig. 7.3 Development of resistant bacteria through (**a**) evolution, (**b**) external stress, and (**c**) plasmid-mediated resistant gene acquisition

elements between organisms, such as plasmid, an extrachromosomal DNA molecule that has self-replication properties (Van Hoek et al. 2011; Linkevicius et al. 2013). There are two ways by which susceptible strains acquire resistant genes: vertical and horizontal gene transfer. Mutation in the genomic DNA and transmission of the mutated genome to the offspring are responsible for vertical gene transfer (Laws et al. 2019). On the other hand, horizontal gene transfer refers to the transfer of resistance genes from a non-parental source to susceptible bacteria through conjugation, transformation and transduction which is carried out mainly by plasmids (Fig. 7.3c) (Brito 2021).

The key mechanisms involved in microbial resistance development are (i) mutation of the antimicrobial targets (e.g. enzymes); (ii) reduction in drug uptake; (iii) inactivation of the antimicrobial agents; and (iv) excretion of the antimicrobial agents by efflux pumps (Fig. 7.4). Moreover, the availability of similar enzymes which are not affected by the antimicrobial agent also influences development of microbial resistance. Therefore, the effectiveness of antimicrobial drugs lowers significantly.

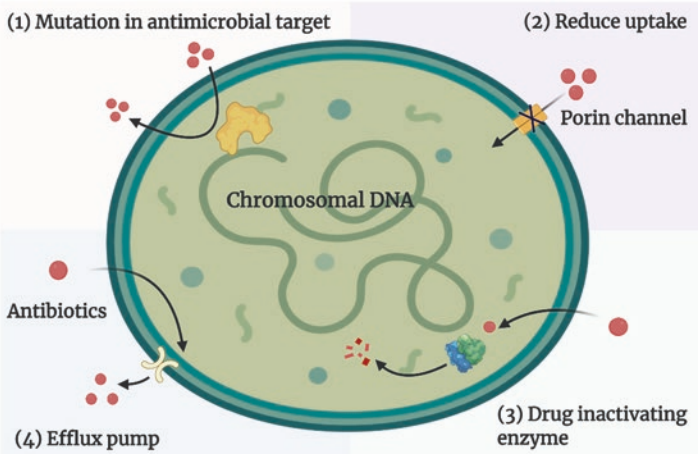


Fig. 7.4 Main ways bacteria develop resistance against antimicrobial agents

7.3.1 Mutation in Antibacterial Target

Errors during DNA replication and exposure to mutagenic substances cause mutation in the genome sequence. These phenomena result in a change of antimicrobial targets which play a critical role in resistance development. For example, mutation in the penicillin-binding protein (PBP) results in β-lactam resistance (Sun et al. 2014), while mutation of *gyrA* encoding for DNA gyrase structure results in quinolone resistance (Gruger et al. 2004). Similarly, alteration in the 30S ribosomal subunit causes resistance to streptomycin (Demirci et al. 2013). Though mutation is a natural phenomenon, frequent exposure to antibiotics boosts up spontaneous mutation development in the bacterial genome. Therefore, misuse of antibiotics may enhance mutation rate in target cells by accelerating adaptation of pathogens in presence of antibiotics.

7.3.2 Reduction in Drug Uptake

A membrane-penetrating drug is able to reach the cytoplasm and display better action. The OM of Gram-negative bacteria built upon lipopolysaccharides provides the first line of defence against drugs (Nikaido 1989). Under this, a thin peptidoglycan layer forms the cell wall. Gram-positive bacteria are lack of outer membrane while their cell wall presents as a thicker multilayered peptidoglycan envelope. The lipopolysaccharide layer of the outer membrane in Gram-negative bacteria provides an efficient physical barrier (Blair et al. 2014) and restricts the entry of hydrophilic molecules while bypassing hydrophobic drugs (e.g. rifampicin, fluoroquinolone) are less impacted (Kumar and Schweizer 2005). Thus, Gram-negative bacteria show

intrinsic resistance to vancomycin, β-lactams, aminoglycosides, chloramphenicol and tetracyclines due to their impermeable outer membranes (Munita and Arias 2016). However, there are examples, where a mutation in *Neisseria gonorrhoeae* OM protein results in changes in the lipid bilayer organisation and its permeability, leading to resistance to various hydrophobic antibiotics (Guymon et al. 1978). One of the most relevant causes of reduced drug uptake is reduction or alteration in the porin channels. Porins are transmembrane proteins and are made of antiparallel β-strands in a cylindrical tube (Schirmer 1998). It works as a 'molecular sieve' and allows the diffusion of small hydrophilic molecules into the cytoplasm. They can be classified based on activity (i.e. specific or non-specific) and structure (i.e. mono-, di- or trimeric porins) (Pagès et al. 2008). Revolution in bacterial outer membrane (OM) layer reduces porin number and declines diffusion of small hydrophilic molecules (e.g. β-lactams, fluoroquinolones, tetracycline and chloramphenicol) (Hancock and Brinkman 2002). Thus, mutations in porin proteins have a negative correlation with the susceptibility of bacteria to antimicrobials.

7.3.3 Drug Inactivation

Antibiotics interfere with bacterial protein synthesis routes, interact with the metabolites and induce apoptosis (Wilson 2014). Resistant bacteria acquire genes that encode antibiotic-degrading enzymes. One very common enzyme is β-lactamases which hydrolyse and inactivate the β-lactam ring in penicillins, cephalosporins and carbapenems (Tooke et al. 2019; Santajit and Indrawattana 2016). Other drug-inactivating enzymes are aminoglycoside-modifying enzymes (AMEs) and chloramphenicol acetyltransferases (CATs). They irreversibly modify the active site of the antibiotics and inhibit their functions. Therefore, antibiotics lost their potential to kill bacteria. The AMEs covalently modify the hydroxyl or amino groups of aminoglycoside molecules (Almaghrabi et al. 2014). They are classified based on the type of reactions they perform at the site of modification. For example, aminoglycoside 3′-phosphotransferase or APH (3′) is responsible for kanamycin and streptomycin resistance. Aminoglycoside 6′-phosphotransferase or APH (6′) is found in Gram-negative bacteria only which inactivates amikacin and gentamicin (Ramirez and Tolmasky 2010). Some AMEs also have bifunctional activities such as AAC(6′)-APH(2′) which is responsible for aminoglycoside resistance (Chow et al. 2001). CATs bring a chemical modification of chloramphenicol (Schwarz et al. 2004).

7.3.4 *Efflux Pumps*

Efflux pumps (EPs) are cytoplasmic membrane proteins that drain out intracellular drugs (Soto 2013). This is often found in Gram-negative bacteria where both chromosome and plasmid encode genes of EPs (Blair et al. 2015). Hence, some are expressed constitutively, and the rest of them are overexpressed under certain environmental stimuli (Li et al. 2015). This exclusion mechanism lowers active drug concentration in cytosol and is responsible for drug resistance (Poole 2005). A proton (H^+) motive force plays a key role in controlling drug movement in and out of the cells. The EP is made of three classes of proteins, i.e. energy-dependent transporter protein in the inner membrane, membrane fusion protein in the periplasmic space and outer membrane protein. Most often EPs are classified into five families based on their structural composition, energy source and substrate specificity (Fig. 7.5). The role of the efflux pump on some commonly used antibiotics is summarised in Table 7.2.

ATP-binding cassette (ABC) transporter is made up of six α-helix transmembrane segments (TMS) and worked in conjunction with cytoplasmic ATPases (Wilkens 2015). They typically transport amino acids, ions, polysaccharides and drugs (e.g. fluoroquinolones, tetracyclines) (Garvey et al. 2011; Lubelski et al. 2007). However, the major facilitator super (MFS) family has the greatest substrate diversity and is made up of 14 TMS. They can transport anions, metabolites, sugars and drugs (e.g. macrolides, tetracycline) via solute/cation symport or solute/H^+ antiport (Blair et al. 2014). The small multidrug resistance (SMR) transporters efflux

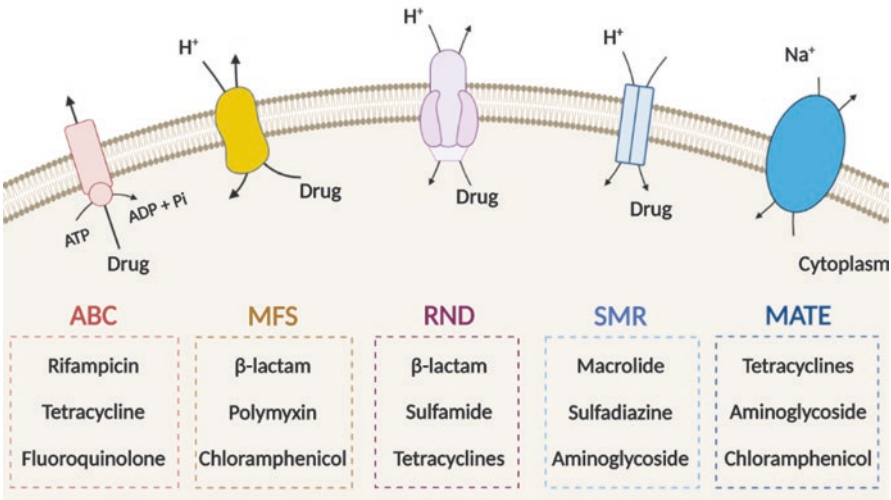


Fig. 7.5 Schematic representation of five major efflux pump families, e.g. ATP-binding cassette (ABC) family, major facilitator super (MFS) family, resistance-nodulation-division (RND) family, small multidrug resistance (SMR) family and multidrug and toxic compound extrusion (MATE) family. (Adapted and modified from Blanco et al. (2016). Copyright 2016 MDPI)

Table 7.2 Role of efflux pumps in association with different antimicrobial resistance mechanisms

Drug	Drug limitation	Mechanism	Efflux pump(s)	Reference
Carbapenems	Alteration of porin selectivity	Phosphate binding protein alteration	RND	Fernández and Hancock (2012)
Chloramphenicol	Reduction of porins	Ribosomal modification	RND, MFS	Long and Porse (2003)
Cephalosporins	Alteration of porin selectivity	Phosphate binding protein alteration	RND	Kocaoglu and Carlson (2015)
Penicillins	Alteration of porin selectivity	Alteration of PBPs	RND	Kocaoglu and Carlson (2015)
Fluoroquinolones	Reduction of porins	DNA gyrase and topoisomerase modification	MATE, MFS, RND	Hooper and Jacoby (2015)
Glycopeptides	Cell wall thickness	Peptidoglycan modification	RND	Blaskovich et al. (2018)
Macrolides	Reduction of porins	Ribosomal mutation	ABC, MFS, RND	Golkar et al. (2018)
Oxazolidinones	Reduction of porins	Ribosomal methylation	RND	Stojković et al. (2016)
Tetracyclines	Reduction of porins	Ribosomal shield	RND, MFS	Li et al. (2013b)
Sulphonamides	Reduction of porins	Reduction of dihydropteroate synthase binding	RND	Yun et al. (2012)

Abbreviation: *ABC* ATP-binding cassette family, *MFS* major facilitator super family, *RND* resistance-nodulation-division family, *MATE* multidrug and toxic compound extrusion family

lipophilic cations and are responsible for *β*-lactams and aminoglycosides resistant (Reygaert 2018). Another common pump is a resistance nodulation division (RND) that is made up of 12 TMS and contains 2 large periplasmic loops between TMS 1 and 2 and TMS 7 and 8 (Nikaido 2011). They utilise substrate/H^+ antiport mechanism to efflux substrates and confer resistance to tetracyclines, chloramphenicol and β-lactams (Piddock 2006). Finally, the multidrug and toxic compound extrusion (MATE) family is made up of 12 TMS and uses Na^+ gradient as the energy source to efflux fluoroquinolone, aminoglycosides and cationic dyes and confer tetracycline resistance mostly (Poole 2005; Chopra and Roberts 2001). To overcome this, EP inhibitors are often used to restore the antimicrobial efficacy of a drug against a resistant species. Shriram et al. discussed briefly on plant-based EP inhibitors and recent developments including future prospects (Shriram et al. 2018).

7.4 Current Approaches to Mitigate AMR

De-escalating AMR takes multidisciplinary and global efforts. Antimicrobial surveillance strategies are created to tackle the overuse of antimicrobial agents via, for example, prompt diagnostics and therapeutic choices, improving prescription habits, eliminating antimicrobial use in agricultural activities and better wastewater handling. Parallel to these, the development of classical small molecular drugs as antibacterial therapeutic tools is ever relevant. Beyond the classical drug discovery, a few alternative approaches are (re-)emerging as tools to develop antimicrobial tools with novel mechanisms of action or to reactivate/potentiate existing antimicrobial agents.

One of the ancient but still relevant approaches is the use of plant-based materials (phytochemicals) (Shriram et al. 2018). These plant-derived secondary metabolites can also be extracted and utilised for human medicine. Phytochemicals having intrinsic antibacterial activity are classified into several groups based on their chemical structures (e.g. phenolics, terpenoids, alkaloids and polypeptides) (Khameneh et al. 2019). Among these, most of them act as an antioxidant agent to induce cell death. Here they interfere in enzyme activities and signal transduction pathways (Barbieri et al. 2017). For example, green tea aqueous extract showed a higher bactericidal effect against clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug-resistant *Pseudomonas aeruginosa* (Radji et al. 2013). Though they offer fewer side effects compared to more potent synthetic drugs, the therapeutic activity of phytochemicals is relatively weak. The required bioavailable concentration is very high, and therefore they are rarely used as therapeutic agents. However, with the evolution of modern genomics and bioinformatics tools, high-throughput screening of phytochemicals to find new molecules to combat resistance is more efficient than ever before (Dos Santos et al. 2016; Kebede et al. 2021).

Other potential agents are antimicrobial peptides (AMPs). These evolutionarily conserved peptides are made of short-chain amino acids and become very effective against microbial species (Mahlapuu et al. 2016). They can originate from natural sources or be synthetically produced. Ideally, cellular uptake of AMPs happens specifically by bacterial cells via the formation of transient pores on the outer membrane resulting in internalisation into the cytosol. This uptake pathway is generally selective toward microbial membranes and thus has no or negligible effect on the host cells (Ebenhan et al. 2014; Wimley 2010; Souza et al. 2020; Jenssen et al. 2006; Lima et al. 2021). Some AMPs like buforin II can diffuse into cells without damaging the cell membrane and bind to DNA and RNA (Park et al. 1998). MRSA is sensitive to nisin, a polycyclic antibacterial peptide, by blocking the cell wall synthesis mechanism (Brumfitt et al. 2002). Though AMPs have broad-spectrum activity, some potential toxicity issues remain unknown till now.

Targeted protein degraders (TPDs) seize cellular metabolic events and activate knockdown of protein of interest (Powell et al. 2021; Gopal and Dick 2020; Izert et al. 2021). Morreale et al. developed a small-molecule degrader (called

BacPROTACs) which is able to degrade protease in mycobacteria (Morreale et al. 2021). Designing an appropriate targeted degradation system may have broad spectra of targeting bacterial pathogens with minor modifications in currently available drugs. Therefore, TPDs have the potential to become effective antimicrobial protein degraders in future.

Cationic transition and heavy metal complexes stimulate oxidative stress on bacteria and disrupt the microbial cell membrane (Frei et al. 2021). Transition metals exhibit different oxidation states and can interact with negatively charged molecules. Nandanwar and Kim outlined and discussed the broad antimicrobial activity of ruthenium- and copper-based antimicrobial drugs (Nandanwar and Kim 2019).

Vaccines and therapeutic monoclonal antibodies play a strategic role in limiting the increase of antibiotic-resistant microorganisms. Vaccines play a major role in controlling resistant bacteria. Vaccines have multiple targeting antigens and thus decrease the emergence of AMR. Recombinant DNA technology boosts the progress in effective vaccine development (Micoli et al. 2021). However, antibacterial antibodies work by neutralising the virulence property of bacteria via complement-mediated lysis (Rosini et al. 2020; Raj et al. 2021). Although vaccines and antibodies against all AMR species are still not available, the predictions of their impact suggest the potential benefit in controlling resistance.

Inorganic nanoparticles (NPs), versatile nanoscale particulate materials with unique chemical and physical properties, are broadly explored for their use in the fight against multidrug-resistant microbes. Metal (silver, gold, iron) (Zhang et al. 2008; Li et al. 2014; Kailasa et al. 2019; Arias et al. 2018; Nakamura et al. 2019), metal oxide (magnesium oxide, copper oxide, zinc oxide) (Nguyen et al. 2018; Meghana et al. 2015; Tiwari et al. 2018), carbon-based (fullerene, carbon nanotubes and graphene oxide) NPs (Maleki Dizaj et al. 2015; Yousefi et al. 2017) and mesoporous silica NPs (Liu et al. 2019) have already been reported displaying antimicrobial activity either on their own or with suitably appended surface functionalisation. Antibacterial activity of inorganic NPs is of course highly dependent on the size, shape, surface charge and chemistry of the particles in direct interaction with the bacterial cell wall. NPs exhibit stronger antimicrobial activity than microscale particles as a larger specific area of small particles offers better interaction with bacteria (Stanić and Tanasković 2020). More often than not, these particles act via mechanical contact with the microorganisms damaging the bacterial cell membrane integrity and morphology (Maleki Dizaj et al. 2015). It was shown on surface-deposited metal nano- and microparticles that size of sharp morphologies is able to lyse/spike bacterial cells dead, while no affect human cells (e.g. red blood cells) (Li et al. 2014). Furthermore, positively charged NPs interact electrostatically with the negatively charged cell membrane, while functionalisation with hydrocarbon moieties can result in increased cellular uptake due to the lipophilic nature of the outer membrane (Ranjan Sarker et al. 2019). With inorganic NPs that offer good antimicrobial activity, there is always a risk of disproportionate release of ions they are composed of that may affect healthy cells and result in poor biocompatibility.

Drug carriers such as liposomes, dendrimers, hydrogels, cubosomes, polymeric NPs and micelles are examples of organic nanoparticles in delivering antimicrobial

agents (Tang et al. 2021; Boge et al. 2019). The liposome is mostly used as an antimicrobial drug carrier that resembles the cell membrane and is able to fuse with microbes. Therefore, the drug-loaded liposomes can readily fuse with the bacterial membrane and release the drug directly into the bacteria (Drulis-Kawa and Dorotkiewicz-Jach 2010).

Most of the drug carriers used to tackle AMR are either purely organic or inorganic. Metal-organic frameworks (MOFs) are emerging hybrid nanocarrier systems that offer several advantages over conventional amicrobial drugs. Notably, several MOFs have inherent antimicrobial properties as well as they are able to carry other antimicrobial drugs to exert a better antimicrobial effect. The following sections of this chapter will summarise the antimicrobial effect and mechanism of MOF action.

7.5 Metal-Organic Frameworks

Metal-organic frameworks (MOFs) are porous nanomaterial composed of two major components: metal clusters (e.g. Zn, Cu, Fe, Co, Zr) and electron-rich organic ligands/linkers (e.g. imidazole, carboxylate) which is connected via coordinative bond formation. In general, metal clusters are considered as inorganic secondary building units which join organic moieties as supporting units within MOF structures. Therefore, MOFs are classified as inorganic-organic hybrid materials. Their structural diversity is ever increasing. A vast diversity of metal and linkers ensure continuous expansion of MOF for a wide range of applications. Depending on the starting materials and the synthetic routes used, they can have remarkable physicochemical properties including crystallinity, porosity, high surface area and thermal stability. Moreover, chemical and structural tunability, charge conductivity, synthetic versatility and often enhanced electrochemical stability make them versatile functional material in biomedical applications (Baumann et al. 2019). Properties of individual MOFs depend on the choice of metal and linker compound. Among different classes, zeolitic imidazolate frameworks (ZIFs) are the most studied MOFs which have zeolite-like topology. In ZIFs, transition metal ions (e.g. Fe, Co, Cu, Zn) connect with imidazole derivatives to build a metal-imidazole-metal coordination network (Park et al. 2006). Their potential in biomedical applications is facilitated by the variety of synthetic techniques and the potentially mild condition under which they can be prepared.

MOFs can incorporate/carry/be loaded with drug molecules when acting as drug delivery systems in a variety of ways: (a) in situ encapsulation, carried drug is present during MOF assembly (Suresh and Matzger 2019; Cunha et al. 2013); (b) infiltration, MOF hosts the guest drug molecules during a post-synthetic loading step, where pores and cavities uptake the guest based on their size and charge density profiles driven by secondary interactions (Chen et al. 2021); (c) surface modification, MOF surface is appended with active pharmaceutical ingredients (APIs) via covalent conjugation or secondary, weak interactions (Horcajada et al. 2010); and (d) core-shell formulation, where layered MOF spheres are created by stepwise

quenching and re-initiation of nucleation resulting in core-shell structures carrying active ingredients and/or cytotoxic species in an otherwise sheltering shell (Li et al. 2013a). While this chapter is not intended toward detailed synthetic aspects of various MOF types, presented below is a brief account of the utility of MOFs as antimicrobial agents.

7.6 Physicochemical Properties Lending Antimicrobial Effect to MOFs

MOFs are basically coordination polymers formed by the self-assembly of organic ligands and metal cations. In addition to the most prominent metal coordination bonds, some other bonds like hydrogen bonding, electrostatic interactions and π–π stacking are also found in the MOF building units (Carné et al. 2011). Structural and chemical diversity, mild synthesis conditions, high loading efficiency and biodegradability offer benefits in biomedical applications including antimicrobial effects. The favourable physicochemical properties of MOFs allow to immobilise or encapsulate a wide range of biomolecules to delivery. The antimicrobial effect of MOFs is responsible for drug/biomolecule incorporation and on-demand release of loaded drug/biomolecule as well as metal ions. Therefore, both the loaded compounds and metal and ligand composition of the framework itself regulate the antimicrobial potency.

7.6.1 MOF as Antimicrobial Drug Delivery System

Resistant bacterial species acquire distinct drug exclusion/inactivating mechanisms to protect themselves. MOFs have been utilised as drug carriers mainly due to their tuneable porous structure and the high surface area that allows for hosting of hydrophobic drugs owning low bioavailability and cellular uptake or molecules that otherwise would not be taken up by the cells or rendered inactive by resistance mechanisms (Soomro et al. 2019; Au-Duong and Lee 2017). Table 7.3 summarises the recent studies on the role MOF as antimicrobial drug delivery systems. While native surface properties of MOF-based drug delivery systems can be designed for enhanced cellular uptake, surface modification strategies are also possible aimed at the improvement of cellular uptake or the selectivity thereof. Polymer-coated MOFs are well-studied for their bacterial cellular uptake (Ma et al. 2018; Raafat et al. 2008).

Once inside the cells, triggered drug release can be induced by previously mentioned conditions resulting in the dissolution of the MOFs: pH, temperature, phosphates, thiols and photosensitisers.

Table 7.3 Antimicrobial effects by MOFs

MOF	Loaded compound	Loading efficiency	Coating element	Size	Antimicrobial assay	Tested microbes	Remarks	Reference
A. Delivery of photosensitiser								
ZIF-8	Curcumin	10.89%	HA and chitosan	107.1 ± 9.5 nm	MIC, MBC, live/dead staining, ROS detection	*S. aureus, E. coli*	MIC and MBC: *S. aureus* (0.625μg/mL) and *E. coli* (2.5μg/mL)	Duan et al. (2020)
ZIF-8	MB	N/A	PAA, AgNPs, vancomycin, PEG	110 nm	Live/dead staining, SEM morphology, CFU count	*E. coli, S. aureus,* MRSA	Decrease in bacteria after (5 min 202 mW) laser treatment	Chen et al. (2019a)
Fe-101 Al-101 Fe-88	ICG	18.17% 16.93% 10.57%	N/A	50–90 nm	CFU count, biofilm	*Enterococcus faecalis*	Prevent biofilm formation (18.6%, 23.5% and 22.8%, respectively in ICG@ Fe-101, Al-101, Fe-88)	Golmohamadpour et al. (2018)
MIL-100	D-AzAla	N/A	N/A	120 nm	In vivo imaging	MRSA	D-AzAla carrier significantly reduced bacterial count in infected tissues. The selective integration of the carrier into bacterial cell walls was confirmed by fluorescence signals	Mao et al. (2018)
Cu-BTC	N/A	N/A	Fe_3O_4	5μm	Growth curve, CFU count, live/dead staining, morphology, hydroxyl radical detection	*E. coli, S. aureus*	≥ 90% bacteria killing rate and boost in intracellular ROS levels	Zhang et al. (2020)

(continued)

Table 7.3 (continued)

MOF	Loaded compound	Loading efficiency	Coating element	Size	Antimicrobial assay	Tested microbes	Remarks	Reference
PCN-224 (Zr-MOF)	N/A	N/A	HA and ag^{+}	85 nm	Growth study, morphology 15 min under visible light irradiation	MRSA Drug-resistant *E. coli*	15 min visible light irradiation resulted in better electrostatic interaction with bacterial membrane	Zhang et al. (2019)
B. Delivery of small molecular antibiotics								
ZIF-8	Ciprofloxacin	93%	Fe_3O_4 and PAA	–	Disk diffusion	*E. coli, S. aureus*	ZOI: 32 and 15 mm for *E. coli*, *S. aureus*, respectively	Esfahanian et al. (2019)
ZIF-8	Ciprofloxacin	21%	N/A	80 nm	Disk diffusion	*E. coli, S. aureus*	ZOI: 46 and 49 mm for *E. coli*, *S. aureus*, respectively. pH-responsive drug release	Nabipour et al. (2017)
ZIF-8	Gentamicin	19%	N/A	200 nm	Disk diffusion	*E. coli, S. aureus*	(*E. coli* ≤ 14 mm, *S. aureus* = 19 mm)	Soltani et al. (2018)
ZIF-8	Ceftazidime	~10.9%	N/A	N/A	Growth curve	*E. coli* K12	pH-responsive drug release	Sava Gallis et al. (2019)
ZIF-8	Ampicillin and ZnO	1.55%	N/A	450–500 nm	MIC, membrane disruption, agar well diffusion	*E. coli*, *S. aureus*	MIC: *E. coli* 12.50μg/mL and *S. aureus* 48μg/mL. Membrane disruption by ZnO-generated ROS	Mohanta et al. (2019)

MOF	Loaded compound	Loading efficiency	Coating element	Size	Antimicrobial assay	Tested microbes	Remarks	Reference
MIL-53	Vancomycin	~11.5%	Chitosan	402 ± 14 nm	MIC, IC_{50}, MBC	*S. aureus* (sensitive and vancomycin-resistant)	Cell wall disruption via Fe^{3+}-mediated electrostatic interaction MIC: sensitive (3.81 ± 1.13μg/mL) and vancomycin-resistant (8.92 ± 0.69μg/mL) IC_{50}: sensitive (3.81 ± 1.13μg/mL) and vancomycin-resistant (24.06 ± 1.18μg/mL) MBC: sensitive (127.81 ± 2.66μg/mL) and vancomycin-resistant (169.34 ± 2.58μg/mL)	Ghaffar et al. (2019)
ZIF-8	Physcion	11.49%	N/A	165 nm	Agar well diffusion	*P. putida*, *E. coli*, engineered *E. coli* (QH4), *S. aureus*	ZOI: *P. putida* (13 mm), *E. coli* (23 mm), engineered *E. coli* (QH4) (18 mm), *S. aureus* (20 mm)	Soomro et al. (2019)

(continued)

Table 7.3 (continued)

MOF	Loaded compound	Loading efficiency	Coating element	Size	Antimicrobial assay	Tested microbes	Remarks	Reference
MIL-53	Vancomycin	20%	N/A	500 nm	CFU count, morphology	*S. aureus*	Released Fe^{3+} at acidic pH to damage bacteria at the site of infection. Membrane corrugation and significant decrease in CFU number in concentration-dependent manner.	Lin et al. (2017)
IRMOF-3, MOF-5, Zn-BTC	Ampicillin, kanamycin	N/A	N/A	5–10μm	MIC	*E. coli*, *S. aureus*, *S. lentus*, *Listeria monocytogenes*	Amp@IRMOF-3 8–16μg/mL Kan@IRMOF-3 4–8μg/mL Amp@MOF 16–24μg/mL Kan@MOF-5 8–16μg/mL Amp@Zn-BTC 8–24μg/mL Kan@Zn-BTC 16μg/mL	Bhardwaj et al. (2018)
C. Delivery of inorganic active agents								
ZIF-8	N/A	N/A	Ag nanowires	220 nm	Growth kinetic, disk diffusion	*Bacillus subtilis*, *E. coli* BL21	Synergistic antibacterial effects of Ag core and ZIF-8 shell 100 and 90% growth inhibition in *Bacillus subtilis* and *E. coli* BL21, respectively	Guo et al. (2018)

MOF	Loaded compound	Loading efficiency	Coating element	Size	Antimicrobial assay	Tested microbes	Remarks	Reference
$Zn_{50}Co_{50}$-ZIF	N/A	N/A	N/A	100 nm	Growth curve, CFU count	MRSA	Sustained dissolution and photoinduced ROS generation, reduction of 45% colonies in presence of light	Ahmed et al. (2019)
Zn-BIF	N/A	6.67%	Ag NPs	(100μm)	MIC, CFU count, live/dead staining	*E. coli*, *S. aureus*	Mechanical damage MIC: *E. coli*, 30–50μg/mL and *S. aureus*, 30–50μg/mL	Qi et al. (2020)
ZIF-8	N/A	20%	ZnO	N/A	MBC	*E. coli*, *Klebsiella pneumoniae*, *Proteus mirabilis, S. aureus*	0.25 mg/mL	Redfern et al. (2018)
Ni-MOF	N/A	N/A	Ag NPs	N/A	MIC, inhibition effect, cell growth, viability testing	*B. subtilis, E. coli, P. aeruginosa, C. albicans*	Mechanical damage MIC: 27–136μg/mL Viability (after 48 h): *B. subtilis* (93.85%), *E. coli* (92.15%), *P. aeruginosa* (87.43%), *C. albicans* (84.07%)	Abd El Salam et al. (2018)

(continued)

Table 7.3 (continued)

MOF	Loaded compound	Loading efficiency	Coating element	Size	Antimicrobial assay	Tested microbes	Remarks	Reference
CuTCPP MOF	N/A	N/A	Ag NPs	550–600 nm	CFU assay, disc diffusion, MIC, MBC	*E. coli, S. aureus, B. subtilis*	max ZOI in Ag-CuTCPP MOFs MIC: *E. coli* 12.5 ug/ml, *B. subtilis* 6.25 ug/ml, *S. aureus* 6.25 ug/ml) MBC: *E. coli* 25 ug/ml, *B. subtilis* 12.5 ug/ml, *S. aureus* 12.5 ug/ml Inhibition ratio: *E. coli* 82.18%, *B. subtilis* 72.8%, *S. aureus* 89.1%	Ximing et al. (2017)
HKUST-1	Cellulose fibres	N/A	AgNPs	200 nm to few μm	Growth inhibition	*S. aureus*	99.41% of growth inhibition	Duan et al. (2018)
ZIF-8	N/A	N/A	GO nanosheet	40–80 nm	MIC, bacteriostasis rate	*E. coli*	MIC: 128μg/mL	Wang et al. (2016)
ZIF-8	N/A	17%	Iodine	530 ± 105 nm	Live/dead staining, disk diffusion	*E. coli, S. epidermidis, S. aureus*	100% killing effective rate at 2 min for both *Staphylococcus* genus and at 3 min for *E. coli*	Au-Duong and Lee (2017)
D. Delivery of biomolecules								

MOF	Loaded compound	Loading efficiency	Coating element	Size	Antimicrobial assay	Tested microbes	Remarks	Reference
ZIF-8	N/A	N/A	BSA	153.9 nm	MIC, disk diffusion, morphology	MRSA, resistant *E. coli*	MIC: MRSA 25μg/mL, resistant *E. coli* 50μg/mL ZOI: MRSA (12.7 mm) and resistant *E. coli* (9.4 mm)	Wu et al. (2018)
ZIF-8	N/A	N/A	Lysozyme	350 nm	Temperature-dependent growth kinetic	*Micrococcus lysodeikticus*	Encapsulation retained the lysozyme activity up to 45 °C	Liu et al. 2017a
ZIF-8	NIF	27.8%	Sodium alginate	A	Growth curve, disk diffusion, morphology	*S. aureus, E. coli*	Cell membrane damage	Luo et al. (2020)
Zn@MOF	Thymol	3.96%	N/A	N/A	Growth study growth inhibition	*E. coli* O157:H7	Thymol-loaded Zn@MOF showed maximum microbial growth inhibition, 4.4 log reduction in 24 h	Wu et al. (2019)
HKUST-1	CMC	N/A	N/A	15μm	ZOI, CFU count *E. coli* were 12.8 ± 1.4 mm at 7 days, and *S. aureus* were 17.4 ± 0.1 mm	*S. aureus, E. coli*	ZOI: *E. coli* 12.8 ± 1.4 mm and *S. aureus* 17.4 ± 0.1 mm	Huang et al. (2020)

Abbreviation: *N/A* not available in literature, *HA* hyaluronic acid, *MB* methylbenzene blue, *D-AzAla* 3-azido-D-alanine, *NIF* niflumic acid, *ICG* indocyanine green, *PAA* polyacrylic acid, *PEG* polyethylene glycol, *CD* γ-cyclodextrin, *GRGDS* Gly-Arg-Gly-Asp-Ser (a fibrinogen-mimetic peptide), *GO* graphene oxide, *ZOI* zone of inhibition, *CMC* carboxymethyl chitosan

7.6.1.1 Small Molecular Antibiotics

The high porosity, loading efficiency, and surface functionalisation properties of MOFs enable them to load and deliver a wide range of antibiotics including ciprofloxacin, gentamicin, nalidixic acid, vancomycin etc. (Sava Gallis et al. 2019; Soltani et al. 2018; Nabipour et al. 2017). These studies explain the synergistic antimicrobial effect of MOF precursors and loaded antibiotics. The released metal ions of MOFs enhance greater interaction with bacteria, cause severe membrane damage and allow higher uptake of loaded antibiotics (Shen et al. 2020; Polash et al. 2021). In a comprehensive study, ampicillin and kanamycin were loaded into three different Zn-based MOFs (i.e. IRMOF-3, MOF-5 and Zn-BTC) which lowered 25–50% MIC values compared to the use of free antibiotics and MOFs against Gram-positive *(Staphylococcus aureus*, *Staphylococcus lentus*, *Listeria monocytogenes)* and Gram-negative *(Escherichia coli)* bacteria (Bhardwaj et al. 2018).

One-pot preparation of vancomycin- and folic acid-loaded ZIF-8 showed better antibacterial efficacy against MRSA (Chowdhuri et al. 2017). Following that, a pH-jump reagent (2-nitrobenzaldehyde) was modified into ZIF-8 by Song et al. where it acted as a gatekeeper to produce acid in situ in response to UV light and resulted in the release of loaded antibiotics (i.e. rifampicin). This light-irradiated system was effective in controlling MRSA infection and speeding up the wound healing process significantly (Song et al. 2018). Moreover, polysaccharide coating enhances the interaction between drug carrier and targeted bacteria and offers to enhance cellular uptake of the drug. For example, the chitosan coating of vancomycin-loaded MIL-53, an iron-based MOF, showed a better antibacterial effect than the free antibiotic against resistant *S. aureus* (Ghaffar et al. 2019).

7.6.1.2 Inorganic Active Agents

MOF is often used to co-deliver inorganic compounds such as copper, zinc, silver and gold NPs alongside encapsulated antimicrobial drugs. The benefit of using the MOF system is the porous structure and high surface area properties offer to tag these inorganic agents for additional applications like sensing and target specificity. Qi et al. developed a metal (M = Ag/Cu) NPs-coated zinc-based boron imidazolate framework (M@Zn-BIF) which exhibited ~99.9% antibacterial activity via synergistic action with excellent photocatalytic efficiency (Qi et al. 2020). This system offers a facile method for preparation of a MOF-based composite which is able to detect and remove the organic element and inactivate bacteria in water. The availability of metal ion on the surface of MOFs enhances better electrostatic interaction with the outer cell membrane and facilitates membrane disruption (Abd El Salam et al. 2018; Duan et al. 2018). Upon interference of these metal ions with the membrane, permeability and cellular uptake of drug@MOFs increased. In other studies, such composite systems were also shown to induce ROS generation, to inhibit transcription and protein synthesis (Rtimi et al. 2019). The enhanced antibacterial activity of NPs@MOFs is often explained by the synergistic bactericidal effect of NPs

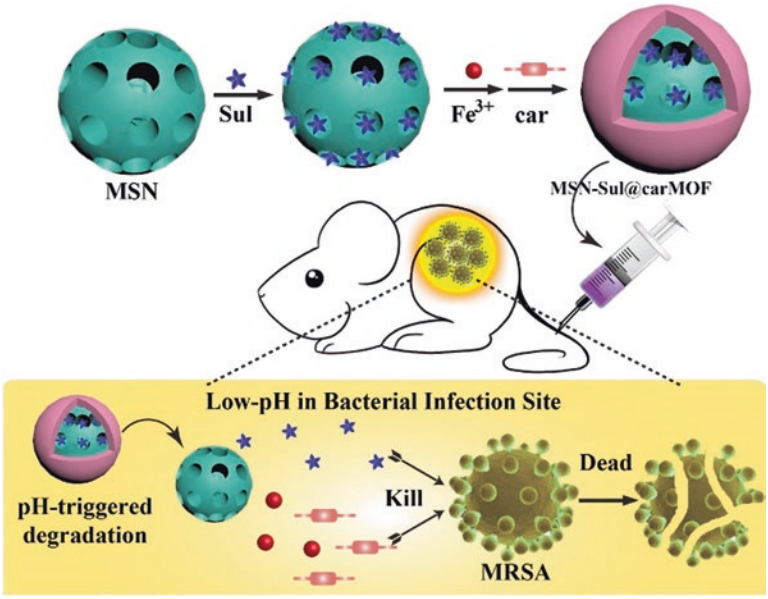


Fig. 7.6 Design of carbenicillin-Fe^{3+} MOF coated with mesoporous silica nanoparticles (MSN) and the synchronous release of antibiotic molecules and inhibitors at the bacterial infection site at low pH. (Reproduced with permission from Duan et al. (2017). Copyright 2017 Elsevier)

and MOF-derived metal ions or the prolonged release of the silver ions in a controlled manner for prolonging antibacterial action. A pH-sensitive Fe-based MOF was used to deliver β-lactam antibiotics in methicillin-resistant *Staphylococcus aureus* (MRSA) in both in vitro and in vivo study (Fig. 7.6) (Duan et al. 2017). Here, carbenicillin, a β-lactam antibiotic, was used as an organic ligand that coordinates with Fe^{3+} to form a metal-carbenicillin framework and coated with mesoporous silica nanoparticles (MSN).

7.6.1.3 Biomolecules

MOFs can protect biomolecules against rough conditions such as temperature, pH and reactive species that would be able to corrupt the biomolecular activity. This can be exploited in the antimicrobial fight. For example, the bactericidal property of lysozyme (a natural antimicrobial enzyme) was protected by ZIF-8 even at high temperatures (45 °C) that are detrimental to enzyme activity (Liu et al. 2017a). The encapsulation ability of MOFs offers an opportunity to load and deliver AMPs through AMP-loaded MOFs as an effective drug carrier system to mitigate AMR. Therefore, favourable physicochemical properties of MOF make them an excellent candidate in carrying a wide range of biomolecules for therapeutic applications. MOFs and their functionalised derivatives can be a good source in

delivering sensitive AMPs and other biomolecules that cannot be delivered by other existing tools. A wide scope is available in this field to explore and study the MOF-based antimicrobial effect using clinical samples.

7.6.2 MOF Itself as Antimicrobial

Though MOF is mostly developed as a drug carrier to deliver antibiotics and compounds, MOF itself has been shown to cause antibacterial activity. MOF exerts antimicrobial action by releasing the framework ingredients in response to certain stimuli (e.g. acidic pH and light). Most often the metal constituents play a vital role in causing bacterial death via physical disruption of the membrane, generation of reactive species and finally the intracellular release of disruptors.

7.6.2.1 Disruption of Cell Membrane Integrity

MOFs, based on their charge-carrying constituents or surface chemistries, are able to disrupt cell membrane integrity. The presence of -COOH groups is responsible for the negative surface charge of bacteria which absorb the cationic metal constitution of MOFs via coulombic attraction. This results in irreversible cell wall damage. Similar to metal NPs, MOFs act as a reservoir of metal ions. Early studies claimed that there is a strong positive correlation between the released amount of metal ions and antibacterial activity (Lu et al. 2014b; Berchel et al. 2011). Close contact with bacterial membrane and environmental stimuli boost up the release of metal ions from the framework architecture where the released ions penetrate the bacterial cell wall, replace cations on the cell membrane surface and finally destroy the structural integrity of the membrane (Pettinari et al. 2021; Bhardwaj et al. 2018) (Wyszogrodzka et al. 2016). Hence, the cell membrane loses its biological function leading to cell death.

As with other nano- and microparticulate systems, the morphology of framework (e.g. spherical, leaf type flakes, cubic) can lead to membrane interruption and eventually cell lysis (Esfahanian et al. 2019; Yuan and Zhang 2017; Abdelhameed et al. 2019). Suresh et al. (2013) explained the impact of nanomaterial size and shape on bacteria (Suresh et al. 2013). The authors found that decreasing the particle size meant increasing the surface area to the mass ratio which result in a change in properties of the nanoparticle. As particle morphology is closely connected with its physical-chemical properties (e.g. size, surface area, charge), these parameters should be considered in preparing therapeutic nanomaterials like MOFs which play a crucial role in interacting with a biological system, uptake and toxicity (Ray et al. 2021).

7.6.2.2 Generation of Reactive Species and Photodynamic Effect

Reactive oxygen species (ROS) are highly reactive and unstable chemicals that react easily with other components in the cell and promote bacterial death (Dryden 2018). Examples of ROS include superoxide, peroxides, hydroxyl radical, singlet oxygen, etc. Besides these, a family of reactive nitrogen-carrying species called reactive nitrogen species (i.e. nitric oxide, nitrogen dioxide) also offer antimicrobial effects (Fang 2004). Therefore, these reactive species have been considered a key factor in controlling the microbial infection. Host immune cells (mostly macrophages and neutrophils) release these cytotoxic effectors which irreversibly oxidise and trigger cellular damage (Shekhova 2020). Similar to other nanoscale materials, MOFs also have inherent reactive species triggering capability and display an antibacterial effect. Liu et al. developed zirconium-based MOFs (UiO-66 and MOF-525) mixed with membrane matrix and biocompatible binder called poly(ε-caprolactone) (Liu et al. 2017b). These stable and biocompatible polymer-mixed Zr-MOFs generate ROS under LED (λ = 510 nm) exposure and had been utilised as effective antimicrobial 'filter' against *E. coli* as it generates ROS under LED (λ = 510 nm) exposure.

One of the most popular modes of ROS generation is through photodynamic therapy (PDT). Antimicrobial PDT becomes a promising non-invasive tool to treat infectious pathogens. In principle, light stimuli are able to activate non-toxic photosensitiser to exert bactericidal effect (de Freitas et al. 2018). Alternatively, in photothermal therapy, photoexcitation of absorbing molecules/nanomaterials can induce hyperthermia that can also be targeted at bacteria (Qing et al. 2019; Han et al. 2020a). The sudden imbalance caused by the in situ-generated, highly reactive free radicals and anions starts a reaction cascade leading to cellular damage and/or cell death often due to the triggered imbalance in ion channel homeostasis (Chen et al. 2017; Ong et al. 2017). Therefore, light-irradiated singlet oxygen prevents bacterial growth.

A pH-sensitive ZIF-8-polyacrylic acid (PAA) material is constructed to deliver a broad-spectrum photosensitiser antibacterial agent called methylbenzene blue (MB). It was further modified by AgNP and vancomycin/NH_2-polyethylene glycol to construct a ZIF-8-PAA-MB@AgNPs@Van-PEG composite which was found to inhibit the growth of *E. coli, S. aureus* and MRSA when subjected to 650 nm laser irradiation (Chen et al. 2019a). Qian et al. demonstrated a facile preparation of antimicrobial electrospun mats using photosensitive drug-loaded MOFs and co-electrospinning of biodegradable poly (ε-caprolactone (PCL) matrix. Rose bengal (RB), an effective photosensitiser, was encapsulated into ZIF-8. The resulting photodynamic RB@ZIF-8 exerted dose and time-dependent in vitro bactericidal effect of Gram-positive *S. aureus* and Gram-negative *E. coli* with 515 nm irradiation (Qian et al. 2020). Similarly, ZIF-8 was used to encapsulate curcumin (CCM, a light-sensitive photosensitiser) and further modified with biocompatible polymers hyaluronic acid (HA) and chitosan (CS) through a layer-by-layer method to fabricate an effective antibacterial agent. The local high-positive charge density of resulting CCM@ZIF-8@HA@CS NPs allows effective binding with bacteria, and

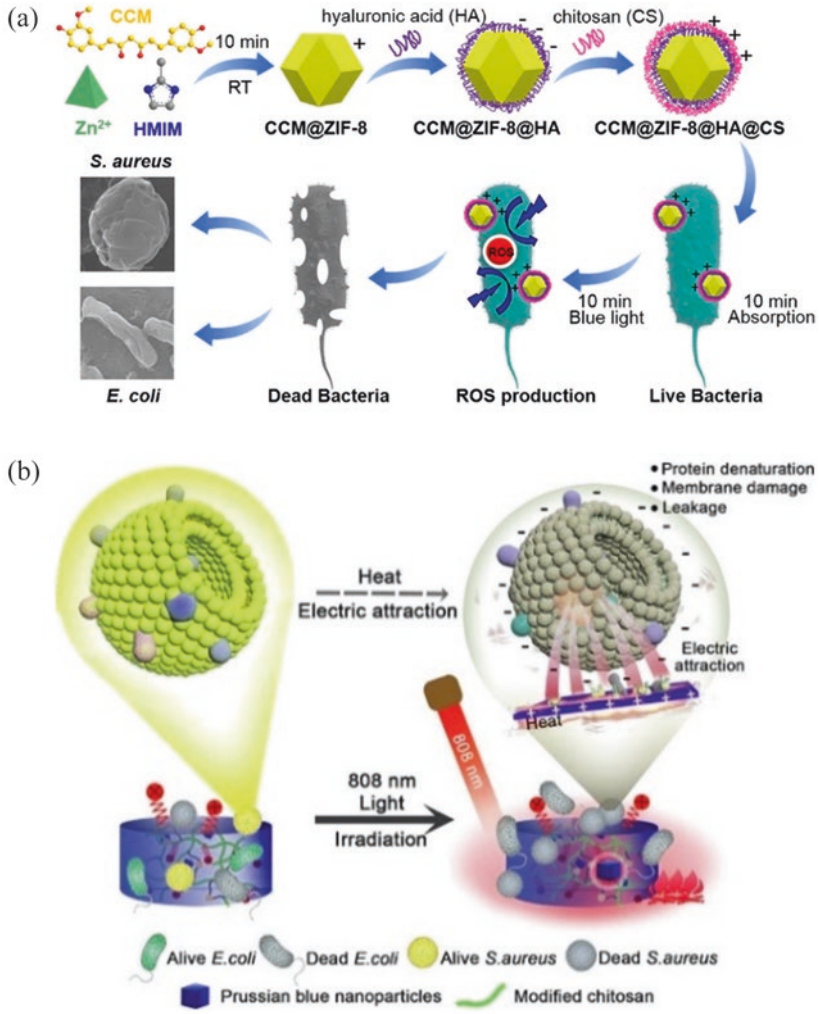


Fig. 7.7 MOFs-mediated photodynamic effect. (**a**) Curcumin-loaded ZIF-8 decorated with hyaluronic acid (HA) and chitosan (CS) and photodynamic action against Gram-positive (*S. aureus*) and Gram-negative (*E. coli*) strains under blue-light exposure. (Reproduced with permission from ref. Duan et al. (2020). Copyright 2020 American Chemical Society). (**b**) Antibacterial mode of action of the Prussian blue and chitosan-modified photothermal hydrogel. The electropositive hydrogel could capture bacteria and kill them combined with heat under 808 nm near-infrared light irradiation. (Reproduced with permission from Han et al. (2020b). Copyright 2020 Elsevier)

photodynamic property allows dissociation and antimicrobial effect under blue light irradiation (Fig. 7.7a) (Duan et al. 2020).

In addition to zinc-based MOF, UiO-66 (a zirconium-based framework) is also an efficient multifunctional drug delivery system (Lv et al. 2020). Carboxylic zinc phthalocyanine, a broad-spectrum photosensitiser, was connected to porous

UiO-66-NH_2 by amidation (a distinct reaction to form amine link between aromatic carboxylic acid and aliphatic amide) to construct an efficient drug carrier system. Then antibacterial agent linezolid (a class of oxazolidinones antibiotic) and lysozyme (a biocompatible and heat resistance protein) were loaded in the pores and coated on the surface by electrostatic interactions. The (Li@UiO-66-H_4Pc)@lysozyme construct showed a synergistic photodynamic and chemotherapeutic effect when irradiated with a 670 nm laser against *S. aureus*, *E. coli* and MRSA. Han et al. prepared a photosensitive bactericidal hydrogel using Prussian blue nanoparticles and chitosan-modified MOFs (Fig. 7.7b) (Han et al. 2020b). Electrostatic absorption and photothermal property allow capture and rapid killing of bacteria under 808 nm light irradiation, respectively. The synergistic role of the hydrogel offered 99.97 and 99.93% cell death of *S. aureus* and *E. coli*, respectively, under light irradiation. This MOF significantly promoted would healing process and offered potential therapeutic efficacy.

Recently, Fe_3O_4@Cu-BTC (a copper-based MOF), a recyclable and magnetic antibacterial material, has been reported to boost intracellular ROS levels. The slowly released copper ions from this MOF adhere to the negatively charged bacterial cells, destroy membrane integrity and then generate ROS to facilitate cell death. Transfer of photoexcitable electron-hole pairs within the MOF played a significant role in the antibacterial activity of Fe_3O_4@Cu-BTC (Zhang et al. 2020). Taken together, these emerging examples highlight the potential of MOF to deliver antimicrobial drugs with non-toxic photosensitiser which offer light-sensitive and better antimicrobial effects.

7.6.2.3 Intracellular Release of Disruptors

MOF particles aimed at reaching the intracellular space can also be tailored via the coordinate bonds between the organic linkers and metal ions and result in biodegradable structures. When biodegradable MOFs collapse/dissolve into small molecular weight constituents under particular circumstances, they can display two distinct effects: (a) cellular compatibility, sustained slow release of metal ions and loaded drug allow to respond appropriately regarding the biological requirement, or (b) toxicity, cause burst release of metal ions and organic ligands from unstable MOFs to increase the local concentration of metal and organic ligand which exerts toxic effects depending on the nature and concentration of leached species.

As the trigger for biodegradation, pH is one of the most often exploited factors to stimulate the dissolution of the MOF skeleton. As pH at the site of bacterial infections is lower than the physiological pH, this can trigger the disintegration of coordination bonds between MOF precursors (Poddar et al. 2019) or the release of carried antimicrobial agents when loaded (Chen et al. 2019b; Wyszogrodzka et al. 2016). A recent study found that MOFs can also be degraded in phosphate-buffered saline media (PBS) where the high affinity of phosphate groups for the Zn^{2+} cations leads to form amorphous zinc phosphate (Fig. 7.8) (Velásquez-Hernández et al.

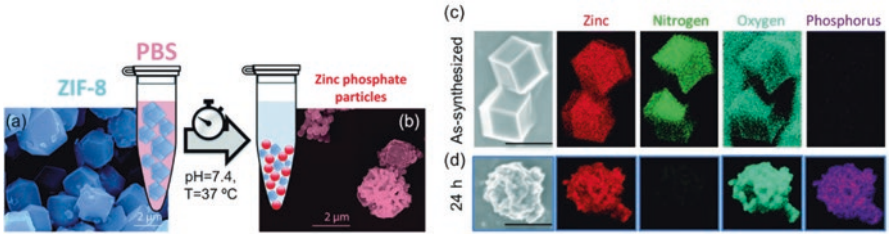


Fig. 7.8 The fate of ZIF-8 particles under physiological conditions (**a**) before and (**b**) after incubation in PBS. SEM image and EDX elemental maps of (**c**) as-synthesised ZIF-8 particles and (**d**) particles recovered after the incubation process in PBS for 24 h. (Reproduced with permission from Velásquez-Hernández et al. (2019). Copyright 2019 The Royal Society of Chemistry)

2019). This phenomenon led to developing ATP-responsive MOFs biocomposite where chelation of Zn^{2+} cations triggers the release of loaded cargo (Yang et al. 2019).

7.7 Future Perspectives of MOF as Antimicrobial

MOF is a recent addition to the list of nanomaterial with multidimensional therapeutic applications. Simple preparation, availability and variation in precursor make it easier to control the properties of MOF. Their exceptional loading capacity enables the delivery of wide range of biomolecules and drugs. pH sensitivity and photodynamic amiability enable the on-demand release of antimicrobial drugs and infection control. Furthermore, MOF can be functionalised with biocompatible polymer to achieve better stability, aqueous solubility and protection of the drug from unwanted release. The antimicrobial action of MOF largely depends on the release of metal ions from the framework skeleton and loaded drug. That offers better antimicrobial performance than the free drug. Distinct mode of action empowers irreversible physical damage to the cell membrane and inhibits the development of resistance. The antibacterial mechanism of MOFs is yet to be extensively studied. The use of organic ligands for antimicrobial MOFs synthesis is debateable as the use of a high concentration of ligands may have a negative impact. Scaling up the drug@MOF enables the industrial feasibility of MOF for mass production and distribution. Systemic in vivo antimicrobial and toxicity studies should be explored before taking the MOFs-based antimicrobial therapy in the clinical study. The field is still growing and should expand systemically to mitigate antimicrobial resistance. In future, it is expected that MOFs-based system would be explored for better targetability, specificity, loading and delivery of antimicrobial elements.

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Chapter 8
Silver-Based Nano-formulations for Treating Antibiotic-Resistant Microbial Strains

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Abstract Nosocomial infections represent the most severe complications among hospital patients and coworkers upon exposure to antibiotic-resistant microbial strains. Such healthcare-associated infections (HAIs) are one of the leading causes of morbidity and mortality, inflicting enormous social and economic burdens worldwide. Whether a patient's body, hospital floors, or medical textiles and medical devices/instruments, these pathogens find surfaces to attach/grow and deceive the rigorous implementation of adopted hygiene practices. Hence, there is an utmost need to develop new antibiotic alternatives, such as engineered nanoparticles, which could offer a better biocidal response to almost all surfaces while minimizing the antimicrobial resistance crisis. Due to its broad-spectrum features and multitude of mechanisms of action, nano-silver has emerged as the most deserving candidate for designing such antimicrobial strategies. Nano-silver formulations are equally amenable to be utilized as surface coatings, thin films, polymeric layers, hydrogels, nanocomposites, and blend mixtures, other than its usage as a colloidal suspension. Treatments to curb antibiotic overuse by formulating new antibiotics/nano-silver blends are also gaining importance for preventing and controlling microbial infections. The current chapter thus summarizes various nano silver-based antimicrobial strategies, their action mechanism, and efficacy against antibiotics/multidrug-resistant microbial strains.

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Keywords Multidrug-resistant strains · Silver nanoparticles · Silver nanocomposites · Healthcare-acquired infections · Antibacterial mechanism

8.1 Introduction

The emergence of several new antibiotic resistance bacterial strains poses alarming threats to contemporary advancements in healthcare sector. A wide range of biomedical devices and antibiotics used to treat patients are ineffective in eradicating microbial infections due to the resistance development against them (Jernigan et al. 2020; Thorpe et al. 2018; Cassini et al. 2019; Tacconelli et al. 2018). While bacteria are constantly evolving to develop resistance against new antibiotics, virtually all hospital-related surfaces, e.g., human skin, floor, tiles, sutures, bandages, biomedical implants, surgical instruments, and devices, act as natural "niches" to microbes to attach, grow, and proliferate in the form of biofilm, which eventually protects them against harsh conditions (Høiby et al. 2010). Microbial biofilm provides additional resistance against antibiotics and host immune factors; microbes can survive and multiply quickly over the host surface. Considering the consequences, developing an immediate solution is the current need of the hour, which has gained substantial attention to combat this threat for global health. Researchers have been exploring various materials and approaches to make a universal antimicrobial agent with a broad spectrum of bactericidal activity and its applicability for different applications. The reluctant and robust nature of multidrug-resistant microbes has made most of them ineffective against conventional biocidal agents. A blooming field has suggested some interesting leads, known as nanotechnology (Tacconelli et al. 2018; Gupta et al. 2019; Doughari et al. 2009).

Nanomaterials have witnessed exponential growth in developing new antimicrobial biomaterials with superior characteristics (Sillu and Agnihotri 2020; Agnihotri et al. 2018; Singh et al. 2016, 2021; Chauhan et al. 2019). At the nanoscale, particularly between 10 and 25 nm, materials exhibit remarkable physiochemical attributes such as the high surface area to volume ratio that dictates their antimicrobial potential (Agnihotri et al. 2014). Metal and metal oxide nanomaterials based on silver, gold, copper, zinc oxide, and titania have witnessed their antimicrobial character against various strains (Mukherji et al. 2012; Agnihotri et al. 2015; Yadav et al. 2016; Sirelkhatim et al. 2015). Silver is the most effective nano-weapon due to its innate bacterial killing capacity. It requires low inhibitory concentration to achieve complete bacterial inhibition. Nano-silver has shown its therapeutic arsenal against multidrug-resistant (MDR) strains such as methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Staphylococcus aureus* (VRSA), ampicillin-resistant *Escherichia coli*, and erythromycin-resistant *Streptococcus pyogenes*. The bactericidal activity of silver nanoparticles against biomaterial-associated infections, i.e., *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Staphylococcus*

haemolyticus, *Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Proteus vulgaris*, *Candida albicans*, and *Propionibacterium acnes*, has also been thoroughly investigated by many scientists.

For biomedical application viewpoints, nano-silver-based antimicrobial therapies can safely be applied during surgical treatments to prevent pathogenic growth without causing any adverse effects on neighboring healthy tissues or interacting with the biomedical apparatus. Although the exact mechanism of antimicrobial action of nano-silver against antibiotic-resistant bacteria is debatable, a series of possibilities, including its direct interaction with the cell wall membrane, production of reactive oxygen species, disrupting cellular respiration machinery, and rupturing signal transduction pathway, have been elucidated as the leading causes (Ahmad et al. 2020; Crisan et al. 2021). The attachment of silver nanoparticles with bacterial cell walls is the first point of interaction, which can be augmented via the functionalization of silver nanoparticles easily. Various studies have established the vital role of nanoparticles' size, as penetration through the cell membrane is a critical step in inhibiting bacterial growth (Agnihotri et al. 2014).

Besides benefits, silver nanoparticles suffer limitations, and their employability for biomedical use is often restricted. It is evident that being extremely unstable, silver nanoparticles experience poor colloidal stability and thus are prone to get aggregated, losing their actual antimicrobial efficacy. Moreover, humans' direct uptake of nano-silver may induce severe cytotoxicity and inflammatory response that worsens the condition for critically ill patients. This issue can be overcome by anchoring silver nanoparticles onto a suitable support material such as porous networks, thin films, hydrogels, polymeric nanocomposites, and even fibrous materials (Agnihotri et al. 2012, 2013, 2015; Agnihotri and Dhiman 2017). These strategies have been investigated to fabricate novel nano-silver-based biomaterials as antimicrobial agents combating MDR (Dhiman et al. 2019). A synergistic impact of host material with nano-silver may result in superior antibacterial surfaces with good cytocompatibility with human cells. Recent advances in the field prove their potential for direct applications in biomedical research as wound healing material, implant surfaces, etc. This chapter thus focuses on two modes of utilizing nano-silver-based formulation in healthcare, either by using the colloidal form or as immobilized/impregnated state in the form of silver-nanobiomaterial.

8.2 Colloidal Nano-silver Formulations

Silver nanoparticles (AgNPs) exist as ultra-smallAg^0 metal in several defined shapes, e.g., spherical, triangular prism, tetrahedral, cubical, prismatic, and octahedral, with various sizes ranging from 5 to 100 nm (de Lacerda et al. 2020; Wahab et al. 2021a). A variety of AgNPs complexes have primarily been employed as a part of the therapeutics to treat various diseases and infections. Also, their non-lethal nature to the human body at low fixation is alluring for obvious medical applications (Talapko et al. 2020). AgNPs are used extensively in food packaging, food

additives, electronics, textiles, household appliances, cosmetics, and water disinfectants, owing to their antibacterial properties (Ferdous and Nemmar 2020). Silver nanoparticles (AgNPs) are shown to be a potential solution against antimicrobial resistance as they present relevant physicochemical characteristics such as stability and good chemical interaction necessary to combat microorganisms (Wahab et al. 2021a).

Despite having a thorough investigation, the exact mechanism of antimicrobial action for AgNPs is still unclear. As per the available literature, three primary hypotheses have been general proposed: (1) degradation of the cell membrane/wall, (2) internalization within the cell and mediating subcellular disruption, and (3) oxidative stress damage (Salleh et al. 2020), as illustrated in Fig. 8.1. Bacterial cell walls and membranes primarily protect them from stress and other harmful localized environments. However, the ultrafine nature of AgNPs and their high surface area help them have better contact with either bacterial cell walls or membranes (Wahab et al. 2021a). AgNPs can easily bind to integral/transmembrane proteins present on the cell wall/membrane of microbes, restricting their vital functions (Wahab et al. 2021a; Salleh et al. 2020; Ijaz et al. 2020). AgNPs attachment may cause severe loss in lipid bilayer integrity and disturb the cell membrane permeability. Reports indicate that such undesirable changes in membrane permeability cause leakage of reducing sugars and proteins across them, leading to cell death. Additionally, the mechanical damage induced through "direct contact" with AgNPs may also result in the extrusion of cytoplasmic fluid. The interactions between AgNPs and cell wall components, especially proteins, inhibit bacterial growth, suggesting that the changes in cell membrane permeability result in the death of bacterial strains (Ghodake et al. 2020).

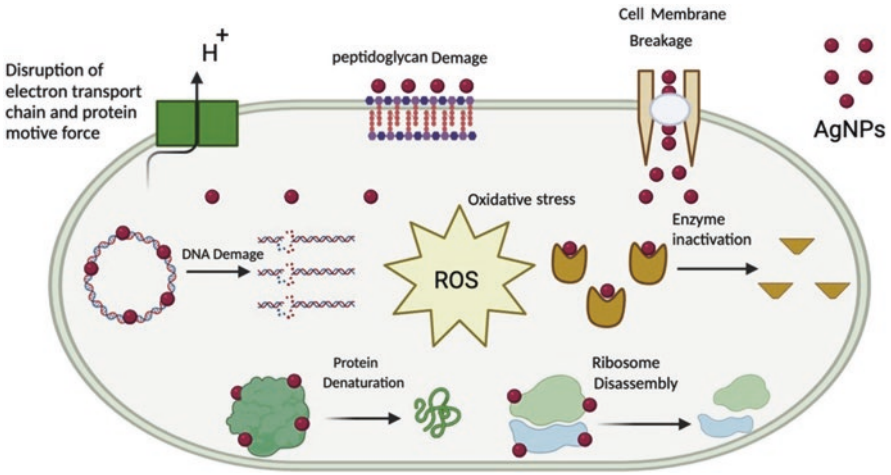

Fig. 8.1 A schematic representation of the probable mechanism of antimicrobial activity of AgNPs. (Reprinted with permission from Wahab et al. (2021a), Elsevier)

A second mechanism, AgNPs may enter the cell through internalization and initiate interaction with subcellular components present, i.e., mitochondria, ribosomes, and nucleus, making them either nonfunctional or disrupting their activities (Wahab et al. 2021a). The ongoing variation in membrane permeability primarily facilitates AgNPs entry into the cell, which interacts with sulfur and phosphorus compounds present in DNA, ultimately resulting in cell death. Furthermore, AgNPs exhibit genotoxic effects that cause severe damages to DNA sequences, where microbes lose their ability to replicate (Wahab et al. 2021a). Another way to inhibit microbial growth is producing reactive oxidative species (ROS) (Salleh et al. 2020). The intercellular ROS prompted by AgNPs may induce lipid damage and leakage of cellular biomolecules and eventually lead to cell apoptosis. The ROS are generated at the particle surface, damaging the cell membrane, protein, and DNA. In a recent study (Khan and Ali 2020), the introduction of AgNPs showed an enhanced quantity of ROS, which subsequently revealed superior antibacterial potential against multidrug-resistant strains such as *Xanthomonas citri*, *S. aureus*, and *Erwinia carotovora*. The mechanism of action also depends on the strain type, i.e., gram-negative bacteria are comparatively more sensitive to AgNPs than gram-positive bacteria (Ahmad et al. 2020). Gram-negative bacteria have more tapered cell walls when compared to gram-positive strains. The thick cell wall of gram-positive bacteria comprises various peptidoglycan coats limiting the permeation of the silver ions (Ag^+) through the cytoplasmic membrane. On the other hand, gram-negative pathogens are mostly consist of only a single peptidoglycan coat. Thus Ag^+ ions easily penetrate into their cytoplasm and cause cell lysis (de Lacerda et al. 2020). While in gram-positive strains, the AgNPs limit the release and uptake of proteins, sugars ions, and other essentials for vital cellular activities (Ahmad et al. 2020). However, regardless of the bacterial cell wall's composition, the penetration of AgNPs will occur, causing cell lysis of both gram-positive and gram-negative bacteria (Ahmad et al. 2020; Salleh et al. 2020).

The routes of AgNPs synthesis also play crucial role in dictating their antibacterial efficacy. Chemical route is often considered to be the most suitable way to synthesize AgNPs as it offers good control on nucleation and growth stages of silver nuclei, resulting in generation of various silver-shaped nanostructures. The ingredients employed in chemical synthesis of AgNPs are referred to as reducing and stabilizing agents. While reducing agent, e.g., trisodium citrate, sodium borohydride ($NaBH_4$), ascorbic acid, and Tollens' reagent, facilitates an instantaneous conversion of Ag ions into silver nuclei (Ag^0), the presence of stabilizing agents like polvinyl alcohol, polyvinylpyrrolidone, polyethylene glycol, and polymethylmethacrylate ensures enough segregation of formed Ag nuclei, thereby controlling shape and size of nanoparticles (Ahmad et al. 2020; Das et al. 2020). For instance, in a study by da Silva et al. (2020), citrate-capped AgNPs were synthesized chemically, and their antibacterial potential was assessed against *Pseudomonas aeruginosa*, one of the most refractory organisms to antibiotic treatment. The local surface plasmon resonance simulation of synthesized AgNPs displayed a significant reduction in bacterial activity. In a similar study, citrate-capped AgNPs were synthesized using silver nitrate ($AgNO_3$) precursor in the presence of sodium citrate as reducing and

protecting agent. The results demonstrated that the capping of citrate on silver surface plays an essential role in enhancing the antibacterial activity of AgNPs against both antibiotic-resistant *S. aureus* and *E. coli* (Kaur et al. 2019). Farouk et al. (2020) synthesized AgNPs (6.8 ± 2.28 nm) using trisodium citrate and sodium borohydride reducing agents. The synthesized AgNPs at various concentrations showed MIC ranging between 0.002 and 0.313μg/mL and MBC of 0.078 and 1.250μg/mL against multidrug-resistant *Salmonella.* Another group of researchers synthesized polyvinyl pyrrolidone (PVP)-coated silver nanowires (25 nm) and silver nanocubes (80 nm) using a modified polyol method. The synthesized NPs were tested for their antimicrobial potential against carbapenem-resistant *Escherichia coli* and vancomycin-resistant *Staphylococcus aureus* strains to treat bloodstream infections in cancer patients. An almost 100% death rate of both the aforementioned strains was achieved using nanowires and nanocubes. They concluded that this nanomaterial could be considered for further biomedical applications to control the growth of multiple drug-resistant strains (Jose et al. 2019). Even though chemical synthesis provides broad control over size and shape; however, it results in chemical sediment on their surfaces, which might be toxic and harmful, thereby increasing AgNPs toxicity towards human cells, discouraging their use (Ahmad et al. 2020; Moradi et al. 2021).

Among all the AgNPs synthesis methods, biological synthesis is the most economical and environmentally sustainable alternative, which could possibly overcome the before-mentioned limitations (Moradi et al. 2021). The reducing and stabilizing agents used in this type of synthesis are molecules produced by bacteria, fungi, yeasts, plants, and algae (Wahab et al. 2021a; Das et al. 2020; Moradi et al. 2021). The typical microbial synthesis process of AgNPs includes incubating the cultured microorganisms with silver precursor salt and monitoring the production of AgNPs visually (Ahmad et al. 2020). In case of the intracellular biosynthesis process, an ultrasonication process is needed to break the cell wall and release the AgNPs (de Lacerda et al. 2020). A plethora of studies suggests that the cellular machinery in microbes mainly aids them in the improved assembly of highly stable AgNPs. In a recent study by (Skóra et al. 2021), *Saccharomyces cerevisiae* was used as a promising tool for synthesizing silver nanoparticles ranging between 17.5 and 20.1 nm. The polydispersity index of 0.397 suggested that the nanoparticles are stable as they do not exhibit any considerable aggregation. The green-synthesized AgNPs showed significant bactericidal activity against multidrug-resistant *S. aureus*, *P. aeruginosa*, *E. coli*, *and C. albicans*. Also, they exhibited biofilm reduction by 53% in *E. coli* and 36% in *P. aeruginosa* for 2 mg/mL AgNPs concentration. The strong inhibition efficacy of AgNPs on migration was observed in cancer cells, suggesting that silver nanoparticles may have a potential function in the inhibition of metastasis. In another study, AgNPs were synthesized using an actinomycete, *Nocardiopsis* sp. *GRG1 (KT235640)* biomass. The relatively well-dispersed AgNPs possessed a size ranging from 20 to 50 nm and showed 91% of inhibition against biofilm-forming methicillin-resistant coagulase-negative *Staphylococcus* at a concentration of 55μg/mL. In conclusion, biosynthesis of AgNPs using microorganisms demonstrates significant antibacterial activity against a variety of antibiotic-resistant microorganisms. However, the pre-synthesis necessities, for

instance, biomass culturing and sometimes low-reaction kinetics, push the need to strive for other bioresources with distinguished privileges (Moradi et al. 2021).

Biogenic synthesis of AgNPs using plants is privileged having a simple, hazardous chemicals-free procedure along with high efficiency and short reaction times. The synthesis method is comparatively nontoxic, reliable, and ecologically supportive (Moradi et al. 2021). The reducing and stabilizing agents naturally available in plants help in synthesizing biocompatible AgNPs. In general, the primary and secondary metabolites like phenols, alkaloids, terpenoids, flavonoids, carbohydrates, and proteins present within the extract are used to reduce Ag^+ into AgNPs (Wahab et al. 2021a; Ijaz et al. 2020). The as-synthesized nanoparticles have been explored for their antibacterial efficacy, for instance, (Choi et al. 2021) employed *Areca catechu* extracts to synthesize AgNPs ranging between 20 and 30 nm. The as-synthesized AgNPs (360μg/mL) showed ZOI of 12.3 ± 0.8 mm, 16.3 ± 1.5 mm, and 17.7 ± 1.2 against vancomycin-resistant *Enterococcus faecalis*, multidrug-resistant *Pseudomonas aeruginosa*, and multidrug-resistant *Acinetobacter baumannii.* They proposed that the AgNPs directly adhered to bacterial cell membranes and caused the subsequent bacterial destruction. Besides, silver ions (Ag^+) may also release from AgNPs restricting the microbial growth. In a similar study, the polyphenol-rich extract of *Origanum vulgare* leaf was used for synthesis of AgNPs (30.20–58.81 nm). The synthesized nanoparticles exhibited significant antibacterial activity against ampicillin-resistant *E. coli* and kanamycin-resistant *E. coli*. It was observed that AgNPs damaged the cell membrane and altered the permeability of the cell membrane, thus disrupting the functioning of the bacterial respiratory chain or the proton FOF1-ATPase, causing cell death. Moreover, AgNPs displayed a genotoxic effect that damages DNA sequences. The shapes of NPs also influence their antibacterial efficacy. AgNPs obtained by reducing plant origin substances extracted from the *O. vulgare* leaves possess round shapes. As per the previous studies, round-shaped AgNPs are more active than the other forms (Hambardzumyan et al. 2020). Similar results have also been referred by (Aghajanyan et al. 2020) by using the *Artemisia annua* extract as reducing agents. When compared with round-shaped AgNPs synthesized using *O. vulgare* leaf extracts, the Artemisia extract's synthesized AgNPs displayed seven to nine times lower activity. These nanoparticles can potentially be applied for various antimicrobial biomedical and biotechnological applications.

In another study, the *Cinnamomum zylinicum* bark extract was employed to biosynthesize AgNPs to combat the MDR gram-negative bacteria *Acinetobacter baumannii*, *Klebsiella pneumonia*, and *Pseudomonas aeruginosa* strains and gram-positive bacteria *Staphylococcus aureus*. The results indicated that obtained AgNPs when compared with antibiotics are more efficient in inhibiting both gram-positive and gram-negative bacteria, giving a zone of inhibition of 22 mm against *A. baumannii*, 24 mm against *K. pneumonia* and *P. aeruginosa*, and 25 mm against *S. aureus,* respectively. The results are demonstrating the excellent potential of AgNPs in the development of robust antimicrobial products for clinical use (Almalah et al. 2019). Similarly, in a recent study (Maghimaa and Alharbi 2020), AgNPs were synthesized from the aqueous extract of *Curcuma longa* leaf and later coated on cotton fabric. The highest inhibition zone of 24 mm against antibiotic-resistant

P. aeruginosa was displayed, followed by the 22 mm for *S. aureus*. Thus, the AgNPs-loaded cotton fabrics revealed potential as an effective antimicrobial textile fabric for hospital patients and medical workers. Generally, Ag-infused textiles are advertised with the antibacterial effect and have also evidenced reduction of bad odors after sweating. Poly drug, Triphala powder, a combination of powders of three myrobalans, amalaki (*Emblica officinalis*), haritaki (*Terminalia chebula*), and bibhitaki (*Terminalia belerica*), in equal proportion was used for the synthesis of nano-antibacterial agents to combat against biofilm-forming, antibiotic-resistant pathogen. The synthesized AgNPs of hydrodynamic diameter 242.2 nm at various concentrations revealed a 70–86% reduction in the growth rate of biofilm-forming *Klebsiella pneumonia.* The researchers concluded that Triphala-based AgNPs could be incorporated in different formulations to control pathogenic bacteria causing life-threatening infections in humans and animals (Ranjani et al. 2019). The *Tectona grandis* and *Sisymbrium irio* plants were also explored for the biosynthesis of AgNPs with potential antibacterial agents to reduce, spread, and manage the multidrug-resistant bacterial infections (Mickymaray 2019; Rautela et al. 2019). Conclusively, the plant-derived AgNPs possess great antibiotic and antibacterial potential; hence there is a huge interest in the biosynthesis of AgNPs using plants. The most recent studies with their specific key data along with the synthesis routes have been summarized in Table 8.1.

AgNPs in conjugation with existing antibiotics possesses some novel characteristics granting them enhanced antibacterial potential (Mohamed et al. 2020). When attached with antibiotics, AgNPs showed improved stability, selectivity, and functionality (Kingsley et al. 2006). Some studies have also demonstrated that the antibacterial activities of kanamycin, ampicillin, chloramphenicol, and erythromycin were elevated when conjugated with AgNPs against various pathogenic bacteria (Ahmad et al. 2020). For example, (Li et al. 2021a) have synthesized a combination of AgNPs and kanamycin to perform biofilm-triggered on-demand drug release in situ. The combination of kanamycin and AgNPs displayed superior antibacterial activity against multidrug-resistant test strains (*Staphylococcus aureus*, *Streptococcus pneumonia*, *Pseudomonas aeruginosa*, *and E. coli)* than when used separately. In a study by (Anjum et al. 2018), gentamycin-conjugated AgNPs exhibited significant antibacterial activity against antibiotic-resistant *S. pneumoniae* and *S. aureus*. The antibiotic-NPs conjugate system inhibited the cell replication and caused cell lysis. Mohammed et al. (2021) evaluated the combination of various antibiotics with AgNPs to obtain the most efficient antibacterial agent. Among ciprofloxacin, cefotaxime, and ceftazidime, the combination of ciprofloxacin with AgNPs displayed the highest synergistic antimicrobial activity against multidrug-resistant extended-spectrum beta-lactamase (MDR-ESBL) *E. coli.* However, against MDR-ESBL *K. pneumoniae*, combination of AgNPs and cefotaxime was found to be superior, followed by ceftazidime and ciprofloxacin. The least synergistic combination against both the strains was that of ampicillin and AgNPs. Another reason behind the progressive usage of AgNPs-antibiotics conjugate is the toxicity concerns regarding AgNPs. The efforts are made to reduce the effective antimicrobial dose of AgNPs against multidrug-resistant (MDR) pathogens. In a study reported

Table 8.1 Antimicrobial activities of colloidal nano-silver against antibiotic-resistant bacterial strains

Synthesis	Size of NPs	Pathogens tested	Evaluation parameters	Application area	References
Chemical	~5 nm	Methicillin-resistant *Staphylococcus aureus*	MIC: 1–128μg/mL	Antibacterial material for medical applications	Liu et al. (2018)
Biological	~28.30 nm	Methicillin-resistant *staphylococcus Aureus*, methicillin-resistant *Staphylococcus epidermidis*, and vancomycin-resistant *enterococci*	ZoI: 9.6–24.5 mm	Bactericidal support with pharmacodynamic and pharmacokinetic properties	Muthukrishnan et al. (2019)
Plant based	10–78.9 nm	MDR *Acinetobacter baumannii, Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*	ZoI: 22–25 mm, and MIC: 2.8–4.5μg/mL	Antibacterial products for clinical use	Almalah et al. (2019)
Chemical	25–30 nm	Vancomycin-resistant *E. coli*	ZoI: 7 ± 0.3 mm	Antibacterial nano-drug complex	Kaur et al. (2019)
Biological	30–50 nm	Methicillin-resistant coagulase-negative *staphylococcus*	ZoI: 15.5–18 mm, and 91% of biofilm inhibition	Drug development and healthcare settings	Rajivgandhi et al. (2019)
Chemical	25 nm, 80 nm	Carbapenem-resistant *E. coli,* vancomycin-resistant *S. aureus*	100% death rate	Biocompatible antimicrobial products for biomedical applications	Jose et al. (2019)
Plant based	242.2 nm	*MDR Klebsiella pneumoniae*	70–86% reduction in bacterial growth rate	Antibacterial formulations for clinical use	Ranjani et al. (2019)
Plant based	30–50 nm	Ampicillin-resistant *E. coli*, kanamycin-resistant *E. coli*	MIC 9.16–18.35μg/mL	Antimicrobial support for biomedical applications	Hambardzumyan et al. (2020)

(continued)

Table 8.1 (continued)

Synthesis	Size of NPs	Pathogens tested	Evaluation parameters	Application area	References
Chemical	20 nm	Antibiotic-resistant *Pseudomonas aeruginosa*	MIC:5–10μg/mL, and 100% cell death	Antimicrobial agent for the infection treatment	da Silva et al. (2020)
Biological	6–24 nm	Multidrug-resistant pathogens, *Escherichia coli and Pseudomonas aeruginosa*	MIC: 6.25–50μg/mL	Antibacterial agents to inhibit multidrug-resistant microorganisms	Akter et al. (2020)
Biological	10–50 nm	Multidrug-resistant *Pseudomonas aeruginosa*	ZoI:15 mm	Antimicrobial for treatment of drug-resistant pathogens	D'Lima et al. (2020)
Chemical	6.8 ± 2.28 nm	MDR *salmonella*	MIC: ≤0.002–0.313μg/mL MBC: 0.078–1.250μg/mL	Antibacterial agents for therapeutic purpose	Farouk et al. (2020)
Biological	4–17 nm	MDR *Staphylococcus aureus* and *Escherichia coli*	Growth inhibition of 97.5–96.7%	Antibacterial material for surface decontamination	Ghodake et al. (2020)
Biological	8–30 nm	Antibiotic-resistant *Vibrio parahaemolyticus* and *salmonella typhimurium*	MIC: 3.12–6.25μg/mL, and MBC: 12.5–25μg/mL	Potent antibacterial material against pathogenic bacterial strains	Huq (2020)
Biological	17.5–20.1 nm	MDR *Pseudomonas aeruginosa, Staphylococcus aureus*, and *Escherichia coli*	53–65% eradication of biofilm	Antibacterial potential for dental implants and wound healing	Skóra et al. (2021)
Plant based	20–30 nm	Vancomycin-resistant *enterococcus faecalis,* multidrug-resistant *Pseudomonas aeruginosa*, and multidrug-resistant *Acinetobacter baumannii*	ZoI: 6.0–17.7 mm, MIC: 5.6–11.25μg/mL, and MBC:11.25–22.5μg/mL	Antibacterial application for treating major cause of nosocomial infections	Choi et al. (2021)

by (Mohamed et al. 2020), the AgNPs-vancomycin combination effect was remarkable on the antibiotic (gentamicin, ampicillin, erythromycin, amoxicillin and vancomycin)-resistant *P. aeruginosa* and *K. pneumonia* strains which can be explained by the high surface-to-volume ratio of the AgNPs and their hydrophobic nature. The AgNPs make transport and entrance of antimicrobial agents inside the bacterial cell more effective by damaging the bacterial cell membrane. Inclusively, the synergistic treatments were more effective than either treatment alone; hence, the utilization of nanoparticles in combination with antibiotic is highly regarded.

Halawani et al. (2020) biosynthesized AgNPs from *Rosa damascenes*, and conjugated the same with cefotaxime; the resultant antibiotic-AgNPs conjugate displayed maximum antibacterial activity against methicillin-resistant *Staphylococcus aureus* and cefotaxime-resistant *E. coli* compared to when used separately. Such bio-fabricated AgNPs-antibiotics conjugate provides enormous antibacterial potential against several multidrug-resistant microbes. In a similar study, the combination of AgNPs with a broad-spectrum ciprofloxacin (Cipro-AgNPs) revealed better antibacterial activity when compared to AgNPs and ciprofloxacin separately. The Cipro-AgNPs exhibited zones of inhibition of 33 mm, 35.5 mm, 35.5 mm, and 38.5 mm against pathogenic multidrug-resistant *S. sonnei*, *S. typhi*, *C. amalonaticus*, and *E. coli*, respectively. Hence, it can be suggested that the AgNPs and ciprofloxacin synergistically resulted in better antibacterial activity against pathogens when used in conjugation (Adil et al. 2019).

Even though AgNPs have broad applicability in various fields, certain concerns need to be addressed: First, colloidal AgNPs tend to get aggregated and eventually lose their original antibacterial efficacy. Second, colloidal AgNPs cannot be reused further, making the process uneconomical and unrealistic (Salleh et al. 2020; Agnihotri et al. 2019). In a study performed by (Menazea 2020), the AgNPs were tested in different mediums. The result revealed that these nanoparticles tend to aggregate in an organic medium such as dimethylformamide and tetrahydrofuran solutions. In contrast, immobilized AgNPs represent more stability as they are less prone to aggregation and oxidation when exposed to the aqueous medium. Also, the immobilization of silver nanoparticles on a support matrix further escalates their reusability and minimizes toxic effects associated with their inevitable disposal in the environment (Dhiman and Agnihotri 2020). When immobilized on several organic and inorganic substrates such as graphene, Fe_2O_3, SiO_2, and zinc oxide, silver nanoparticles have displayed enhanced antibacterial performance over long-term use (Agnihotri et al. 2013). Hence, immobilization of AgNPs on a support matrix would allow controlled silver release to realize an effective antimicrobial system against several multidrug-resistant strains.

8.3 Silver Nanocomposites as Antimicrobials

The elimination of limitations and disadvantages associated with colloidal silver nanoparticles has become integral to global research with an aim to promote their reusability, enhancing stability and diversifying their utilization in various applications along with minimizing leaching to reduce ecotoxicological impacts. Alternative strategies such as immobilization and/or incorporation of nanoparticles onto a support matrix are particularly considered to eradicate mainstream issues (Agnihotri et al. 2015; Dhiman et al. 2019; Zheng et al. 2016). The resultant stabilized multi-element structures arising from such strategies are known as nanocomposites. Silver nanocomposites consolidate functionality between silver nanoparticles and matrix materials through enhancing the useful abilities while downplaying the detrimental characteristics. Therefore, the creation of novel silver nano-formulations with modifiable active properties has been enabled by utilization of nanocomposites. In recent years, the unique characteristics of such materials have garnered substantial and widespread interest in extensive applications than single silver nanoparticles.

Most important aspect of nanocomposites is the type of support matrix which is responsible for intended stabilization, and nowadays, there has been availability of wide range of support materials. The incorporation of silver nanoparticles onto some support matrices like electrospun nanofibers, polymeric hydrogels, ceramics, and metal organic frameworks has shown to improve the recovery and reusability of resulting nanocomposites. Other than acting as a template, these support materials may contain some inherent antimicrobial features which work synergistically to enhance antimicrobial performance and broaden their potential applications to combat against the antibiotic resistance in biomedical use. Hence, the current portion will be focused on various silver-based nanocomposites with a support matrix employed for eradicating antibiotic-resistant strains in medical implant-associated infections, biomedical, wound dressings, and other relevant domains.

In biomedical applications, the utility and importance of implants have become indispensable for the replacement and repair of injured tissues/organs. However, implant-associated infections which are caused by bacterial adhesion followed by biofilm formation may result in implant failure, immune system dysfunction, and revision surgeries. Ultimately, the removal of an infected implant followed by a series of antibiotic treatments remains the only way to completely remove infection before implanting new device. As a result, treatments require extended antibiotic therapy and 1000 times higher antibiotic dosage to kill biofilm bacteria than non-sessile planktonic bacteria (Duan and Wang 2006; Xu et al. 2017). This has become progressively difficult with the introduction and increasing amounts of multiple drug/antibiotics-resistant pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA), which are causing high failure rates of current treatments (Vuong et al. 2016).

The blend composites involving metal/metal oxide nanoparticles along with silver nanoparticles have been investigated for their antibacterial efficacy against antibiotic-resistant bacteria to circumvent bacterial proliferation on implants. The

incorporation of other nano-entity could restrain the cytotoxicity of silver nanoparticles while registering a synergistic impact on the antibacterial potential. For instance, (Van Hengel et al. 2020) employed silver and zinc nanoparticles-biofunctionalized laser-melted titanium implants to prevent implant-associated infections caused by antibiotic-resistant bacteria. The antibacterial activity was determined against MRSA using zone of inhibition, minimal inhibitory concentration (MIC), and minimal bactericidal concentration (MBC). The implants' surfaces containing 75% silver (7–25 nm) and 25% zinc (40–60 nm) nanoparticles demonstrated a complete eradication of both adherent and planktonic bacteria in all experiments performed using murine femora within 24 h. The zone of inhibition for the same composition was around 0.6 cm^2, demonstrating excellent potential for biomedical applications. The addition of Zn reduced the cytotoxicity caused by Ag alone while preserving the synergistic antibacterial behavior against the antibiotic-resistant bacterial infection.

The use of biogenically synthesized silver nanoparticles while designing biomedical coatings could be beneficial to improve hemocompatibility with improved functions. For example, Neethu et al. (2020) fabricated a bionanocomposite coating for the central venous catheter (CVC) using mycogenerated AgNPs and polydopamine. Antibiofilm activity of the fabricated material was investigated against multidrug-resistant *Acinetobacter baumannii*. The antibacterial potency of the surface-modified central venous catheter was evident by the zone of inhibition (ZoI) 23.9 ± 0.8 mm against *Acinetobacter baumannii*. The minimum inhibitory concentration and minimum bactericidal concentration were recorded as 15.6 and 31.2μg/mL, respectively. Study establishes that AgNPs (10–15 nm) at minimum bactericidal concentration were able to disrupt bacterial biofilm on the central venous catheter via lysing the adherent cells. While authors evidenced a significant change in biofilm appearance just after 1 h of treatment, the biofilm was completely removed from catheter's surface after 24 h of treatment. This indicates a promising aspect of using green-synthesized silver nanoparticles in the formation of nano-antimicrobials, which is expected to prevent the infections caused by MDR strains in coming future.

In another study, Guan et al. (2019) fabricated polydopamine (PDA) coatings with silver nanoparticle-loaded TiO_2 nanorods on Ti alloy. The antibacterial activity of the coatings was tested against methicillin-resistant *S. aureus* using disc diffusion and colony-counting methods. Ag-TiO_2/PDA coatings displayed significant antimicrobial action with ZoI of 13.5 mm and bacterial inhibition rates of 95.4%, 88.6%, and 80.1% at 1, 7, and 14 days along with satisfactory biocompatibility against mouse calvarial cells. The authors intended Ag-TiO_2/PDA coatings to combat against infections in orthopedic and dental implants. Silk fibroin derived from *Bombyx mori* silkworm is considered a favorable biomaterial owning to its excellent biocompatibility, biodegradability, mechanical strength, and low inflammatory response. The promotion of tissue integration prior to bacterial adhesion, which prevents colonization of certain bacterial species on the implant, is seen as ideal means to overcome antibiotic resistance in bone tissue engineering. Therefore, a group of authors (Patil and Singh 2019) reported a silk fibroin-based bone tissue scaffold with silver nanoparticles with advanced antimicrobial properties. Silk

fibroin with tyrosine residues that have potent electron-donating capabilities was employed as template for AgNPs biosynthesis, consequent reduction, as well as stabilization. The silk fibroin/AgNPs films at an MIC of 20 nM displayed superior bacterial killing capacity in case of kanamycin-resistant *E. coli* compared to ampicillin-resistant *E. coli* where few colonies could be observed. Nevertheless, AgNPs incorporated presented impressive antimicrobial properties with no detrimental effect on osteogenic differentiation potential of human mesenchymal stem cells.

Chien et al. (2018) strived to create an antibacterial material targeted for prosthetic joint implants (metal or ceramic), the failure of which has become increasingly common due to infections arising from antibiotic-resistant pathogens. The silver nanoparticles were confined to mesoporous silica (MS-AgNPs), and the composite powder was evaluated for its antibacterial activity against methicillin-resistant *S. aureus*. The diameters of the inhibition zones (ZoI) were found to be in the range of 15.7–17.6 mm (Fig. 8.2) with increasing composition ratios of silica/Ag of 100:1 (MS/Ag1), 100:5 (MS/Ag5), and 100:10 (MS/Ag10). Additionally, time-killing assay demonstrated that only composites in the MIC range of 5–20 mg/mL were able to effectively inhibit the growth of MRSA.

Wounds have the tendency to get infected which makes the overall healing process more complicated and impeded with the potential risk of body fluid loss and acute inflammation (Wahab et al. 2021b; Potara et al. 2011). Few bacterial infections require external medication and, more so, dressing pads impregnated with a broad-spectrum antibacterial agent that has a higher bacterial killing rate and curing (Konop et al. 2016). In the context of treating antibiotic-resistant strain infections, AgNPs-bio composites could be an excellent antibacterial material and play a key role in designing materials for wound dressing applications because of their biocompatibility, good flexibility, tear resistance, and broad-spectrum antimicrobial activity (Kumar et al. 2018).

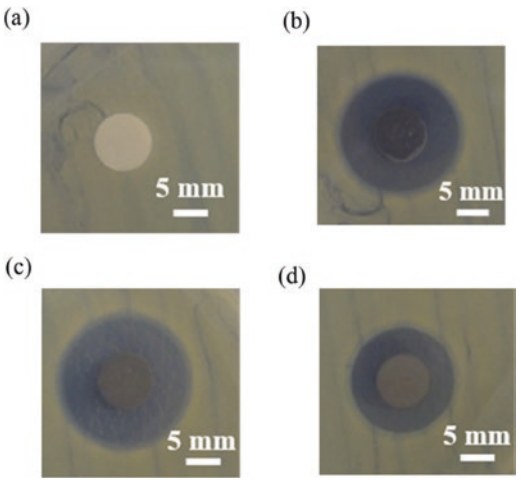

Fig. 8.2 Inhibition zone images of (**a**) mesoporous silica (MS), (**b**) MS/Ag1, (**c**) MS/Ag5, and (**d**) MS/Ag10 against methicillin-resistant *Staphylococcus aureus* (MRSA). (Reprinted with permission from Chien et al. (2018), Elsevier)

Stojkovska et al. (2019) produced an eco-friendly, non-sticky, and bioactive honey-based AgNPs/alginate hydrogel dressing with the aim to target multidrug-resistant bacterial strains causing nosocomial wound infections. The resulting nanocomposites molded into different forms of microbeads, microfibers, and discs were tested for their antibacterial efficacy against multi-resistant hospital strains of *A. baumannii*, *P. aeruginosa*, and *S. aureus*. At the total released silver concentration of ~9μg/mL, the hydrogels exhibited about 99.9–100% bacterial reduction. Bassous and Webster (2019) engineered highly sophisticated and biocompatible polymeric nanovesicles called polymersomes via hydrophobic interactions through self-assembly. The hydrophobic corona of such polymersomes was then employed to incorporate antibacterial formulations consisting of silver nanoparticles and antimicrobial peptides (PR-39, AMPs). These structures were functionalized to aid with wound healing or immunomodulation and combat antibiotic-resistant bacterial infections, caused especially by methicillin-resistant *S. aureus* (MRSA). In vitro testing on MRSA showed that ratio of AgNPs to AMPs of around 1:5.8 corresponding to ≈11.6μg/mL of silver nanoparticles and 14.3×10^{-6} M of peptides worked synergistically to yield 100% MRSA inhibition over time frame of 23 h. These AMP/AgNPs polymersomes could potentially replace antibiotic therapies in the clinical setting on account of its bacteriostatic activity coupled with nominal cytotoxicity towards native human dermal fibroblast cells.

The hybrid composites consisting of natural polymers and metal nanoparticles are promising candidates for biomedical applications. Owing to its various unique features such as biodegradability, biocompatibility, bioactivity, cell adhesion, and ability to form any structure, chitosan has been used widely for biomedical applications. The incorporation of silver nanoparticles with chitosan has been investigated for their antibacterial efficacy. For instance, (El-Aassar et al. 2021) used chitosan-based-crosslinked gelatin/polyvinyl pyrrolidone-embedded silver nanoparticles for formulating novel wound dressing to eliminate multidrug-resistant microbes. Skin healing properties and ability of blood coagulation were among the major aspects for using gelatin, while mechanical strength governed by biocompatible polyvinyl pyrrolidone justifies its role in the composite dressing material. The authors also addressed the use of crosslinker (glutaraldehyde) for modulating mechanical strength and hydrophilicity of the wound dressing. Composite membranes with silver nanoparticle (9.99 nm) concentration ranging from 10μg/mL to 150μg/mL were employed to determine the antibacterial activity against multidrug-resistant microbe strains, i.e., *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus subtilis*. Zone of inhibition (ZoI) was measured to analyze the bactericidal efficiency; at highest concentration of silver nanoparticles (150μg/mL), the widest ZOI of 3.12 cm was found for *S. typhi*, followed by *B. subtilis* (2.78 cm), *S. aureus* (2.55 cm), and *E. coli* (1.97 cm). The detailed investigation of mode of mechanism revealed reduction in resistance genes expression, i.e., *β-lactamase*, *mecA*, and *erm*. The formulated composite membrane resulted in 50% reduction in expression of *mecA* and 97% reduction in expression of *erm* in *S. aureus* (MRSA). In gram-negative bacterial strain *E. coli*, complete elimination of *β-lactamase* and 63% reduction of *erm* expression were reported. Additionally,

observed 12-fold less cytotoxicity of the composite membrane as compared to the control demonstrated its biocompatibility for direct wound application.

Recently, a chitosan-sericin hybrid nanocomposite film containing a unique blend of AgNPs and antibiotic moxifloxacin was prepared for wound dressing applications (Shah et al. 2019). The preliminary experiments performed on Sprague-Dawley rats (burn wound model) for 7 days revealed faster healing pattern in wounds indicated by successful fibrosis, collagen reorganization, and mild epidermal regeneration, while the presence of AgNPs ruled out the chances for contamination. Additionally, the films displayed highest antibacterial activity (ZoI, 37.25–50.75 mm) against all clinical isolates of MRSA. Shah et al. (2018) fabricated functional chitosan silver nanocomposite (CSN) films through in situ chemical reduction using $NaBH_4$. The developed nanocomposite films exhibited significant antibacterial activity against clinical isolates of MRSA strains with ZoI ranging between 12.67 and 24.73 mm as the content of silver increased. This biocidal efficacy was found to be equally and/or even effective than commercially available dressing products such as Aquacel Ag®, Bactigras®, and Kaltostat®. Thus, these nanocomposite films were said to have potential to control antibiotic resistance in healthcare applications such as water disinfection, wound dressing, etc.

Bacterial proliferation control and prevention are essential not only for medical supplies like bandages and dressings but also in case of clothes and domestic use textiles. Such products could easily serve as the niche for obnoxious microbial growth (Karwowska 2017). Therefore, in order to limit contamination from pathogenic microbes and drug-resistant microorganisms, various ventures are being undertaken for development of fibers and textiles with "self-cleaning properties." Nowadays, nanoparticle-based coatings and impregnation particularly involving nano-silver are most recommended for fabric modifications (Dastjerdi and Montazer 2010). Recently, a group of authors to meet the challenge of drug-resistant bacteria made use of highly advocated antimicrobial photodynamic therapy (Chen et al. 2019). In this study, silver nanoparticles (0.91 mg per 100 cm^2 of fabric) combined with a potent photosensitizer, zinc phthalocyanine, were conjugated on a cellulose fabric. The composite material presented highly efficient photodynamic biocidal effect against methicillin-resistant *S. aureus* with 99.96% kill efficacy within minutes under light illumination. Antibacterial mechanism revealed that two active components (AgNPs and photosensitizer) worked synergistically for photoinactivation of bacteria. The photosensitizer activated the silver nanoparticles into silver ions under light illumination, while at the same time, AgNPs enhanced the ROS generation premeditated by the photosensitizer. Moreover, modified cellulose fabrics still displayed about 99% bacterial killing efficacy when used repeatedly for five washing cycles.

In our everyday lives, paper and related products such as books, magazines, office paper, wallpaper, medical records, bank notes, and food items packaging paper are widely used. Since these materials do not have any inherent antimicrobial characteristics, their circulation and distribution in various environments to multiple people can cause alarming rates of transmission of numerous infectious diseases (Angelakis et al. 2014). Furthermore, re-emergence of previously well-controlled

infectious diseases as well as development of new strains of bacteria resistant to currently available antibiotics can arise from such contamination (Ma et al. 1994). Thus, the demand of papers with the ability for inhibition and/or prevention of attachment, establishment, and proliferation of microbes on their surfaces has risen. Consequently, to address this need, many attempts have been made by either preparing antimicrobial pulp for paper or by directly modifying the paper with antimicrobial agents (Amini et al. 2016). Paper with such defense characteristics can be very useful in different applications, including packaging and filtering, etc. Islam et al. (2018), inspired from the biology of the marine mussel, devised an effective method for AgNPs immobilization on cellulose paper (CP). The strategy first involved the succinic acid surface activation of cellulose paper which otherwise lacks any functional groups or provides specific chemical reactivity. Further functionalization of cellulose paper was carried out by dopamine conjugation via coupling reaction which was proven useful in utilizing the tethered catechol groups to effectively immobilize the AgNPs. The antimicrobial properties of the final composites were found to be dependent upon immersion time in ammoniacal $AgNO_3$ solution. The Ag-Dopa-CP at 8 h immersion displayed higher values of ratio between the diameter of the zone of inhibition and the diameter of Ag-Dopa-CP disk (dia_{ZOI}/dia_{disk}) at 2.25, 1.98–2.11 against antibiotic-resistant strains of *Vibrio parahaemolyticus* and *Enterococcus faecalis.* Excellent antimicrobial properties along with exceedingly low leaching of AgNPs from the paper surface would obviously facilitate the use of the fabricated Ag-Dopa-CP as a packaging material. With exceedingly low leaching of AgNPs at 2.6μg/mL (0.21% of total silver content), such modified papers could be employed as antimicrobial packaging materials.

Despite the advancements in many fields in the last several decades, provision of sufficient methods for treatment of freshwater and wastewater to eradicate organic matter, pathogenic microbes, and unwanted chemicals is still among the top vital goals to be accomplished (Lemire et al. 2013). Various traditional approaches have been employed in the past for treatment and still are in effect such as filtration (suspended or microparticle removal), advanced oxidation (organic component degradation), and filtration with UV exposure (disinfection of water from microorganisms). However, there is a requirement for multifunctional systems to decompose moieties and function against multidrug-resistant microbes present in water. Thus, composites of AgNPs provide innovative formulations with multitasking ability against various microorganisms as silver can form nanocrystalline providing better efficacy in water disinfection than regular filters (Deshmukh et al. 2019). These composite systems are feasible and economical as AgNPs can treat water by degrading the organic substances and killing the antibiotic-resistant microbiological species both in one step without affecting the physicochemical properties of water (Villanueva et al. 2014).

Naz et al. (2019) prepared silver nanoparticles (10–15 nm)-embedded graphene oxide (AgNPs-GO) nanocomposites through one-pot synthesis technique which exhibited enhanced photocatalytic and antibacterial activities with improved biocompatibility. The synergism between AgNPs and GO not only completely eradicated the growth of methicillin-resistant *S. aureus* for up to 24 h but also degraded

97–99% of toxic organic dyes such as rhodamine B, methylene blue, and commercial dye, AY, within 12–24 minutes time frame. Furthermore, the nanocomposites with doses up to 160 ppm were nontoxic with ~85% cell viability for human corneal epithelial cells. Similarly, (Guo and Tian 2019) loaded TiO_2 and AgNPs onto graphene oxide to form a novel nanocomposite material for antibiotic resistance control in wastewater systems. The bactericidal capacity of the composites with concentration ranging from 10 to 100 mg/L revealed that growth inactivation rates between 66.6 and 97.8% could be achieved within 10 minutes against tetracycline-, gentamicin-, streptomycin-, kanamycin-, and ampicillin-resistant *E. coli* strains under simulated sunlight irradiation.

Ullah et al. (2018) synthesized water-soluble graphene oxide-based composite loaded with an aminoglycoside antibiotic, tobramycin (TOB), and silver nanoparticles. In the composite TOB/GO/AgNPs, tobramycin served a dual role in assisting the reduction/decoration of silver nanoparticles and creation of synergism for enhanced antibacterial efficacy. The nanocomposite visually reduced the bacterial colonies of drug-resistant *E.coli* pathogen to a significant number compared to pristine individual counterparts (Fig. 8.3). The synergistic effect of the three components where silver nanoparticles produced ROS while GO cuts membrane by physical contact and tobramycin inhibits protein synthesis was collectively attributed to improved antibacterial activity. Analogous antimicrobial performance of silver-based nanocomposites against multidrug-resistant bacterial pathogens has been summarized in Table 8.2.

The broad-spectrum antibacterial activity of AgNPs compared to other common antibiotics will bring benefits to general biomedical applications. Rafael et al. (2019) synthesized a thermosensitive hydrogel based on Pluronic® F127 loaded with AgNPs for in situ chirurgic biomedical applications. The silver nanoparticles with average size of around 22 nm were incorporated into the hydrogel in an attempt to circumvent antibiotic resistance and provide postoperative infection prophylaxis. At MIC of ≥0.125 mM, drug-resistant clinical isolates of *P. aeruginosa* and *S. epidermidis* showed susceptibility towards the loaded hydrogels. Although, it was revealed through in vitro experiments that AgNPs inside the hydrogel demonstrated constrained antibacterial activity and therapeutic effect due to slower silver ion release. However, this sustained release from a positive perspective of clinical applications might allow such hydrogels to have prolonged prophylactic effects over time.

In another report, the effect of zinc oxide and silver nanoparticles (ZnO-AgNPs) on the biofilm of methicillin-resistant *Staphylococcus aureus* (MRSA) and *icaA* gene expression was investigated (Shakerimoghaddam et al. 2020). The composite (ZnO-AgNPs) had reduced minimum inhibitory concentration of 60.8μg/mL as compared to the MIC values of ZnO (393.2μg/mL) and Ag (179.8μg/mL). The reduction in the MIC could be attributed to the increased permeability of silver nanoparticles into the bacterial biofilms and increased free-electron production from zinc oxide due to the combinational effect. The complete inhibition of biofilm was observed at MIC concentration of the ZnO-Ag nanoparticles. Also the expression of *icaA* gene which affects the initial binding of bacteria and biofilm formation was significantly decreased (2.98-fold) under the influence of nanoparticles at

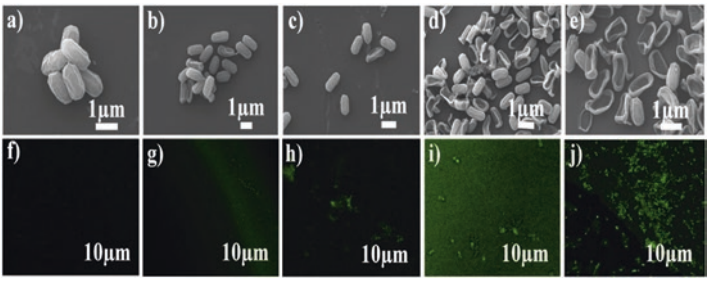

Fig. 8.3 SEM image analysis of treated multidrug-resistant *E.coli* with (**a**) PBS, (**b**) GO, (**c**) tobramycin, (**d**) only AgNPs, and (**e**) TOB/GO/AgNPs. The intracellular production of ROS in (**f**) control, (**g**) GO, (**h**) tobramycin, (**i**) only AgNPs, and (**j**) TOB/GO/AgNPs. (Reprinted with permission from Ullah et al. (2018), Elsevier)

sub-MIC (1/2 MIC). The observed results demonstrated that nanoparticles synergistically prevent early attachment and biofilm formation, thus proving to be a potent antimicrobial agent for biomedical applications.

Recently, antibacterial activity of silver nanoparticles-decorated and mesoporous silica-coated single-walled carbon nanotubes was evaluated against drug-resistant bacteria (Zhu et al. 2020). The MIC and MBC of the composite were determined against multidrug-resistant bacteria *Escherichia coli* and *Staphylococcus aureus*. The MIC of composite (SWCNTs@mSiO_2-TSD@Ag) against gram-negative *E. coli* was 120μg/mL, which was equivalently better than both SWCNTs@mSiO_2-TSD and commercial AgNPs. Similar results were obtained in case of gram-positive bacteria *S. aureus* where MIC of composite was recorded at 140μg/mL. The improved antibacterial activity could be due to two reasons: First, the mesoporous silica due to its hydrophilic nature induced dispersibility, thus increasing the contact area with bacterial cell walls. Second, the small size and uniform distribution of silver nanoparticles (2.78 ± 0.70 nm) in the pores of silica have superior antibacterial potential. Authors further investigated the bacterial growth kinetics in presence of the composite and reported that even at MIC/2 concentration, the propagation of *E. coli* is restrained for the first 12 h, and using the MIC concentration of composite (120μg/mL) resulted in complete inhibition of the growth of *E. coli* up to 24 h. The morphological observations revealed the damage on bacterial cell membranes after treatment with composite material. The release of Ag^+ ions was attributed as the mode of action as the release was faster on the first day, and then followed a sustainable release till 11 days. The cumulative release of silver ions was found to be five times higher in case of composite material as compared to silver nanoparticles. Exposure of Ag^+ to the bacterial cytoplasmic matrix leads to irreversible damage to DNA, proteins, and lipids resulting in death of the bacteria. The cytotoxicity assay of 150μg/mL SWCNTs@mSiO_2-TSD@Ag demonstrated 71% cell viability, which was in the safer limit. The superior bacterial killing, wound healing characteristics, as well as excellent biosafety of the composite present it as an excellent means for treating clinical drug-resistant infections.

Table 8.2 Antimicrobial activities of silver-based nanocomposites against antibiotic-resistant bacterial strains

Silver-based nanocomposites (NCs)	Size of NPs	Pathogens tested	Evaluation parameters	Application area	References
Zeolites/AgNPs	ND	Methicillin-resistant *S. aureus*	MIC: 0.019–0.750 mg/mL	Antibiotic resistance control in medicine	Golubeva et al. (2018)
Dopamine-modified cellulose paper/AgNPs discs	50–60 nm	Antibiotic-resistant *E. faecalis*, *V. parahaemolyticus*, *S. marcescens*	ZoI_{dia}/ZoI_{disc}: 1.66–2.52	Antimicrobial packaging material	Islam et al. (2018)
Chitosan/AgNPs film	ND	Clinically isolated MRSA strains, *S. aureus*, *P. aeruginosa*	ZoI: 11.40–36.83 mm	Multifunctional material for antibiotic resistance control in wound dressing, water disinfection, drug delivery	Shah et al. (2018)
Tobramycin/silver nanospheres/graphene oxide	5 nm	Multidrug-resistant *E. coli*	Colony counting; visual bacterial reduction	Hybrid material to combat MDR bacteria	Ullah et al. (2018)
Mesostructured silica (MS)/AgNPs	2–80 nm	Methicillin-resistant *S. aureus*	MIC: 5–20 mg/mL ZoI: 15.1–17.6 mm	Antibacterial material for resistant pathogens in prosthetic joint infection	Chien et al. (2018)
Polydopamine (PDA)/AgNPs-loaded TiO_2 nanorods	50–100 nm	Methicillin-resistant *S. aureus*	ZoI: 13.5 mm; Inhibition rates: 95.4%–96.7%	Antibacterial orthopedic and dental Ti alloy coatings	Guan et al. (2019)
Chitosan/sericin/AgNPs/moxifloxacin	18.39–96.93 nm	Methicillin-resistant *S. aureus*, 2 clinical isolates of MRSA	ZoI: 37.25–50.75 mm	Antibacterial films for wound healing	Shah et al. (2019)
Alginate/AgNPs/honey hydrogel	5–10 nm	*Multidrug-resistant A. baumannii*, *P. aeruginosa*, *S. aureus*	99.9–100% bacterial reduction	Antibacterial wound dressing	Stojkovska et al. (2019)

Polymersome nanocapsules (PsNPs)/PR-39 peptides/AgNPs	118–136.9 nm	Methicillin-resistant *S. aureus* (MRSA)	100% bacterial growth inhibition	Materials for alternate antibiotic therapies in clinical setting and wound healing	Bassous and Webster (2019)
β-Carboxyphthalocyanine zinc (PS)/AgNPs/cellulose fabric	~100 nm	Methicillin-resistant *S. aureus* (MRSA)	99.96% kill efficacy under light illumination	Wound healing and biomedical textile antibacterial material	Chen et al. (2019)
Pluronic® F127/AgNPs hydrogel	20.06–22.25 nm	Extremely drug-resistant (XDR) clinical isolates of *P. aeruginosa*, *S. epidermidis*	MIC: ≥0.125 mM	Antibacterial product for broad prophylaxis of postoperative infections	Rafael et al. (2019)
Ag/graphene oxide	10–15 nm	*E.coli,* methicillin-resistant *S. aureus*	100% growth inhibition	Antibiotic resistance control material for wastewater treatment	Naz et al. (2019)
AgNPs/silk fibroin scaffold	5–12 nm	Ampicillin and kanamycin-resistant *E. coli*	MIC: 20 nM	Bone tissue engineering	Patil and Singh (2019)
TiO_2/ag/graphene oxide	ND	Tetracycline, gentamicin, streptomycin, kanamycin, and ampicillin-resistant *E. coli* strains	Inactivation rates: 66.6–97.8%	Antibiotic resistance control material for wastewaters	Guo and Tian (2019)
Porous titanium functionalized with silver nanoparticles	7–25 nm	*Methicillin-resistant S. aureus (MRSA)*	ZoI 0.6 cm^2	Treatment of implant-associated infections (IAIs)	Van Hengel et al. (2020)
AgNPs and polydopamine coatings	10–15 nm	*Acinetobacter baumannii*	ZoI 2.39 cm	Development of antibacterial surfaces	Neethu et al. (2020)
ZnO-AgNPs	20 nm	*Methicillin-resistant S. aureus (MRSA)*	MIC 60.8μg/mL	Antimicrobial agent	Shakerimoghaddam et al. (2020)

(continued)

Table 8.2 (continued)

Silver-based nanocomposites (NCs)	Size of NPs	Pathogens tested	Evaluation parameters	Application area	References
Silver nanoparticles-decorated and mesoporous silica-coated single-walled carbon nanotubes	2.78 ± 0.70 nm	*E. coli* and *Staphylococcus aureus*	MIC 120–140μg/mL	Treatment of clinical drug-resistant infections	Zhu et al. (2020)
Fungal chitosan-silver nanoparticle nanoconjugates	13.8–68.9 nm	*S. Typhimurium* and *S. aureus*	MIC 3.50–4μg/mL	Antimicrobial agent	Alsaggaf et al. (2020)
Graphene oxide and silver nanoparticle hybrid composite	9–12 nm	*P. aeruginosa*	Incubation time: 0–10 min (kill efficiency 20–100%)	Antibacterial agents	Lozovskis et al. (2020)
Silver-microfibrillated cellulose bio-composite	140 nm	*S. aureus* and *P. aeruginosa*	MIC (125–1500 ppm)	Development of biomedical instruments and therapeutics	Garza-Cervantes et al. (2020)
Chitosan-based-crosslinked gelatin/polyvinyl pyrrolidone-embedded silver nanoparticles	9.99 nm	*P. aeruginosa, S. typhi*, *S. aureus*, *E. coli*, and *B. subtilis*	ZoI 0.33–3.12 cm	Wound dressing	El-Aassar et al. (2021)
Silver covalently bound to cyanographene	10 and 28 nm	MRSA and ESBL (extended-spectrum *β*-lactamases-producing *Klebsiella pneumoniae*)	MIC 0.2–3.4 mgL^{-1}	Broad-spectrum antibacterial agents	Panáček et al. (2021)
Nano-silver-decorated biodegradable mesoporous organosilica nanoparticles	2–8 nm	*E. coli, P. aeruginosa, S. aureus,* and *E. faecalis*	MIC (12–48μg/mL) MBC (12–48μg/mL)	New generation of antibacterial materials to kill antibiotic-resistant bacteria	Li et al. (2021b)
Silver nanoparticles in nanoporous carbon nitride	4–8 nm	Multidrug-resistant *E. coli*	MIC (16μg/mL)	Potential for biomedical applications including medical devices, food packaging, disinfectant, and wound dressing applications	Wahab et al. (2021b)
Ag/80S bioactive nanocomposite	2.26–5.66 nm	Carbapenem-resistant *K. pneumoniae*	MIC (2.5 to5.0 mg/mL)	Novel antimicrobial agent	Yang et al. (2021)

In another study, (Alsaggaf et al. 2020) used phyco-synthesized silver nanoparticles and nano-fungal chitosan composites against drug-resistant bacterial pathogens of *Salmonella Typhimurium* and *Staphylococcus aureus*. The ZoI and MIC of the composite were found to be 25.9 ± 1.5 mm and 3.50μg/mL against gram-negative strain (*S. Typhimurium*) and 23.8 ± 1.4 mm and 4.0μg/mL against gram-positive strain (*S. aureus*). The morphological observations after 4 h of treatment revealed shrunken and distorted bacterial cells with defects on their surfaces. The complete lyses of bacterial cells were observed after 8 h of treatment. The electrostatic interaction could be the reason for superior antibacterial activity as the positive-charged fungal chitosan provides favorable microenvironment for composite docking onto the bacterial surface, facilitating the interactions of silver nanoparticles with bacterial cell membranes. The fabricated composite could be utilized as antimicrobial agent in biomedical field.

Similar to the resistance developed by various microorganisms against antibiotics, resistance against silver nanoparticles is also evident in some of the bacteria, imposing a greater threat. The resistance against silver is induced via secretion of flagellin, which triggers coagulation of silver nanoparticles, thus restraining their antibacterial activity. The feasible solution for this is to prevent the nanoparticle agglomeration, and silver nanoparticles-based composites are the best suitable candidate. For instance, (Panáček et al. 2021) used densely functionalized graphene (cyanographene) for effective covalent attachment of silver nanoparticles and investigated its use against silver and antibiotic resistance bacterial strains. In case of silver resistance strains (*Escherichia coli* and *Pseudomonas aeruginosa*), silver covalently bound to cyanographene (GCN/Ag) was able to fully eradicate bacteria even at 30-fold lower concentration as compared to only silver required to kill all bacterial cells. The MIC of composite was found to be 1.9 mg/L against *P. aeruginosa* and 3.4 mg/L against *E. coli*. The composite was even more effective against multidrug-resistant bacterial strains, where MIC of 0.5 mg/L against ESBL (extended-spectrum *β*-lactamases-producing *Klebsiella pneumoniae*) and 1.9 mg/L against MRSA (methicillin-resistant *Staphylococcus aureus*) was observed. Cell membrane damage was evident of contact killing mechanism. The composite remained efficient antibacterial activity after 60 bacterial generations, as compared to colloidal AgNPs which lost their activity after 20 generations. The cytocompatibility of GCN/Ag in healthy human cells was also tested, with 60% cell viability composite demonstrated excellent cytocompatibility. The limited silver release was another important factor of the composite where accumulative leaching of only 0.27 mg/L was recorded even after 6 months of storage in water. The effective anchoring of silver nanoparticles onto other materials could also be used to produce broad-spectrum antibacterial agents and restrain the leaching of silver into environment.

In another report, graphene oxide and silver nanoparticle hybrid composite (GO-Ag HN) was investigated for antimicrobial activity against antibiotic resistance *Pseudomonas aeruginosa* strain (Lozovskis et al. 2020). The authors observed that with increasing incubation time period from 0 to 10 minutes with hybrid nanocomposite, the abundant growth of bacterial strain decreased from 80% to 0%. Similar

results were obtained via counting of colony-forming units in similar incubation period. The structural changes in the bacterial cell membrane before and after incubation period revealed appearance of some bulges, sags, and pores on the surface of cells. The change in the cell membrane surface indicates membrane damage that increases permeability and membrane leakage and results in cell lysis. The superior antibacterial efficacy of hybrid nanocomposite against antibiotic-resistant *P. aeruginosa* can be attributed to toxicity of graphene oxide, which increases after addition of silver nanoparticles (9–12 nm). Sharp edges of graphene oxide formed in the final composite may have caused physical damage to the cell membrane upon direct contact with bacteria. Furthermore, material characterization demonstrated that the edges of graphene oxide sheets were densely decorated with silver nanoparticles, causing high penetration of silver nanoparticles into the bacterial cell. These obtained results indicated that GO-Ag HN is a promising antibacterial agent with futuristic potential.

Multidrug-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa* were used to determine antibacterial activity of silver-microfibrillated cellulose bio-composite (Garza-Cervantes et al. 2020). The MIC of gram-positive *S. aureus* was found to be 1500 ppm and gram-negative *P. aeruginosa* was 125 ppm. The high concentration of bio-composite required for complete inhibition of gram-positive bacteria can be attributed to the thicker peptidoglycan layer that could prevent the penetration of bio-composite into the cell cytoplasm. The study highlights the potential of the bio-composite to be applied in the future development of biomedical instruments and therapeutics. Wahab et al. (2021b) investigated the silver nanoparticles-incorporated nanoporous carbon nitride (NCN@Ag) as antibacterial agent against multidrug-resistant *Escherichia coli* pathogens. The highly dispersed AgNPs containing NCN@Ag sample demonstrated superior bactericidal effect as compared to NCN, signifying the role of in situ-incorporated AgNPs to improve the antibacterial activity. The MIC value of NCN@Ag was recorded at 16μg/mL, which was 64-fold lower than the MIC value of NCN (1024μg/mL) for the complete inhibition of multidrug-resistant *E. coli*. Small sizes of NPs (4–8 nm), as well as higher surface-to-volume ratio of NCN@Ag, governed a larger contact area with infectious pathogens which contributed towards the enhanced antibacterial efficacy. The possibility of the presence of silver nanoparticles on the outer surface of pore channels further plays a significant role via direct contact with bacterial cell membrane. In future the similar approach can be used at a wider scale via employing other nanoporous materials with high surface area, large pore volumes, and accessible porosity. The application area of such materials can be expended in various biomedical applications such as medical devices, food packaging, disinfectant, and wound dressing applications.

Porous materials impose as a better attachment matrix for silver nanoparticles owing to their uniform pore sizes, large pore volumes, and large surface areas. Mesoporous materials have the pore size appropriate to act as a reactor for nanoparticle formation and improve their use as antimicrobial agents. For instance, (Yang et al. 2021) used mesostructured materials to confine silver nanoparticles (Ag/80S) and investigated its antibacterial potential against carbapenem-resistant *Klebsiella*

pneumonia. Material characterization revealed 7.5 nm mesopore size and high surface area of 307.6 m^2/g and uniform distribution of silver nanoparticles through the composite. MIC values of the composite were recorded in the range of 2.5–5 mg/mL, with low cytotoxicity. The zone of inhibition study against *K. pneumonia* resulted in 18 mm of inhibition zone for Ag/ 80S disk, whereas no zone of inhibition was observed for 80S alone. The morphological analysis of bacterial cells treated with Ag/80S revealed high pore deformations in membrane of cells treated with 1% Ag/80S as compared to untreated or 0.5% Ag/80S. High surface area of the composite resulted in convenient interaction between silver nanoparticles and membrane surface, where silver nanoparticles penetrated the bacterial membranes and triggered cell death. A time-dependent increase in ROS levels was also recorded which is in accord with increasing interaction between silver nanoparticles and cell membrane surface. Nano-silver-decorated mesoporous organosilica nanoparticles (Ag-MONs) were explored for their antibacterial capacity against various antibiotic-resistant bacterial strains (Li et al. 2021b). Gentamicin-loaded nano-silver-decorated mesoporous organosilica (Ag-MONs@GEN) nanoparticles were also investigated for antibacterial potential and as delivery agent of gentamicin and nano-silver. Ag-MONs@GEN significantly inhibited the growth of antibiotic-resistant *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Enterococcus faecalis* in a dose-dependent manner. The MIC values of Ag-MONs@GEN, against *E. coli*, *P. aeruginosa*, *S. aureus*, and *E. faecalis*, were 12, 12, 24, and 48μg/mL, respectively. The effect was more prominent in gram-negative bacteria than for gram-positive bacteria owing to the selective killing effects of both GEN and nano-silver on gram-negative bacteria. At minimum bactericidal concentration, Ag-MONs@GEN exhibited complete inhibition of the bacterial colonies of *Escherichia coli* (24μg/mL), *Pseudomonas aeruginosa* (12μg/mL), *Staphylococcus aureus* (24μg/mL), and *Enterococcus faecalis* (48μg/mL). The excellent antibacterial effects on resistant bacterial strains demonstrated the synergistic antibacterial capacity of Ag-MONs@GEN as compared to Ag-MONs and GEN alone. The cytotoxicity evaluation revealed excellent biocompatibility with IC_{50} values of 313.6 ± 15.9 and 295.7 ± 12.3μg/mL, in L929 and HUVECs cells. The study can be used in future to develop simultaneous platforms for drug delivery and antibacterial agents with enhanced antibacterial ability against antibiotic-resistant bacteria. Golubeva et al. (2018) modified zeolites comprised of Rho, Beta, and paulingite structures with silver nanoparticles and clusters (3–6 wt.%). The modified structures displayed excellent antimicrobial activity against MRSA which increased with increment in silver content, ultimately demonstrated by decreasing MIC from 0.750 to 0.019 mg/mL. Under identical conditions of silver concentration, type of zeolite matrix and conformation of silver particles gained the controlling aspect of antimicrobial activity. For instance, Rho zeolite displayed higher antibacterial activity on account of stabilized Ag_8 nanoparticles and clusters compared to the least antimicrobial activity in Beta zeolite. These silver zeolites were found to be suitable for antibiotic resistance control in medicine for their pronounced biological activity towards drug-resistant strains and selective cytotoxicity towards tumor cells.

8.4 Conclusions

The need for hygienic living conditions prompts new challenges for the development of affordable and efficacious antimicrobial materials that should be environmentally friendly and absolutely nontoxic towards human beings. The development of antibiotic resistance among individuals is developing at a fast pace, which creates several complications during medical treatments and/or surgical procedures. In the context of reducing the risk of microbial infections, several antibacterial nanoformulations of AgNPs and its associated composites based on supporting matrices have been developed by the researchers. Encouragingly, the existing antimicrobial therapies including antibiotics can work efficiently in combination with different silver nanoparticles for treatment complications either due to antibiotic-resistant or multidrug-resistant microbial strains. Recent investigations and various studies pertaining to antibacterial and biocompatibility activities of AgNPs have provided evidence that these activities may be influenced by many factors such as types of nano-formulations, films, coatings, and hydrogels and concentration or volume ratio of each constituent. By controlling and manipulating such parameters with extensive research on AgNPs cytotoxicity can help these materials earn a marketable commercially available status. The wide applications of silver nanoparticles and their composites towards controlling antibiotic resistance consolidated in this chapter would provide a reference value for future research to construct and implement nano-silver-based biomaterial designs in biomedical applications.

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Chapter 9
Gold Nanoparticles: A Lethal Nanoweapon Against Multidrug-Resistant Bacteria

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Abstract Multidrug-resistant (MDR) bacteria, also called superbugs, pose serious threat to the human health and existence because of their ability to develop resistant mechanism against commercially available antibiotics. Besides increasing the morbidity and mortality rate of patients, MDR bacteria are also putting huge financial stress on health sectors across the globe. It is estimated that approximately 700,000 people are losing their life every year due to MDR bacteria. It has been projected that more than 10 million people around the world will be the victim of MDR bacteria by 2050, if the current trend continues, superseding cancer as the main cause of global mortality. Hence, the mankind is in dire need to find an effective and safe tool against MDR bacteria.

Several metallic nanoparticles including Au, Ag, ZnO, and GO (graphene oxide) have shown antibacterial propensity against a wide range of bacteria. However, gold nanoparticles (AuNPs) received wide attention because of their inertness for the human body, easy surface fabrication properties, and optical properties. AuNPs demonstrate antibacterial activity through direct interaction with bacterial cell wall, generating reactive oxygen species (ROS), passing through the cell membrane and interacting with cellular macromolecules (i.e., DNA and proteins). In this chapter, we shall discuss different types of AuNPs, their role as potent antibacterial agents

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against pathogenic as well as multidrug-resistant bacteria, mechanism of antibacterial activity, biocompatibility, and future prospects in healthcare system.

Keywords Gold nanoparticles · Multidrug-resistant bacteria · Antibacterial activity · Biocompatibility

9.1 Introduction

Gold (Au) is an inert metal with distinct electronic and optical properties. When Au nanoparticles (AuNPs) are excited with light of a particular wavelength, the incident photons interact strongly with the conduction band of electrons and cause them to oscillate with resonant frequency. The collective oscillation is known as localized surface plasmon resonance (LSPR) that creates strong and localized electromagnetic fields and allows sensitive detection of changes in dielectric environment surrounding the surface of nanoparticles. This unique property makes them highly useful in imaging, drug delivery, cancer theranostics, and tackling menace of multidrug-resistant bacteria (Shankar et al. 2004; Mukherjee et al. 2001; Li et al. 2014). Au nanoparticles induce hyperthermia (i.e., increased temperature to kill cancer cells) upon illumination with near-infrared (NIR) light (Dash and Bag 2014; Huang et al. 2011). Furthermore, AuNPs can also be successfully and selectively delivered to malignant as well as benign tumors and can act as carriers for chemotherapeutic drugs. They are used as imaging agents as well as biosensors because of their ability to emit photons upon illumination (Marangoni et al. 2013).

The misuse and overuse of antibiotics have resulted in the emergence of multidrug-resistant bacteria, which is the cause of an additional medical costs of up to billion dollars every year (Rossolini et al. 2014; Li et al. 2014). Hence, suitable antibacterial agents to deal with multidrug-resistant bacteria are urgently needed. The antibacterial activity demonstrated by various nanomaterials including Ag, Au, Cu, Ti, ZnO_2, and MgO_2 could be a suitable alternative to commercially available antibiotics (Vimbela et al. 2017; Hossain et al. 2019; Niloy et al. 2020; Polash et al. 2021; Ranjan Sarker et al. 2019). More specifically, gold nanoparticles (AuNPs) have distinctive properties including their adjustable shape, size, surface properties, optical properties, high stability, biocompatibility, and multiple functionalization potential that make them suitable for different applications in the field of nanomedicine (Ashraf et al. 2016).

Based on the unique physical and chemical properties of nanoparticles, they provide a common platform for therapeutic applications against drug-resistant bacteria (Li et al. 2014). For example, AuNPs have already been used in the treatment of gum disease and dental caries, diagnosis of cancer, and in tissue engineering. Since AuNPs have antifungal and antibacterial activity, they can be conjugated with

biopolymers to improve their efficacy as bioactive materials (Bapat et al. 2020). In addition, AuNPs can also be used as carriers of antibacterial drugs. Antibacterial drugs are conjugated with AuNPs through chemical interactions so that drugs can be released at the desired site of action (Fan et al. 2019). Notably, AuNPs do not show toxic effects to normal cells at certain concentrations (Fang et al. 2019; Chatterjee et al. 2011). Therefore, it is possible to modify AuNPs that exhibit antibacterial activity against standard bacterial strains in general and have unique antibacterial activity against multidrug-resistant bacteria in particular (Su et al. 2020).

The recent development in nanoscience and nanotechnology has helped researchers to design and develop novel biomaterials including AuNPs with excellent bioactivity as well as biocompatibility. Many inorganic (i.e., metal) nanoparticles have been developed including AuNPs. The AuNPs show various colors based on their shape, size, and amount of aggregation of particles (Daniel and Astruc 2004). They are used in medical and pharmaceutical industries for various purposes: antibacterial agents, antibiofilm, diagnostic tools, drug delivery vehicles, personal care products, and for cosmetics development (Su et al. 2020; Abdalla et al. 2020). This chapter summarizes recent research works on the development of AuNPs and their application to tackle the menace of pathogenic and multidrug-resistant (MDR) bacteria.

9.2 Different Types of AuNPs

There are many different types of gold nanoparticles (AuNPs) depending on their size, shape, and physical properties (Fig. 9.1). Important AuNPs include Au nanospheres, nanorods, nanoshells, and nanocages. There is also another type of Au-based nanoparticles known as "SERS nanoparticles" with excellent surface-enhanced Raman scattering property. Most of the AuNPs are produced with well-defined size, shape, and monodispersity due to the continuous development of synthetic, and characterization techniques in the last two decades.

9.2.1 Au Nanospheres

The gold nanospheres, also known as gold colloids of 2 to 100 nm in diameter, can be synthesized through controlled reduction of an aqueous $HAuCl_4$ solution using different reducing agents under varying conditions. Citrate is the most widely used reducing agent that can produce nearly monodisperse gold nanospheres (Turkevich et al. 1951; Frens 1973). The size of the nanospheres can be controlled by changing the citrate to gold ratio. Generally, less amount of citrate generates larger nanospheres. The two major limitations of this method are the low yield and the obligation of using water as the solvent. A two-phase method, introduced by Faraday in 1857, capable of producing stable (irrespective of temperature and air) gold

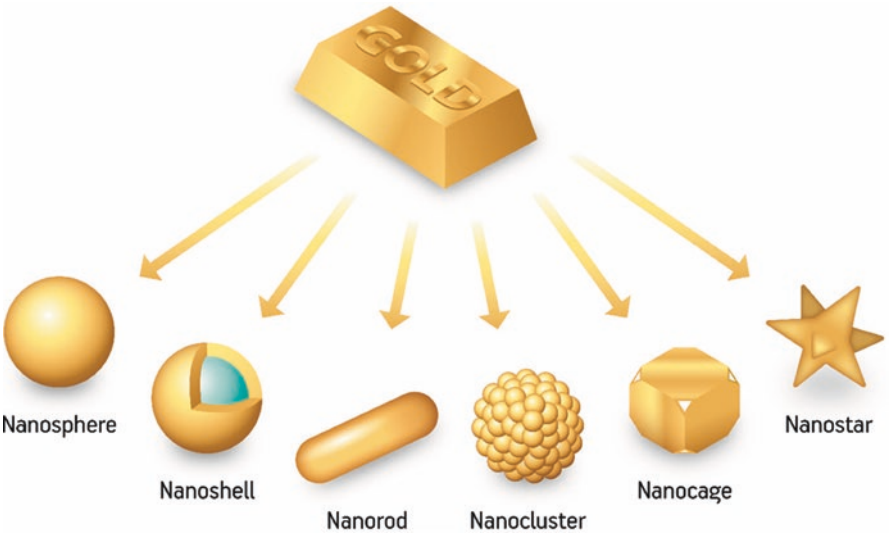

Fig. 9.1 Different types of AuNPs according to their shape and morphology. (Adapted from Freitas de Freitas et al. (2018))

nanospheres of reduced dispersity and defined size (i.e., 10 nm in diameter), was reported in 1993 (Giersig and Mulvaney 1993). The phase transfer reagent such as tetraoctylammonium bromide was used to improve this technique. Moreover, thiol/gold molar ratios can affect the average size of the nanospheres (Brust et al. 1994). Larger thiol-to-gold ratios and rapid addition of cold reductant solutions yield smaller and more monodispersed gold nanospheres. Many other methods have already been investigated for the synthesis of gold nanospheres using other reducing agents or ligands (Leff et al. 1996). On the other hand, dendrimers have been used as templates or stabilizers for the generation of Au nanospheres (Esumi et al. 1998). Biocompatible block copolymers have already been employed for the synthesis of sterically stabilized Au nanospheres in aqueous solution (Yuan et al. 2006). The shape and size of the Au nanospheres could be readily controlled by optimizing the synthesis parameters including block copolymer composition, relative/absolute concentrations of the block copolymer, and $HAuCl_4$. It was also reported that Au nanospheres could be grown in human cells (Anshup et al. 2005). Furthermore, Au nanospheres display a single absorption peak in the visible range between 510 nm and 550 nm. The absorption peak shifts to a longer wavelength with the increasing particle size, and the width of the absorption spectra is related to the size distribution range. Many other types of AuNPs with different size/shape including nanorods, nanoshells, and nanocages have been explored to obtain optical properties suitable for biomedical applications.

9.2.2 Au Nanorods

The Au nanorods have received worldwide attention because of their inimitable shape-dependent optical properties (i.e., different plasmon bands) that make the Au nanorods exclusive materials for biological imaging, sensing, photothermal therapy, and drug delivery (Hainfeld et al. 2008; Kodiha et al. 2014; Nichols and Bae 2014; Choi et al. 2010). Their application is very precise because even a small change in the shape, size, and surface nature alter their properties which in turn affect their biological applications. The surface plasmon resonance (SPR) band of Au nanorods is in the NIR region that makes them suitable for photothermal therapy, biological sensing, and imaging (Martin 1994). The Au nanorods can be synthesized using the template method. The diameter of the template membrane pore can be used to determine the diameter of the Au nanorods, while the length of the nanorods can be controlled through the amount of gold deposited within the pores. The main disadvantage of this method is low yield since only one monolayer of nanorods is prepared. The formation of Au nanorods through electrochemical synthesis has been also reported (Reetz and Helbig 1994; Yu et al. 1997; Chang et al. 1999). According to this approach, many experimental parameters determine the length of the Au nanorods that influence their aspect ratio which is defined as the length divided by the width. On the other hand, the seed-mediated synthesis, the most established method for Au nanorod preparation, provides higher aspect ratio than that prepared by the other methods (Jana et al. 2001a; Busbee et al. 2003). Usually, Au seeds are prepared by chemical reduction of Au salt (i.e., $HAuCl_4.3H_2O$) with a strong reducing agent including sodium borohydride (i.e., $NaBH_4$). These seeds, serving as the nucleation sites for Au nanorods, are then added to the growth solution containing Au salt, a weak reducing agent such as ascorbic acid, and hexadecyltrimethyl ammonium bromide. The aspect ratio of Au nanorods can be controlled by varying the amount of gold seeds with respect to the precursor. Furthermore, Au nanorods can be produced in quantitative yield upon addition of $AgNO_3$ (Jana et al. 2001b, 2002). Besides, several other types of approaches have also been investigated for the fabrication of Au nanorods including bioreduction (Canizal et al. 2001), growth on mica surface (Mieszawska and Zamborini 2005), and photochemical synthesis (Kim et al. 2006).

9.2.3 Au Nanocages

Gold (Au) nanocages are characterized by their ultrathin porous walls and hollow interiors. They are synthesized using silver nanoparticles (AgNPs) as template that participate in the galvanic replacement (GR) reaction (Skrabalak et al. 2007, 2008; Lu et al. 2007). The controllable pores on the surface of Au nanocages have been synthesized via galvanic replacement reaction between silver nanocubes and aqueous $HAuCl_4$ (Chen et al. 2006). The silver (Ag) nanostructures with controlled

morphologies can be generated through polyol reduction where $AgNO_3$ is reduced by ethylene glycol. Sequential addition of Ag atoms to the seeds produces the desired nanostructures by controlling Ag seed crystalline structures in the presence of poly(vinylpyrrolidone), a polymer capable of selectively binding to the surface. Ag nanostructures are used as supportive templates that can be transformed into Au nanostructures with hollow interiors via galvanic replacement (Chen et al. 2005, 2006). The wall thickness and dimension of the resultant Au nanocages could be readily controlled with high precision by adjusting the molar ratio of Ag to $HAuCl_4$ (Cai et al. 2008). The light penetration ability in soft tissues can be maximized by restricting the light source to near infrared (NIR) region (i.e., 650 to 900 nm). This is because the light absorption ability of hemoglobin and water is negligible in the NIR region. The LSPR peaks can be concisely tuned throughout the visible and NIR region to make the Au nanocages suitable for this application (Kwon et al. 2012; Mahmoud et al. 2010; Au et al. 2008; Mahmoud and El-Sayed 2010). Au nanocages can also be functionalized with the bioactive molecules to target cancer cells for diagnosis and photothermal therapy at an early stage (Dreaden et al. 2012; Chen et al. 2012).

9.2.4 Au Nanoshells

The Au nanoshells are composed of silica core coated by a thin Au metallic shell. The most interesting property of Au nanoshells is their unique surface plasmon resonance property that can be finely tuned from visible to NIR region. There are multiple templates employed for the formation of hollow Au nanoshells including silica particles (Averitt et al. 1997), metal particles (Oldenburg et al. 1998, Oldenburg et al. 1999a, b; Tuersun and Han 2013), and so on. The Au nanoshells have been applied in various biomedical applications such as whole-blood immunoassays and photothermal cancer therapy (Loo et al. 2005; Park et al. 2008; Bickford et al. 2010). Cancer cells were successfully ablated in vitro by Au nanoshells as observed through magnetic resonance thermal imaging. Furthermore, the use of Au nanoshells for the photothermal ablation of tumors in mice showed complete regression of tumors while the mice remain healthy (Gobin et al. 2007, 2008; Hirsch et al. 2003; Lowery et al. 2006). Optical imaging techniques including those that use AuNPs as the contrast agents have limited applications in human studies. On the other hand, in the NIR region (i.e., 700–900 nm), the absorbance of all the bioactive molecules is negligible which provides clear window for optical imaging (Frangioni 2003). Au nanoshells can be designed and fabricated to control the location of surface plasmon resonance (SPR) peaks from visible to the NIR region of the electromagnetic spectrum by varying the composition and dimensions of the layers (Oldenburg et al. 1999a). The SPR peak can also be tuned by changing the ratio of the core size to its shell thickness for a given composition of Au nanoshells. Furthermore, Au nanoshells with SPR peaks in the NIR region can be prepared by coating Au shells with silica or polymer beads of variable thickness (Oldenburg et al. 1998; Caruso

et al. 2001). The silica cores are grown according to the Stöber process, that is, the reduction of tetraethyl orthosilicate in basic ethanol. A seed-mediated growth technique is typically used to coat the silica nanoparticles with Au in an aqueous environment. The small Au nanospheres, such as 2–4 nm in diameter, can be attached to the silica core using an amine-terminated silane as a linear molecule that allows additional Au to be reduced until the seed particles coalesced into a complete shell (Oldenburg et al. 1999b). The diameter of the gold nanoshells is mainly determined by the diameter of the silica core, and the shell thickness can be controlled through the amount of gold deposited on the surface of the core. Gold nanoshells have also been synthesized via in situ gold nanoparticle formation using thermosensitive core-shell particles as the template (Suzuki and Kawaguchi 2005). The use of different microgels as the core offers significantly reduced particle aggregation. The thickness of Au nanoshells was also controlled through electroless deposition of Au. Recently, a virus scaffold has been used to assemble Au nanoshells which may potentially provide cores with a narrower size distribution and smaller diameters (i.e., 80 nm) than that of silica (Radloff et al. 2005).

9.2.5 Au Nanostars

Gold (Au) nanostars belong to the anisotropic AuNPs category. Au nanostars are composed of a core and several branches with sharp tips. The unique characteristics of Au nanostars originate from these branches and their interaction with the core. The attractive features of Au nanostars are localized surface plasmon resonance (LSPR), surface-enhance Raman Scattering (SERS) activity, and catalytic activity. The Au nanostars are used for catalysis, nanosensing assays, thermal therapy, and drug delivery. The Au nanostars have multiple sharp branches that demonstrate significant electromagnetic field enhancement and have unique plasmon bands which can be tuned from visible to NIR region. The synthesis of Au nanostars has been driven by the interest on the localized surface plasmons (LSPs) response to the environment, especially on sharp tips and edges, where light can be highly concentrated (Hao et al. 2007; Rodríguez-Lorenzo et al. 2009; Hrelescu et al. 2009; Dondapati et al. 2010). They serve as effective tools in the field of nanomedicine due to their unique properties. The Au nanostars also display stronger surface-enhanced resonance activity than Au spheres or even rods (Mukherjee et al. 2001; Cai et al. 2008).

9.3 Antibacterial Activity of AuNPs

Although gold nanoparticles (AuNPs) are not strong antimicrobial agent as AgNPs, they have been reported to demonstrate antibacterial (Lima et al. 2013) as well as antifungal activity (Wani and Ahmad 2013; Zawrah et al. 2011). Furthermore,

AuNPs have been used as an alternative tool to high-dose antibiotics against infectious diseases including antibiotic-resistant bacteria (Podsiadlo et al. 2008). The nanoparticle toxicity and antibacterial activity mainly depend on the intrinsic properties, surface modification, and tested bacterial species. The AuNPs of smaller diameter can penetrate the bacterial cells and cause cellular damage followed by death (Bindhu and Umadevi 2014a). Antibacterial properties of triangular-shaped AuNPs demonstrate better activity toward both gram-positive and gram-negative bacteria than that of spherical AuNPs (Smitha and Gopchandran 2013). The sharp-faced triangular NPs, irrespective of their surface chemistry, size, and composition, can pierce the endosomal membrane before translocating to the cytoplasm where they can be retained. This feature makes them preferable to round-shaped NPs for drug delivery, gene delivery, subcellular targeting, and long-term tracking (Chu et al. 2014). Recently, it has been reported that very small AuNPs (i.e., less than 2 nm) showed excellent antibacterial activity against gram-positive and gram-negative bacteria (Kundu 2017).

9.3.1 Antibacterial Activity Against Pathogenic Bacteria

The AuNPs synthesized using citrate, polyvinylpirrolidone (PVP), or other commonly used stabilizers usually do not show antibacterial activity (Amin et al. 2009). More specifically, the AuNPs (size: 20–30 nm) stabilized by PVP and/or sodium dodecyl sulfate (SDS) did not show antimicrobial activity against *Staphylococcus aureus* ATCC 6538, *Escherichia coli* K12 NCTC 10538, and fungi *Candida albicans* ATCC 10231 at a concentration of 0.0016 wt% (Mukha et al. 2010). Chatterjee et al. (2011) found that AuNPs showed no concentration-dependent antibacterial activity while stimulating the level of cell division. AuNPs are biologically inert and usually do not show antibacterial activity (Allahverdiyev et al. 2011) that can be convinced from their high MIC value and small zone of inhibition (ZOI) value when compared to AgNPs. Hernández-Sierra et al. (2008) compared the antibacterial activity of AgNPs (size: 25 nm) with that of AuNPs (size: 80 nm) against *S. aureus* ATCC 25923. The AgNPs had a MIC (minimum inhibitory concentration) value of 4.86 ± 2.71 μg/ml and MBC (minimum bactericidal concentration) value of 6.25 μg/ml. On the other hand, AuNPs showed antibacterial propensity at a very high concentration (197 μg/ml). Shankar et al. (2014) synthesized AuNPs, AgNPs, and Au-AgNPs having hydrodynamic size 140 ± 13, 74 ± 6, and 128 ± 15 nm, respectively. The growth of *S. aureus* and *E. coli* was inhibited at 16 μg/ml and 8 μg/ml of AgNPs, respectively. Similar pattern of inhibition was also observed for Au-AgNPs having MIC value 16 and 32 μg/ml against *S. aureus* and *E. coli,* respectively. On the other hand, AuNPs did not show any antibacterial activity at the tested concentration (i.e., 128 μg/ml). Sreelakshmi et al. (2011) compared the antibacterial activity of AuNPs and AgNPs (size: 10 nm) synthesized using natural honey as a source of stabilizing as well as reducing agent. Their MIC values confirmed that honey-capped AgNPs exhibit very good antibacterial activity, whereas AuNPs exhibit

moderate activity against the tested strains. On the other hand, PVP-coated AuNPs have ~10 times higher MIC value than that of AgNPs synthesized using the same polymer (Mukha et al. 2010; Hernández-Sierra et al. 2008). Hence, AuNPs are not usually used as antibacterial agents. In addition, incorporation of AuNPs (2 nm), methylene blue (MB), and toluidine blue (TBO) with polyurethane polymer, a polymer used to prepare catheters, enhanced the killing of *S. aureus* suspension under white illumination with a hospital light source (Naik et al. 2011). However, many other studies have revealed that AuNPs synthesized under certain conditions showed efficient antibacterial activity and they can be functionalized with different biopolymers to make an effective antibacterial agent. The size, shape, surface modification (coating and capping agents), and purification methods of AuNPs are the key factors that determine their antibacterial activity. However, CTAB-coated AuNPs of 1~2 nm and 1~20 nm in size were reported to show similar zone of inhibition (~22 mm) against *E. coli* (ATCC 25922 strain) without any size dependence (Azam et al. 2009; Arshi et al. 2011).

Furthermore, Badwaik et al. (2012) investigated the antibacterial activity of dextrose-coated AuNPs having different hydrodynamic diameters such as 25, 60, and 120 ± 5 nm. AuNPs with hydrodynamic diameter of 120 and 60 nm inhibit the proliferation of *E. coli* in a concentration-dependent manner and the MIC values were 16×10^{10} and 16×10^{11} particles/ml, respectively. On the other hand, AuNPs having 25 nm hydrodynamic diameter did not show any significant effect against the proliferation of *E. coli* even at concentration as high as 128×10^{12} particles/ml. Hence, they concluded that the antibacterial activity of AuNPs increases as the particle size increases, for example, in the order of 25 < 60 < 120 < nm. On the other hand, Ahmad et al. reported that smaller AuNPs (i.e., 7 nm) showed excellent antifungal activity and greater biocidal effect against *Candida* species when compared to that of relatively large AuNPs (i.e., 15 nm) (Ahmad et al. 2013). The capping agent is also an important determinant of the antibacterial activity of AuNPs. Zhang et al. (2008) synthesized hyperbranched poly(amidoamine) having terminal dimethylamine groups (HPAMAM-N $(CH_3)_2$) to prepare AuNPs and they found that the cationic dimethylamine contributes to the antimicrobial activity through strong ionic interactions with bacteria. As shown in Table 9.1, AuNPs stabilized or modified by various coating agents have distinct antibacterial effects. Many small molecules were also used for the synthesis of AuNPs and were investigated for their antibacterial potential. For example, 4, 6-diamino-2-pyrimidinethiol (DAPT), an analogue of 2-pyrimidinethiol that is present in *E. coli*; two positively charged and amino-substituted pyrimidines 4-amino-2-pyrimidinethiol and 2,4-diamino-6-pyrimidine thiol (iDAPT) (Chatterjee et al. 2011); and one negatively charged pyrimidine 4,6-dihydroxyl-2- pyrimidine thiol (DHPT) (Zhao et al. 2010) were used to fabricate AuNPs. The MIC values of DAPT, APT, and iDAPT fabricated AuNPs against *Pseudomonas aeruginosa* were 16, 18, and 24 μg/ml, respectively. DHPT-coated AuNPs did not inhibit the growth of both *E. coli* and *P. aeruginosa* even at high concentration (80 μg/ml).

The methods used for the purification of AuNPs were neither mentioned nor adequately carried out before performing antibacterial assay. There could be debate

Table 9.1 A summary of the antibacterial activity of AuNPs synthesized using different types of reducing agents and capping agents

AuNPs (reducing agents and capping agents)	Size (if present)	Test bacteria	Effect of antibacterial activity	Ref.
Citric acid Polyallylamine HCl	20–30 nm	*B. Calmette-Guérin* *E. coli*	Effect and mechanism depend upon composition and surface modifications	Zhou et al. (2012)
Citrate/CTAB	1–22 nm	*E. coli*	AuNPs show high antibacterial activity with zone of inhibition of 22 mm	Zhang et al. (2015)
Acridine derivatives	15–20 nm	*E. coli* *Bacillus subtilis*	Acridine-AuNPs has stronger antibacterial effect than acridine alone	Mitra et al. (2014)
Citrate	20–30 nm	*E. coli*	Dose-dependent inhibition, 0.1–5 μg/mL	Zhou et al. (2012)
Phosphine	–	*S. aureus*	Antibacterial activity of two ligands are compared	Borah et al. (2011)
Pyrimidine thiols	3 nm	*P. aeruginosa*	MICs of Au-DAPT, Au-APT, and Au-iDAPT are 16, 18, and 24 μg/mL	Zhao et al. (2010)
Thioguanine	–	*Micrococcus luteus* *S. aureus* *P. aeruginosa* *E. coli*	AuNPs are more potent than thioguanine	Selvaraj et al. (2010)
PVP/SDS	20–30 nm	*S. aureus* *E. coli* K12	AuNP of 0.0016% wt. showed no effect on tested strains	Mukha et al. (2010)
Gallic acid	14, 39, 77 nm	*S. mutans*	MICs of 12.31, 12.31, and 49.25 μg /mL for 13.7, 39.4, and 76.7 nm AuNPs	Moreno-Álvarez et al. (2010)
Reduced by lysozyme at 40 °C	–	*Acinetobacter baumannii* *Enterococcus faecalis*	Broad-band labeling agents for pathogenic bacteria	Chen et al. (2010)
Citrate, CTAB	1 ~ 22 nm	*E. coli*	Zone of inhibition of 22 mm	Arshi et al. (2011), Zhang et al. (2015)
Polyamidoamine	7.7, 4.6, 3.9 nm	*E. coli* *S. aureus* *B. subtilis* *Klebsiella mobilis*	Inhibit up to AuNP (2.8 μg/mL) Ionic interaction with bacteria	Zhang et al. (2008)
PVP	80 nm	*S. aureus*	MIC, > 197 μg/mL	Hernández-Sierra et al. (2008)

(continued)

Table 9.1 (continued)

AuNPs (reducing agents and capping agents)	Size (if present)	Test bacteria	Effect of antibacterial activity	Ref.
Polythiophene composite	–	Common bacterial pathogens	Efficient antibacterial effect was observed	Adhikari et al. (2013)
Citrate or PAH	2–30 nm	*E. coli*	*E. coli* growth inhibited at 0.1, 1, 5 μg/mL citrate-AuNPs	Zhou et al. (2012)
Capped by amine or polyacrylate	–	*E. coli*	99.999% killing in 10 min. Coating agents are responsible for antibacterial activity	Wan and Yeow (2012)
Dextrose	25, 60, and 120 nm	*E. coli*	For 120 and 60 nm AuNPs with MIC 16 × 1010 and 16 × 1011 NPs/mL for 25 nm AuNPs, no inhibition at 128 × 1012 NPs/mL	Badwaik et al. (2012)
Cationic peptides	1.2–2.5 nm	*S. aureus* *B. subtilis* *E. coli* *P. aeruginosa*	MIC higher with AuNP than without	Pal et al. (2011)
Zeolite (2.3–2.8%)	5 nm	*E. coli* *Salmonella typhi*	Eliminate 90–95% of *E. coli* and *S. typhi* colonies at short time	Lima et al. (2013)
Polyoxometalates and lysine	–	*E. coli*	5 μM causes 80% bacterial death	Daima et al. (2013)
Cationic monolayer	2 nm	MDR strains and MRSA	MIC values dependent on side chain functional groups and chain length	Li et al. (2014)
Zeolite	–	*E. coli* *S. Typhi*	AuNPs dispersed on zeolites eliminate *E. coli* and *S. typhi* at short times	Zhang et al. (2015)

on the antibacterial activity of AuNPs because of the presence of capping agents or other reagents used for the synthesis of nanoparticles that were not purified properly. It might be, therefore, the presence of Au(III) ions as well as unreacted reagents interfere with the antibacterial results, thereby producing false results. Several studies have reported the purification of chemically synthesized AuNPs prior to investigating their antimicrobial activity. For example, Daima et al. synthesized tyrosine-functionalized AuNPs and dialyzed to remove free ions and unbound tyrosine prior to investigating their antibacterial activity to avoid any interference on their toxicity bacteria (Daima et al. 2013). Furthermore, Zhou et al. (2012) prepared citrate-stabilized AuNPs followed by purification through centrifugation to test antibacterial activity against *E. coli* and *Bacillus Calmette-Guérin.* Nazari et al. also reported the synthesis and purification of AuNPs before testing their antibacterial activity against *P. aeruginosa, S. aureus*, and *E. coli* (Nazari et al. 2012).

On the other hand, AuNPs were also synthesized and fabricated using natural polymers extracted from either microorganisms or plants, or directly in the presence of microorganisms. They are summarized in Table 9.2. These processes are known as green or eco-friendly synthesis or biosynthesis. For instance, Nagaraj et al. (2012) synthesized spherical AuNPs (size: 10–50 nm) using *Caesalpinia pulcherrima* flower extract as the reducing agent that showed efficient antimicrobial activity against *Aspergillus*, *E. coli*, and *Streptobacillus* sp. Das et al. (2009) synthesized AuNPs (size: 10 nm) on the surface of *Rhizopus oryzae* MTCC 262 through in situ reduction of $HAuCl_4$ that showed high antibacterial activity against several gram-positive and gram-negative pathogenic bacteria. Mishra et al. (2011) synthesized AuNPs (size: 50–70 nm) and AgNPs (size: 10–20 nm) via extracellular synthesis using yeast *Candida guilliermondii* and the highest antibacterial activity for both AuNPs and AgNPs was found against *Staphylococcus aureus*. AuNPs were also synthesized using various plant extracts including *Mentha piperita* (MubarakAli et al. 2011*)*, root extract of *Trianthema decandra,* (33–65 nm) (Geethalakshmi and Sarada 2012), *Helianthus annuus* flower extracts (Geethalakshmi and Sarada 2012), and dried flower extract of *Carthamus tinctorius* (Liny et al. 2012). Interestingly, all the green synthesized AuNPs demonstrated efficient antibacterial activity against several bacteria strains that were unaffected in the presence of chemically synthesized AuNPs (Mishra et al. 2011). Finally, the question is what is responsible for the antibacterial activity of green-synthesized AuNPs. It may be due to the extracts alone, AuNPs or their combination with plant extracts, since several plant extracts including *Euphorbia hirta* plant alone demonstrated antibacterial activity (Annamalai et al. 2013). Hence, the antibacterial activity may also be due to the synergistic effect of the combination of AuNPs and extracts (Annamalai et al. 2013).

9.3.2 Antibacterial Activity Against Multidrug-Resistant (MDR) Bacteria

Metallic gold is inert and non-toxic that may change when shifts from metallic to oxidation states (0, I, and III) (Merchant 1998). The antibacterial mechanism of AuNPs is associated with (i) the collapse of membrane potential that inhibits the ATPase activity and causes deterioration of the cellular metabolism and (ii) inhibition of the binding subunit of ribosomes to tRNA (Cui et al. 2012). Also (iii) Shamaila and co-workers showed that AuNPs disrupt the bacterial respiratory chain by binding to the thiol group of enzymes including nicotinamide adenine (NADH) dehydrogenase and produce oxidative stress resulting in the cellular death (Shamaila et al. 2016). Since AuNPs are non-toxic to the host (Li et al. 2014; Conde et al. 2014; Rajchakit and Sarojini 2017), the possibility of fine-tuning their conjugation ability to act as carriers of antibiotics or other antibacterial moieties may enhance their bactericidal effect as well as potentiate the effect of antibiotics (Baptista et al. 2018). Functionalization of AuNPs with cationic and hydrophobic polymers was

Table 9.2 A summary of antibacterial activity of AuNPs synthesized using "green" method

AuNPs (green method)	Size (if present)	Test bacteria	Effect of antibacterial activity	Ref.
Black tea extract	2–100 nm	*P. aeruginosa* *S. aureus* *E. coli*	AuNPs did not increase antibacterial activity at concentration of 40 µg/disc	Nazari et al. (2012)
Shewanella oneidensis	12 ± 5 nm	*E. coli* *S. oneidensis* *B. subtilis*	No bactericidal effect for the tested strains at concentrations of 150 µM	Suresh et al. (2011)
Punica Granatum	5–17 nm	*S. aureus* *S. typhi* *Vibrio cholerae*	MIC values for the tested bacteria – 0.33, 0.37 and 0.41 mg/mL	
C. zeylanicum leaf broth	>100 nm	*E. coli* *S. aureus*	Efficient antibacterial activity	Smitha and Gopchandran (2013)
Memecylon umbellatum leaf	15–25 nm	*B. subtilis* *E. coli* S. *pneumoniae* *S. aureus* *S. typhimurium* *K. aerogenes*	Inhibited bacterial growth	Arunachalam et al. (2013)
E. hirta	6–71 nm	*E. coli* *P. aeruginosa* *K. pneumoniae*	Complete inhibition at a concentration of 200 µg/mL	Annamalai et al. (2013)
T. decandra	33–65 nm	*P. vulgaris* *E. coli* *S. aureus* *S. faecalis*	Excellent activity at a concentration of 10 mg/L on each disc	Geethalakshmi and Sarada (2012)
Solanum nigrum	18–20 nm	*B. subtilis* *E. coli* *P. aeruginosa*	Inhibited bacteria growth	Balagurunathan et al. (2011)
Phyllanthus emblica	2–4 nm	*S. aureus* *E. coli*	Zone of inhibition, 0.8–1.0 cm	Balasubramanian (2014)
Gracilaria corticata	45–57 nm	*S. aureus* *E. faecalis* *E.coli* *Enterobacter aerogenes*	Antimicrobial activity observed for tested bacteria at 24, 21, 19 and 14 mm	Naveena and Prakash (2013)
Phytochemicals	15–35 nm	*E. coli* *B. subtilis* *S. aureus* *Enterococci*	AuNPs showed zone of inhibition against all the studied bacteria	Mahitha et al. (2013)
Soybean Polyphenols	7–12 nm	*S. aureus* *P. aeruginosa* *A. baumannii*	Gram-negative bacteria with thin cell wall were more susceptible to cell wall damage compared to gram-positive bacteria	El-Batal et al. (2013)

(continued)

Table 9.2 (continued)

AuNPs (green method)	Size (if present)	Test bacteria	Effect of antibacterial activity	Ref.
Rhizopus oryzae MTCC 262	10 nm	*P. aeruginosa* *E. coli* *B. subtilis* *S. aureus*	Inhibition starts at 50 μg/mL caused rupture of cell membrane	Das et al. (2009)
Saururus chinensis leaf	–	*S. aureus* *E. coli*	27.6% inhibition with AuNPs of 0.2 mM and >90% inhibition after 24 h exposure at AuNPs of 0.8–1 μM	Sreekanth et al. (2012)
Reduced by *C. tinctorius* flower	–	*S. aureus* *E. coli* *B. subtilis*	Efficient antibacterial effect was observed	Nagajyothi et al. (2012)
Treatment with root extract of *T. decandra*	33–65 nm	*E. faecalis* *S. aureus* *S. faecalis* *B. subtilis* *Y. enterocolitica* *P. vulgaris* *E. coli* *P. aeruginosa*	Inhibition areas (mm) for *E. faecalis* 10.5, *S. aureus* 14.5, *S. faecalis* 13.5, *B. subtilis* 9.5, *Y. enterocolitica* 15.5, *P. vulgaris* 15.0, *E. coli* 9.5, *P. aeruginosa* 11.5	Geethalakshmi and Sarada (2012)
Ananas comosus	–	*E. coli* *Streptobacillus sp*	Effective on *E. coli* & *Streptobacillus sp*	Basavegowda et al. (2013)
Euphorbia hirta	6–71 nm	*E. coli* *P. aeruginosa*, *K. pneumoniae*	Inhibited 88% *E. coli*, 86% *P. aeruginosa*, and 94% *K. pneumoniae* at 200 μg/mL; plant *E. hirta* has antibacterial activity itself	Annamalai et al. (2013)
Solanum torvum	–	*E. coli* *Pseudomonas* *Bacillus*	AuNPs showed strong and fair zone of inhibition	Ramamurthy et al. (2013)
Dioscorea batatas	18–56 nm	Gram-positive and gram-negative bacteria	AuNPs inhibited *S. aureus*, *S. epidermidis*, *E. coli*; 21.5% inhibition by 0.2 μM and >50% by 0.8–1 μM AuNPs	Sreekanth et al. (2015)
LD fruit peel	140, 74, 128 nm	*S. aureus* *E. coli*	No antibacterial activity at >128 μg/mL for AuNPs, but for AgNPs and Au-Ag-NPs	Shankar et al. (2014)

(continued)

Table 9.2 (continued)

AuNPs (green method)	Size (if present)	Test bacteria	Effect of antibacterial activity	Ref.
Abelmoschus esculentus	14 nm	*B. subtilis* *B. cereus* *P. aeruginosa* *M. luteus* *E. coli*	AuNPs (0.2 mg/mL) showed inhibition zones of 26, 24, 15, 35 and 21 mm against *B. subtilis*, *B. cereus*, *E. coli*, *M. luteus*, *P. aeruginosa*	Mollick et al. (2014)
Trichoderma viride, Hypocrea lixii	61 nm	*E. coli* *Shigella sonnei* *P. syringae*	Inhibition of the growth of *E. coli*, *S. sonnei*, and *P. syringae* up to 53%, 47%, 55%	Mishra et al. (2014)
Punica granatum	5 and 20 nm	*S. aureus* *S. typhi* *V. cholerae*	MICs against *S. aureus*, *S. typhi*, *V. cholerae* are 0.33, 0.37, 0.41 mg/mL	Lokina et al. (2014)
Solanum lycopersicums	14 nm	*S. aureus* *P. aeruginosa*	Effective inhibition of growth of all tested bacteria	Bindhu and Umadevi (2014b)
Mentha piperita	150 nm	*E. coli* *S. aureus*	Effective against *aureus* *E. coli,* but not *S. aureus*	MubarakAli et al. (2011)
Candida guilliermondii	50–70 nm	Five pathogenic bacterial strains	Highest inhibition against *S. aureus*; chemically synthesized AuNPs showed no effect	Mishra et al. (2011)
Trianthema decandra or saponin	37.7–79.9 nm	10 different bacteria	Zones of inhibition of 8.2 mm to 11.5 mm; excellent activity against *Y. enterocolitica*, *P. vulgaris*, *E. coli*, *S. aureus*, *S. faecalis*	Geethalakshmi and Sarada (2013)
Grapes fruit	–	*P. aureus* *S. typhi* *V. cholerae*	Excellent antibacterial activity toward most of the tested bacterial strains	Lokina et al. (2014)

shown to be effective against both gram-negative and gram-positive uropathogens including MRSA. These AuNPs exhibited low toxicity to mammalian cells, and development of resistance to these nanoparticles was very low (Li et al. 2014). Vinoj et al. demonstrated that the conjugation of AuNPs with N-acylated homoserine lactonase proteins (AiiA AuNPs) resulted in a nano-composite with greater antibacterial activity against MDR species when compared to AiiA proteins alone (Vinoj et al. 2015).

The integration of AuNPs on the shell of PVA-lysozyme microbubbles demonstrated better antibacterial potential against *E. coil* than that of only microbubbles (Mahalingam et al. 2015). Galic acid–capped AuNPs have also been found to be active against gram-negative and gram-positive bacteria (Kim et al. 2017). Recently, Ramasamy et al. reported one-pot synthesis of cinnamaldehyde-immobilized gold

nanoparticles (CGNPs) having more than 80% effectivity against biofilm formation of gram-positive (methicillin-sensitive and -resistant strains of *S. aureus*, MSSA, and MRSA, respectively) and gram-negative (*E. coli and P. aeruginosa*) bacteria (Ramasamy et al. 2017a, b). The incorporation of AuNPs with ultrathin graphitic carbon nitride (g-C_3N_4) generates peroxidase activity and demonstrates excellent antibacterial potential against drug-resistant (DR) gram-positive and gram-negative bacteria. They also exhibit high efficiency in eliminating existing DR biofilms and preventing the formation of new biofilms in vitro (Wang et al. 2017a). The conjugation of antibiotics (e.g., vancomycin and methicillin) to AuNPs increases their intrinsic activity against MDR strains (Baptista et al. 2018). Recently, Payne et al. developed a single-step synthesis technique for kanamycin-capped AuNPs (Kan-AuNPs) that exhibit high antibacterial activity against both gram-positive and -negative bacteria including kanamycin-resistant bacteria. The authors observed a significant reduction in the MIC value against all the bacterial strains tested when compared to free drug. This higher efficacy was due to the disruption of the bacterial envelope that resulted in the leakage of cytoplasmic content and thereby cell death (Payne et al. 2016). Pradeepa et al. synthesized AuNPs using bacterial exopolysaccharide (EPS) and functionalized them with antibiotics (e.g., levofloxacin, cefotaxime, ceftriaxone, and ciprofloxacin). They observed that antibiotic-conjugated AuNPs exhibited excellent bactericidal activity against MDR gram-positive and -negative bacteria when compared to free drugs. *E. coli* was the most susceptible MDR bacteria followed by *K. pneumoniae* and *S. aureus* (Vidya et al. 2016). Recently, Yang et al. described the effect of small molecule (6-aminopenicillanic acid, APA) coated AuNPs to inhibit MDR bacteria (Yang et al. 2017). They conjugated AuNPs with electrospun fibers of poly(ε-caprolactone) (PCL)/gelatin to produce materials that inhibit wound infection by MDR bacteria and also demonstrated that APA-AuNPs reduce MDR bacterial infections (Yang et al. 2017). Shaker et al. evaluated the surface functionalization of AuNPs with carbapenems [i.e., imipenem (Ipm) and meropenem (Mem)] and investigated their antibacterial activity against carbapenem-resistant gram-negative bacteria isolated from an infected human. Both Ipm-AuNPs and Mem-AuNPs (size: 35 nm) showed significant increase in their antibacterial activity against all the tested isolates (Shaker and Shaaban 2017). Recently, Shaikh et al. described the synthesis and characterization of cefotaxime-conjugated AuNPs to target drug-resistant CTX-M-producing bacteria. The authors inverted resistance in cefotaxime-resistant bacterial strains (i.e., *E. coli* and *K. pneumoniae*) by using cefotaxime-AuNPs. Hence, the conjugation of unresponsive antibiotics with AuNPs can restore their antibacterial activity against drug-resistant bacterial strains (Shaikh et al. 2017).

One of the most important properties of AuNPs is their ability to generate heat upon illumination with laser (Mendes et al. 2017; Mocan et al. 2017). This property is very important because it can be exploited to develop photothermal nano-vectors to destroy MDR bacteria at the molecular level (Mocan et al. 2017). For example, Khan et al. showed that the combination of Concanavalin-A (ConA)-directed dextran capped AuNPs (GNP_{DEX}-ConA) conjugated with methylene blue (MB) (MB@GNP_{DEX}-ConA)-mediated photodynamic therapy (PDT) enhanced the efficacy and

selectivity of MB-induced killing of MDR clinical isolates including *E. coli, K. pneumoniae, and Enterobacter cloacae* (Khan et al. 2017). Gil-Tomas et al. reported that the covalent conjugation of AuNPs with toluidine blue O–tiopronin forms an enhanced and exceptionally potent antimicrobial agent when activated by white light or 632 nm laser light (Gil-Tomás et al. 2007).

Hu et al. modified the surface of AuNPs with pH-responsive mixed charged zwitterionic self-assembled monolayers composed of weak electrolytic 11-mercaptoundecanoic acid (HS-C10-COOH) and strong electrolytic (10-mercaptodecyl) trimethylammonium bromide (HS-C10-N4) that exhibited an enhanced photothermal ablation of MRSA biofilm without any damage to the healthy tissues around the biofilm when illuminated with near infrared (NIR) laser (Hu et al. 2017). Furthermore, the antibacterial activity of glucosamine-gold nanoparticle-graphene oxide (GlcN-AuNP-GO) and UV-irradiated GlcN-AuNP-GO was evaluated against *E. coli* and *E. faecalis*. UV irradiation of GlcN-AuNP-GO demonstrated higher antibacterial activity than antibiotic kanamycin (Govindaraju et al. 2016).

Ocsoy et al. developed DNA aptamer-functionalized AuNPs (Apt@AuNPs) and gold nanorods (Apt@AuNRs) to kill methicillin-resistant *Staphylococcus aureus* (MRSA) through photothermal therapy (PTT) (Ocsoy et al. 2017). They found that both Apt@AuNPs and Apt@AuNRs attached to MRSA and inactivated cells by 5% and > 95%, respectively, through PTT. The difference in the induction of cell death was based on the relatively high longitudinal absorption of NIR radiation and strong photothermal conversion capability of the Apt@AuNRs compared to Apt@AuNPs. Recently, a new approach based on the conjugation of AuNPs with antimicrobial peptides (AMPs) has shown promising results (Rajchakit and Sarojini 2017). For example, Kuo et al. mixed synthetic peptides containing arginine, tryptophan, and cysteine termini [i.e., (DVFLG) 2REEW4C and (DVFLG) 2REEW2C] with aqueous tetrachloroauric acid to generate peptide-immobilized AuNPs [i.e., (DVFLG) 2REEW4C-AuNPs and (DVFLG) 2REEW2C-AuNPs] that were effective against *Staphylococci*, *Enterococci*, and other antibiotic-resistant bacterial strains (Kuo et al. 2016). Conjugation of AMPs with AuNPs usually involves the formation of Au-S coordinate covalent bond between the amine and thiol groups of peptides or conjugating linkers as well as terminal (N- or C-terminal) cysteine of AMPs which help in their conjugation with gold (Tielens and Santos 2010; Xue et al. 2014). However, there is one example when covalent conjugation of an AMP to AuNPs has been achieved via Au-O bond (Lai et al. 2015). Other approaches used a polyethylene glycol linker to covalently attach AMPs with AuNPs that showed significantly increased antibacterial and anti-biofilm activity against antibiotic-resistant gram-negative bacteria (Casciaro et al. 2017). Yeom and co-workers demonstrated the most advanced clinical application for AuNPs@AMP using infected mice in vivo that resulted in the inhibition of *Salmonella typhi* colonization in the organs of the animals (Yeom et al. 2016). The reason behind the increased antimicrobial activity of AuNPs@AMP over the free AMPs is that AuNPs can get a higher concentration of the peptides at the site of action. These NPs interact with lipopolysaccharides (LPS) and proteins of bacterial membrane and, in some cases, penetrate the

bacterial membrane through the porin channel. Thus, the nanoparticles interact with the bacterial inner membrane making the AuNPs@AMP conjugate more efficient than the non-conjugated form (Baptista et al. 2018). Rai et al. demonstrated the conjugation of cecropin-melittin (CM-SH), a known peptide with inherent antibacterial propensity (Boman et al. 1989), with the surface of AuNPs through Au-S bond. The CM-SH-AuNPs showed greater antimicrobial activity in a systemic (i.e., animal) model than CM-SH (Rai et al. 2016) (Table 9.3).

Table 9.3 Mechanism of antibacterial activity of AuNPs against multidrug-resistant (MDR) bacteria

Type of antibiotic resistance	Targeted bacteria	Mechanisms of antibacterial activity of AuNPs	Ref.
Methicillin resistant	*S. aureus*	Photothermal therapy with ROS generation	Hu et al. (2017), Ocsoy et al. (2017), Kuo et al. (2009), Millenbaugh et al. (2015), Mocan et al. (2016)
Methicillin resistant	*E. faecalis*	Combination with vancomycin	Lai et al. (2015)
Ampicillin resistant	*S. aureus, E. coli, P. aeruginosa, Enterobacter aerogenes*	Combination with ampicillin lead to entry into the bacterial cell	Brown et al. (2012)
Carbapenems resistant	*Klebsiella pneumoniae, Proteus mirabilis, A. baumannii*	Disturbance of osmotic balance and disruption of the integrity of bacterial cell wall	Shaker and Shaaban (2017)
Cefotaxime resistant	*E. coli, K. pneumoniae*	Disruption of the bacterial cell wall, DNA damage	Shaikh et al. (2017)
Kanamycin-resistant	*Streptococcus bovis, S. epidermidis, E. aerogenes*	Disruption of the bacterial cell wall	Payne et al. (2016)
Biofilm formation	*P. aeruginosa*	Interaction with cell surface	Yu et al. (2016)
Biofilm formation	*S. aureus*	Laser excitation of the near IR LSPR led to an efficient photothermal response with efficient killing of bacteria biofilms	Pallavicini et al. (2014)
Biofilm formation	*S. epidermidis, S. haemolyticus*	Combination with antibiotics	Roshmi et al. (2015)
Biofilm formation	*E. coli, P. aeruginosa, S. aureus*	Penetration through biofilm layers and interaction with cellular components	Ramasamy et al. (2017a, b)

(continued)

Table 9.3 (continued)

Type of antibiotic resistance	Targeted bacteria	Mechanisms of antibacterial activity of AuNPs	Ref.
Biofilm formation	*E. coli*, *P. aeruginosa*, *S. aureus*, *B. subtilis*	ROS generation	Wang et al. (2017a)
Biofilm formation	*Proteus* species	Interaction between proteins and NPs	Vinoj et al. (2015)
Multidrug resistant	Gram-negative bacteria	Automated microarray-based system for early identification of pathogen and resistance marker detection	Walker et al. (2016)
Multidrug resistant	*E. coli, S. aureus, K. pneumoniae*	Combination with antibiotics	Vidya et al. (2016)
Multidrug resistant	*E. coli, S. aureus, Salmonella typhimurium*	Depend on coexisting chemicals that were not removed from AuNPs	Zhang et al. (2015, Dasari et al. (2015)
Multidrug resistant	*E. coli*	Interaction between lysozyme micro-bubbles and cell wall	Mahalingam et al. (2015)
Multidrug resistant	*S. aureus, E. coli, P. aeruginosa*	Disruption of bacterial cell wall	Li et al. (2014), Yang et al. (2017)
Multidrug resistant	*E. coli, S. aureus*	Interaction with biomolecules	Kim et al. (2017)
Multidrug resistant	*E. coli*, *K. pneumoniae*, *E. cloacae*	Photodynamic therapy/ photothermal therapy; Photodynamic therapy/ photothermal therapy	Khan et al. (2017)
Multidrug resistant	*S. aureus, E. coli, E. cloacae, P. aeruginosa*	Photodynamic therapy/ photothermal therapy	Mocan et al. (2016)
Multidrug resistant	*Salmonella typhimurium*	Photodynamic therapy/ photothermal therapy	Lin and Hamme II (2015)
Multidrug resistant	*S. aureus*	Photodynamic therapy/ photothermal therapy	Gil-Tomás et al. (2007)
Multidrug resistant	*E. coli*	ROS generation	Zhang et al. (2013)
Multidrug resistant	*E. coli*	Change of membrane potential and inhibition of ATP synthase; inhibition of the subunit of the ribosome for tRNA binding	Cui et al. (2012)
Multidrug resistant	*E. coli*, *K. pneumoniae*, *S. aureus*, *B. subtilis*	Change of membrane potential and inhibition of ATP synthase; inhibition of the subunit of the ribosome for tRNA binding	Shamaila et al. (2016)

(continued)

Table 9.3 (continued)

Type of antibiotic resistance	Targeted bacteria	Mechanisms of antibacterial activity of AuNPs	Ref.
Multidrug resistant	*S. aureus*	Photoacoustic detection and photothermal therapy	Galanzha et al. (2012)
Multidrug resistant	*E. coli, K. pneumoniae*	Not revealed	Bresee et al. (2014)
Multidrug resistant /biofilm formation	*P. aeruginosa*	Conjugation with AMP	Casciaro et al. (2017)
Multidrug resistant/ biofilm formation	*Staphylococci, Enterococci,* and other bacterial strain	Conjugation with AMP	Kuo et al. (2016)
Multidrug resistant /biofilm formation	*E. coli, S. aureus, K. pneumoniae, P. aeruginosa*	Conjugation with AMP	Rai et al. (2017)
Multidrug resistant /biofilm formation	*Salmonella typhimurium*	Conjugation with AMP	Yeom et al. (2016)

9.4 Mechanism of Antibacterial Activity of Au Nanoparticles

Drug-resistant bacteria acquire genetic modification to exclude antimicrobial drugs and become less sensitive to drugs. To find an effective way to control the threat of bacterial drug resistance, a novel approach to enhance antimicrobial activity is urgently needed. AuNPs are the most studied metal nanoparticles (NPs) for antibacterial applications (Borzenkov et al. 2020). They are biologically inert and pure form of Au does not exert any antibacterial activity (Zhang et al. 2015). However, the surface area of AuNPs is suitable for conjugation with antibiotics and other drugs. A wide range of proteins, drugs, and even nucleotides have also been successfully delivered using AuNPs in the recent past (Perzanowska et al. 2021). Though the exact mechanism underlying the antimicrobial propensity of AuNPs remains unclear, several factors including size, shape, and surface functionalization significantly influence the activity of AuNP.

Major pathways through which AuNPs exert antimicrobial activity include (i) direct contact with bacteria (Shaikh et al. 2019), (ii) physical disruption of membrane (Piktel et al. 2021), (iii) generation of reactive oxygen species (ROS) (Yu et al. 2020), (iv) interaction with cellular proteins and genetic elements (Wang et al. 2015), and (v) trigger host-response immunity (Dykman and Khlebtsov 2017) (Fig. 9.2a). The antimicrobial efficacy of AuNP is largely dependent on their size and shape. The size of the AuNPs plays a critical role in their bactericidal activity, and functionalization with hydrophilic molecule enhances their interaction with bacterial membrane. Hayden et al. reported the size-dependent antimicrobial effect

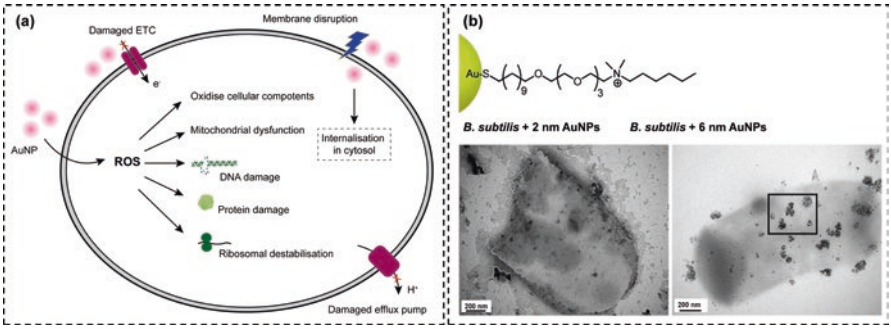

Fig. 9.2 Mechanism of antibacterial activity of AuNPs. (**a**) AuNPs cause irreversible membrane damage and, hence, particles get internalized in the cytosol. AuNPs then interfere with the cellular components and induce the generation of reactive oxygen species (ROS). ROS brings about different kinds of damage and finally promotes cells to apoptosis. Damaged electron transport chain (ETC) and efflux pump change the electron and proton homeostasis in a cell, respectively. (**b**) Size-dependent antimicrobial activity of gold (Au) NPs. TEM images confirmed *B. subtilis* membrane rupture by 2 nm AuNPs but not in case of 6 nm NPs. (Reproduced from Hayden et al. (2012) with permission. Copyright © 2012 American Chemical Society)

of cationic AuNPs (Hayden et al. 2012) (Fig. 9.2b). The larger particles (>100 nm) are unable to cross the bacterial membrane; however, smaller particles do and make pores in the membrane (Zheng et al. 2017; Xing et al. 2018).

The large surface area of AuNPs allows the attachment of a wide range of functional elements to enhance the pristine antimicrobial effect. Hence, both AuNPs and their functionalized derivatives have been used to control infections caused by antimicrobial-resistant (AMR) pathogens (Li et al. 2014; Zhao et al. 2013). In addition to increased bactericidal effect, functionalization of the AuNPs' surface also stabilizes the particle and provides prolonged effective and safe drug delivery (Tao 2018). Multivalent Au atom is conjugated with multiple ligands to enhance its antimicrobial activity (Zheng et al. 2017; Ortiz-Benítez et al. 2019). In addition, capping agents used in the synthesis of AuNPs also disrupt cell membrane via electrostatic interactions. AuNPs capped with a mixture of different small molecules can also enhance their antibacterial propensity. For example, AuNPs of 2 nm size capped with p-mercaptobenzoic acid (pMBA-Au) are not effective against bacteria. When pMBAs on the AuNPs' surface are partially replaced with a mixture of 2-mercaptoethylamine and 3-mercaptopropylsulfonate, the resultant AuNPs show 99.9% growth inhibition against *E. coli* at 0.5 mM concentration (Bresee et al. 2011). Li et al. systematically modified the surface of AuNPs to combat multidrug-resistant (MDR) bacteria. Herein, they functionalized 2 nm size AuNPs using hydrophobic molecules having different chain length (Li et al. 2014). The hydrophobic interaction destroys the integrity of bacterial membrane and allows internalization of metal ions, loaded drugs, and protein inhibitors. On the contrary, it also allows the leakage of cytoplasmic content.

The interaction between cationic AuNPs and negatively charged membrane proteins (mostly teichoic acids) results in the aggregation, protrusion and, therefore,

damage the membrane permanently (Hayden et al. 2012; Zhao et al. 2010). The positive charge of AuNPs is responsible for better interaction with negatively charged bacteria which leads to membrane rupture. However, it has been reported that interaction of AuNPs with bacteria changes the membrane permeability, interrupts the electrolyte balance, and deactivates protein function (Wang et al. 2017b). Payne et al. confirmed that the membrane-attached AuNPs penetrate the cell wall of bacteria using small-angle X-ray scattering (Payne et al. 2016). This phenomenon resulted in the disruption of cytosolic environment and leakage of cellular components (Fig. 9.3).

Ortiz-Benítez et al. found that AuNPs reached the cytosolic environment of resistant *S. pneumoniae* and formed spherical cytoplasmic structures known as inclusion bodies (Ortiz-Benítez et al. 2019). They separated the proteins from the inclusion bodies that are potential candidates to facilitate the uptake of NPs in *S. pneumoniae*.

However, conjugation of antibiotics to AuNPs provides enhanced bactericidal effect than pristine AuNPs (Rattanata et al. 2016). Bagga et al. reported that AuNPs-levofloxacin showed greater bactericidal effect against *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* (Payne et al. 2016; Bagga et al. 2017). XX et al. reported a one-pot, fast synthesis of vancomycin-conjugated AuNP which had 16-fold better antibacterial activity against vancomycin-resistant *Enterococci* when compared to free vancomycin (Wang et al. 2018). Other similar

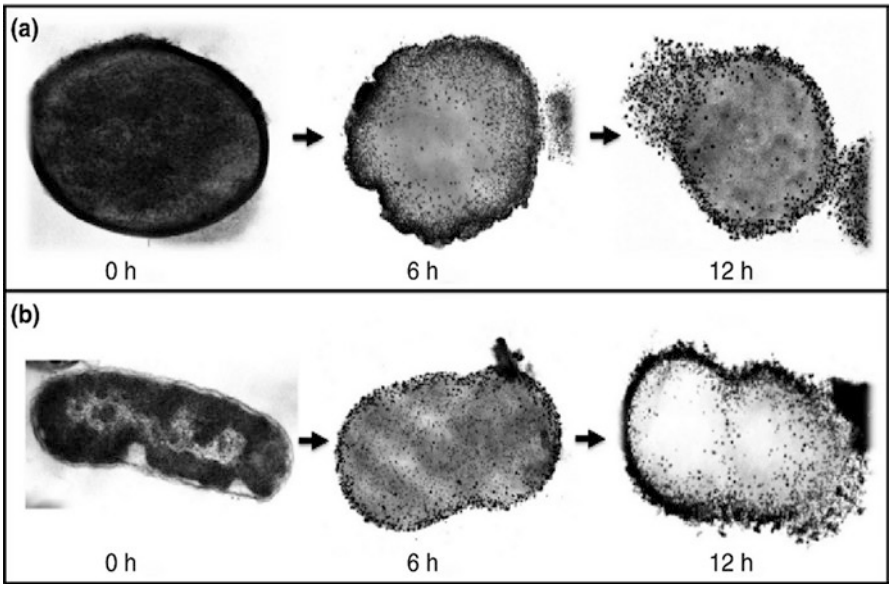

Fig. 9.3 Transmission electron microscopic images of bacteria. Sequential images of gram-positive *Staphylococcus epidermidis* bacteria (**a**) and gram-negative *Enterobacter aerogenes* bacteria (**b**) treated with kanamycin-AuNPs after 0, 6, and 12 h of incubation. (Reproduced with permission Payne et al. (2016). Copyright © 2016)

studies also support the codelivery of antibiotics with AuNP to reduce resistance development (Fuller et al. 2020; Justo and Bosso 2015; Sans-Serramitjana et al. 2016; Wang et al. 2020; Fan et al. 2019). Thus, the delivery of antibiotic-conjugated AuNPs not only improve antibacterial efficacy but also require low dose of free antibiotic. Moreover, they also retard bacteria from developing resistance to antibiotics (Chopra 2007). Antibiotic-conjugated AuNPs exert higher antibacterial activity with minimal toxicity (Huh and Kwon 2011). Though synergistic effect of AuNPs-drug conjugate is always discussed as responsible for enhanced antimicrobial activity, the exact mechanism is still unknown. It is hypothesized that this stable conjugation improves internalization of antibiotics to resistant cells (Gu et al. 2003). Only a stable conjugation offers better antibacterial effect. Major drawbacks of antibiotic adsorption on AuNPs include aggregation and poor dispersity of the nanoparticles leading to compound instability. On the other hand, the conjugation of amphiphilic antimicrobial peptides (AMPs) with AuNPs enhances the antibacterial activity of nanoparticles. The advantage of conjugating amphiphilic AMPs is that they facilitate the interaction of AMP-AuNP with bacterial membrane (Wimley and Hristova 2011; Craik et al. 2013; Wadhwani et al. 2017). For example, Lee et al. conjugated hexahistidine-tagged AMPs with aptamer-bound AuNP (Lee et al. 2017). The resultant conjugate was highly effective against infectious pathogens including *Vibrio vulnificus*.

The photothermal property of AuNPs is advantageous for their biomedical applications (Mahmoud et al. 2019). AuNPs produce heat upon illumination with laser, and the thermal energy damages the neighboring bacteria. It is important to make sure that the AuNPs and bacteria are in close proximity so that the photothermal energy generated in the local environment damages the target cell (Mahmoud et al. 2018). Photothermal therapy (PTT) and photodynamic therapy (PDT) are the two main approaches followed by AuNPs to kill bacteria upon laser irradiation. In case of PTT, AuNPs convert the light energy into heat and enhances local temperature to kill bacteria (Jo and Kim 2013; Zhu et al. 2014; Pallavicini et al. 2017). On the other hand, PDT relies on the irradiation of photosensitizers to generate more reactive oxygen species (ROS) that kill bacteria (Venditti 2019). TEM images confirmed the laser-induced bacterial death after treatment with AuNPs. Bermúdez-Jiménez et al. reported the antibacterial effect of chitosan hydrogel–embedded AuNPs against clinical MDR pathogens upon illumination with laser (Bermúdez-Jiménez et al. 2019) and the minimum inhibitory concentration (MIC) was <4 μg/ml. The low-power infrared diode laser ruptured the bacterial membrane through enhanced ROS production. The principle of PDT is to generate toxic singlet oxygen upon illumination of photosensitizers with visible light. The highly reactive singlet oxygen damages the neighboring bacterial membrane, interferes with the cellular metabolic pathways, and damages the DNA (Bertoloni et al. 2000; Narband et al. 2009). In recent studies, photosensitizer-embedded AuNPs showed effective antimicrobial activity against both gram-positive and gram-negative bacterial strains (Rossi et al. 2019; Jain et al. 2006; Pallares et al. 2016; Darby et al. 2016; Ni et al. 2008). PDT

is very safe and effective; however, it is highly recommended to deliver therapeutic concentration of the photosensitizers.

Cui et al. investigated the molecular mechanism of bactericidal activity of AuNPs using transcriptomic and proteomic approaches. Two major ways of exerting antibacterial activities include decrease in the cellular ATP level that collapses the membrane potential and the inhibition of tRNA-binding ribosomal protein subunit (Cui et al. 2012). Thus, AuNPs interfere with the activities of ATPase and tRNA. Hence, decoding of mRNA responsible for functional protein enhances ROS-mediated chemotaxis in bacteria and leads to apoptosis (Cui et al. 2012). The most supported mechanism for antibacterial activity of the AuNP is the generation of reactive oxygen species (ROS). These highly reactive chemicals enhance oxidative stress and form vacuoles inside cells (Mohamed et al. 2017).

It is important to highlight that commercially available antibiotics do not follow the multidimensional mode of action like AuNPs to inhibit bacterial growth. Hence, AuNPs can be a good alternative tool to control MDR pathogens. The different mode of antibacterial action of AuNPs is due to the structural differences of gram-positive and gram-negative bacteria (Ranjan Sarker et al. 2019). Hence, AuNPs have a promising future in the field of drug-delivery system (DDS) because delivery of drugs using AuNP is not only effective but also less toxic.

In-depth studies are recommended to unravel the precise mode of action involved in AuNPs-bacteria interaction and their inhibition.

9.5 Biocompatibility of Au Nanoparticles

The field of nanomaterials is expanding rapidly with a wide range of applications (Khan et al. 2019). Besides having several medical applications, AuNPs have toxicities associated with them. Hence, it is very important to know the basic information about the nanomaterials, especially composition, variability of size, shape, surface charge, surface area, surrounding media, and aggregation tendency in biological fluid that influence the biocompatibility of NPs. In this section, properties of Au NPs will be discussed to understand their biocompatibility cell.

A range of techniques have been used to investigate the biocompatibility of AuNPs (Fig. 9.4). Table 9.4 summarizes the principle and benefits of each assay. MTT assay is considered as the "gold standard" to investigate the toxicity of AuNPs. It is a colorimetric assay to determine cellular metabolic activity (Stockert et al. 2018). Herein, enzymatic activity of mitochondrial reductase is monitored under a defined condition. In an oxidation reaction, the enzyme reduces a yellow-colored tetrazolium dye (known as MTT or 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) and produces insoluble purple-colored formazan. This irreversible reaction reflects the number of live cells at different time points.

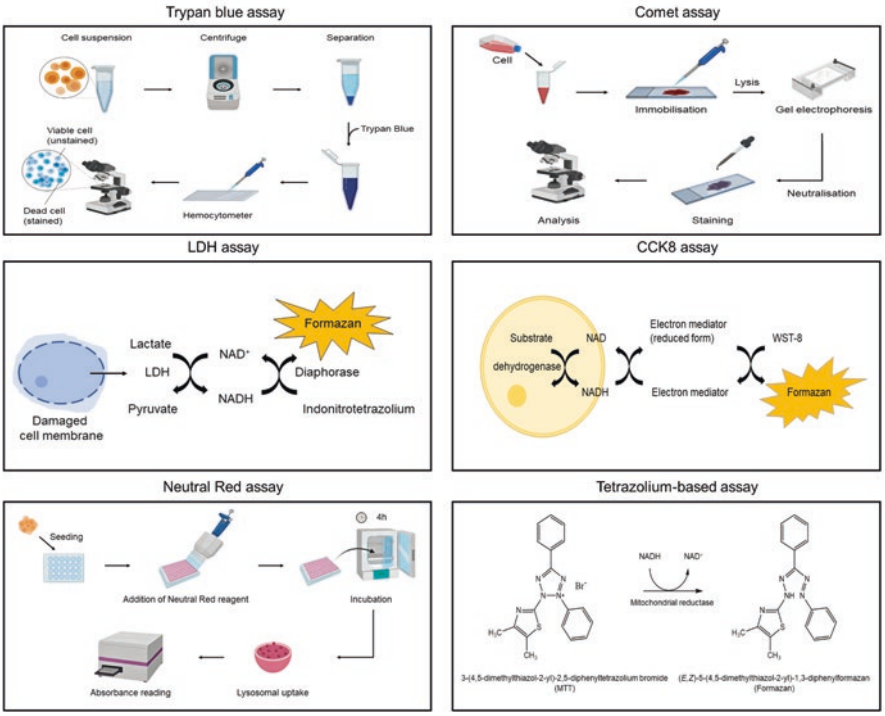

Fig. 9.4 Commonly used assays to determine cell viability

Cell number is quantified by measuring the absorbance of formazan at 570 nm. The degree of formazan production reflects the number of dead cells and is directly proportional to light absorption.

One of the pioneering reports on the biocompatibility assessment of AuNPs was reported by Shukla et al., where the biocompatibility and the uptake of AuNPs by RAW 264.7 macrophage cells were investigated (Shukla et al. 2005). Their findings suggest that AuNPs are highly biocompatible having antioxidation potential at higher doses besides suitable for prolonged treatment. In addition, they found that spherical AuNPs did not induce the secretion of proinflammatory cytokines (e.g., TNFα and interleukin β) by macrophage cells. Chithrani et al. studied the effect of AuNP's shape, size, and toxicity (Chithrani et al. 2006). They concluded that citrate-capped spherical and rod-shaped AuNPs did not cause any significant toxicity to HeLa cells. This study was followed by other research groups and found negligible toxicity for spherical and rod-shaped AuNPs using different cell lines in vitro (Khan et al. 2007; Gu et al. 2009; Villiers et al. 2010). We demonstrated that functionalization of elongated tetrahexahedral (ETHH) AuNPs with α-lipoic

Table 9.4 Summary of commonly used assays to evaluate the cytotoxicity of AuNPs

Assay	Principle	Remarks	Reference
Trypan blue assay	This diazo dye penetrates the cell membrane of dead cells and stains them selectively	Identifies live (unstained) and dead (blue) cells Provides quantitative cell-to-cell count Fast method Long-time incubation may give false positive result	Strober (2001)
Comet assay	Denatured cleaved DNA fragments migrate out of the cell under electrophoresis and the undamaged DNA remains within the cell membrane	Single-cell gel electrophoresis Economical Sensitive method	Fairbairn et al. (1995)
LDH assay	Colorimetric detection of lactate dehydrogenase (LDH)	Depends on LDH detection Qualitative and quantitative cell count Not preferable for multiple samples	Kumar et al. (2018)
Cell Counting Kit 8 (CCK8)	Cellular dehydrogenases cause reduction of water-soluble tetrazolium salt, WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] And produce orange colored formazan	Colorimetric assay No premixing is required Rapid and sensitive Expensive Absorbance interference	Cai et al. (2019)
Neutral Red assay	Incorporation of dye lower in dead cell	Depends on lysosomal function Simple and fast method Quantitative cell count	Repetto et al. (2008)
Tetrazolium-based assays	Conversion of tetrazolium dye to insoluble formazan	Depends on mitochrondiral function Colorimetric assay Simple and fast method Qualitative and quantitative cell count Repeatable Economical Not suitable for suspending cells Seeding amount and assay duration must be optimized	Stockert et al. (2018), Vistica et al. (1991)

acid, a natural antioxidant, increased their hemocompatibility as well as biocompatibility to human red blood cells (RBCs) and HeLa cells, respectively (Ranjan Sarker et al. 2019). Recently, we also reported that functionalization of concave cube AuNPs (CCAu) with α-lipoic acid and glutathione, a tripeptide with antioxidation potential, increased their hemocompatibility to human RBCs and biocompatibility to HeLa cells, L929 fibroblasts, and CHO-GFP cells (Pandala et al. 2021). On the contrary, Patra et al. synthesized spherical (33 nm in diameter) AuNPs and investigated their toxicity using three different cell lines (Patra et al. 2007). They found the particles were toxic to human lung carcinoma cells (A549), but compatible with baby hamster kidney cells (BHK21) and human liver carcinoma cells (HepG2).

The hydrodynamic size of AuNPs plays an important role in terms of their cellular uptake and biocompatibility. Earlier studies found that size variability of AuNPs demonstrates negligible toxicity (Khan et al. 2007; Gu et al. 2009; Villiers et al. 2010; Connor et al. 2005). Large AuNPs are generally stable, inactive, noncatalytic, and biocompatible (Khlebtsov and Dykman 2011). Sophisticated tools including inductively coupled plasma mass spectrometry (ICP-MS) are used to quantify AuNPs taken up inside cell (Merrifield et al. 2018). Moreover, electron microscopy confirmed the presence of Au particles in the treated cells (Shukla et al. 2005; García et al. 2013). Chithrani et al. and others studied the cellular uptake of a wide range of AuNPs and quantified the number of nanoparticles inside each cell using inductively coupled plasma atomic emission spectroscopy (Chithrani et al. 2006; Osaki et al. 2004; Huo et al. 2013; Wang et al. 2010; Heuskin et al. 2017). All these studies reported that the maximum uptake was observed in case of 50 nm AuNPs without any cytotoxic effect. Karakoçak et al. used two independent methods (i.e., electric cell-substrate impedance sensing and MTT assay) to investigate the cellular uptake and biocompatibility of spherical, rod-, and cube-shaped AuNPs (Karakoçak et al. 2016). They concluded that sphere-shaped AuNPs have better biocompatibility when compared to Au rods (Fig. 9.5). However, cube-shaped AuNPs neither entered into cells nor had any cytotoxic effect.

Most often rod-shaped AuNPs are synthesized using a cationic surfactant called CTAB. Wang et al. found that free CTAB up to 1 μM is toxic to mammalian cells (Wang et al. 2008). Therefore, several strategies including chemical exchange or surface functionalization have been chosen to make Au nanorods less toxic. Polyethylene glycol (PEG), a hydrophilic polymer, has been widely used to modify AuNPs' surface. However, PEGylation of AuNPs interferes with the cellular uptake process through reduced endocytosis (Nel et al. 2009; Doak et al. 2009). Chen et al. evaluated intraperitoneally injected eight AuNPs of different size (3–100 nm) and found size dependent toxicity in mice (Chen et al. 2009).

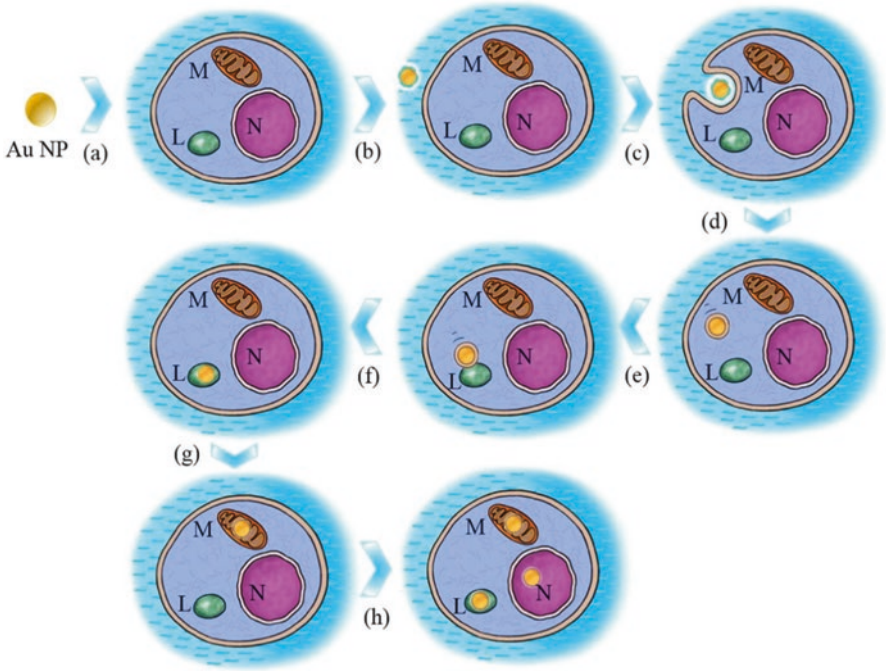

Fig. 9.5 The mechanism of AuNPs-mediated cytotoxicity. (**a**) Exposure of AuNPs to ARPE-19 cell. (**b**) Dynamic bipolymer layer (thick and loose blue color coating) creates a superficial surface exposed to the cell membrane. (**c**,**d**) Uptake of AuNPs by endocytosis. (**e**) Fusion of endocytic vesicle with lysosome. (**f**) Complete internalization of AuNPs by lysosome. (**g**) AuNPs cross the single membrane of lysosome and penetrate the mitochondrial intermembrane space to initiate apoptosis signal. (**h**) AuNPs available in the cytoplasm cause cell death by activating subcellular signaling pathways for apoptosis, initiating cell shrinkage, decreasing cytoplasmic shrinkage, and beginning subcellular fragmentation. Here, L lysosome; M mitochondria; and N nucleus. (Reproduced with permission Karakoçak et al. (2016) Copyright © 2016 Elsevier Ltd.)

9.6 Conclusions and Future Perspectives

Multidrug-resistance bacteria are exerting real threat to the existence of mankind because they have become resistant to almost all the commercially available antibiotics. Overuse and misuse of antibiotics are the main causes of the development of bacterial resistance mechanisms against antibiotics. Gold nanoparticles demonstrate antibacterial activity mainly through the oxidation of bacterial membrane resulting in the formation of pores on the membranes. As a result, AuNPs interact with the cellular DNA, proteins, and other macromolecules and cause bacterial death. Since AuNPs demonstrate antibacterial activity by damaging bacterial membrane and other subcellular organelles, they demonstrate antibacterial activity against a wide range of bacteria including pathogenic bacteria and multidrug-resistant bacteria. Furthermore, AuNPs are highly biocompatible in terms of their

compatibility with various cell lines, red blood cells (RBCs), and mouse model (i.e., in vivo). Hence, in clinical trials, AuNPs can be recommended to be used as an alternative nanoweapon to commercially available antibiotics to tackle multidrug-resistant bacteria.

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Chapter 10
Antimicrobial Potentials of Zinc and Iron Oxide Nanoparticles

Mansee Thakur, Smital Poojary, Kapil Singh Thakur, and Vinay Kumar

Abstract One of the most important issues in global healthcare is the development of antimicrobial or antibiotic resistance (AMR or ABR) in microorganisms. The emergence of antibiotic-resistant strains raised the number of deaths and the severity of bacterial infections dramatically. To overcome this problem, development of metal oxide nanoparticles as an adjuvant or substitute of antibiotics could be one of the most promising strategies to combat AMR in pathogenic bacteria. This chapter in particular has focused on antimicrobial potencies of zinc oxide nanoparticles (ZnO NPs) and iron oxide nanoparticles (IONPs) against multidrug-resistant (MDR) bacteria. It examines the findings of investigations conducted in recent years to investigate the antimicrobial activity of ZnO NPs and IONPs with its mechanism of action. Thus, it will provide a better understanding of using ZnO NPs and IONPs as an alternative to antibiotics and aids in further research and development of antimicrobial agents against MDR strains for healthcare, medicine, and industrial applications.

Keywords Zinc oxide nanoparticles · Iron oxide nanoparticles · Multidrug-resistant (MDR) · Antimicrobial · Antibiotics

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10.1 Introduction

Antimicrobial resistance (AMR) has emerged as a global health issue, posing a threat to the effective prevention and treatment of a growing number of infections caused by bacteria, parasites, viruses, and fungi that are no longer susceptible to commonly used antibiotics (Prestinaci et al. 2015). AMR develops over time, mainly as a result of genetic alterations which can be transmitted from person to person or between humans and animals, as well as through animal-sourced food. There are various reasons that drive AMR including unhygienic sanitation, access to filthy water, lack of sterilized conditions in healthcare facilities, quality and affordability of vaccines and medicines, poor awareness and knowledge, etc. Globalization is also one of the reasons responsible for transmission of AMR by increased migration of humans and animals (WHO 2020). Mutations in microorganisms cause antimicrobial resistance (AMR), according to the World Health Organization (WHO), which makes antibiotics ineffective and diseases linger in the body, raising the risk of spreading to others, and the death rate has found to be more in developing countries as compared to developed ones (WHO 2018). Because of AMR, treating common pathogens becomes more difficult, resulting in longer illness, higher costs, more complications, and more deaths. In the USA, few reports revealed around 2 million patients suffered from antibiotic-resistant infections with nearly 23,000 deaths annually (Munir et al. 2020). It has been predicted by a study that if this condition continues, it is expected to cause 10 million deaths by 2050, whereas another study revealed that AMR may cost the global economy US$100 trillion over the same time span (Mogasale et al. 2021).

The discovery of antibiotics gave rise to hope that infections could be controlled and prevented. Due to the cost-effectiveness and potency, antibiotics have been the favored treatment for bacterial infections. Several studies, however, have shown that widespread antibiotic use has resulted in the proliferation of multidrug-resistant bacterial strains (Wang et al. 2017). The action of antibiotics showed either by restraining or eradicating bacteria, respectively, in a bacteriostatic or bactericidal manner (Fischbach and Walsh 2009). These drugs work by forming a connection with any important microbial metabolic compounds, preventing pathogens from forming functional biological molecules (Linares et al. 2006). Microbial contagious epidemics are increasingly becoming one of the leading causes of morbidity and mortality, with the rapid spread of drug-resistant infectious diseases posing a global health threat. The development of pathogenic resistance to antibiotics occurs through the expression of enzymes that modify or degrade the activity of antibiotic (Poole 2002) and alteration in the composition of cell wall in vancomycin resistance and ribosomes in tetracycline resistance (Jayaraman 2009). Moreover, drug resistance may also evolve as a result of bacterial modifications and improvements to efflux pathways that restrict medication passage (Arora et al. 2020).

Advances in nanotechnology offer a promising solution for AMR as different sizes and shapes of nanoparticles could not only combat against bacteria but also act as drug delivery systems for antibiotics and natural antimicrobial compounds due to

their unique properties and large surface-to-volume ratio. Multitude of mechanisms can exert the antimicrobial activity of nanoparticles (NPs) such as reactive oxygen species (ROS) generation, biofilm inhibition, NP interaction with cell wall of bacteria, induction of innate and adaptive host immunological responses, interaction with DNA and/or proteins, inhibition of signal transduction, etc. Hence, it has become a challenging topic where further study and research is mandatory to understand NPs as antimicrobial agents (Baptista et al. 2018).

This chapter covers recent developments in innovation nanobiotechnology and broad explanation of NPs that shows antimicrobial efficacy against MDR strains as well as mechanisms by which NPs behave as antimicrobial agents along with the future outlook.

10.2 Emergence of Nanomaterials as Effecting Antimicrobial Agents

The challenge of antimicrobial resistance towards antibiotics must be accepted, and novel strategies should be discovered to combat against the pathogens (Ventola 2015). One of the recent advances in technology for this menace is the usage of nanotechnology where novel nano-formulations could be developed with different sizes and shapes of nanoparticles by manipulating materials to treat pathogenic infections (Baptista et al. 2018). Metals and metal oxides of zinc, silver, copper, and titanium are commonly used in pathogen-fighting nanoparticles because they naturally have microbiocidal or microbiostatic properties and have shown bactericidal activity against both Gram-positive and Gram-negative bacteria (Vega-Jiménez et al. 2019). Also some nanoparticles encompass polymeric NMs, liposomes, dendrimers of zinc oxide (ZnO), silver oxide, gold, and solid lipid NMs, whereas few NP gets directly attach to the cell wall of microbes; hence there is no need to penetrate into the cell. There are metal oxide NMs who possess microbicides properties through reactive oxygen species (ROS) (Munir et al. 2020). The most appealing feature of NPs drug delivery systems is their ability to deliver a wide variety of therapeutics to the infection site efficiently and safely, either bound to their vast surface area or enclosed within the structure, having a regulated rate of targeted delivery (Gholipourmalekabadi et al. 2017). The dosage needed to achieve clinical effects can be significantly reduced by enhancing the pharmacokinetic profile and therapeutic index of encapsulated drugs as compared to free drug equivalents which may reduce the toxicity and negative side effects associated with high systemic drug concentrations and repeated dosing (Baptista et al. 2018).

10.3 Antimicrobial Potencies of Zinc/Zinc Oxide Nanoparticles (ZnO NPs) Against Drug-Resistant Microbes (Fig. 10.1)

Zinc oxide (ZnO) is a compound with n-type semiconductor and a band gap of 3.3 eV. The US Food and Drug Administration has classified ZnO as "generally recognized as safe" (GRAS) (21CFR182.8991). It is the most widely used zinc source in the fortification of cereal-based foods as a food additive. ZnO has been incorporated into the linings of food cans in packets for meat, fish, corn, and peas to preserve colors and avoid spoilage due to its antimicrobial properties (Xie et al. 2011). To overcome multidrug resistance, ZnO NPs have been studied by many researchers for the production of next-generation nanoantibiotics against pathogenic microorganisms (Makabenta et al. 2021; Muzammil et al. 2018). ZnO NPs could be synthesized in various forms such as rings, belts, wires, propellers, etc. (Wang 2004). Several reports have proved nano-sized ZnO particles to have more antimicrobial potentials as compared to larger particles because of high surface-to-volume ratio of NPs which allows better interaction with bacteria with selective toxicity to bacteria, whereas they showed minimal effects on human cells (Reddy et al. 2007).

Padmavathy et al. studied antimicrobial activity by enhancing the bioactivity of ZnO NPs using various sizes of NPs suspension through bacteriological tests which includes minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against target organism *Escherichia coli*. It concluded triggered generation of reactive oxygen species on the surface of colony which kills bacteria more effectively using smaller size of ZnO NPs. Hence, ZnO NPs were discovered to be more abrasive as compared to bulk ZnO, which leads mechanical damage to the cell membrane and increased bactericidal activity of ZnO NPs (Padmavathy and Vijayaraghavan 2008). Another study focused on antibacterial activity of

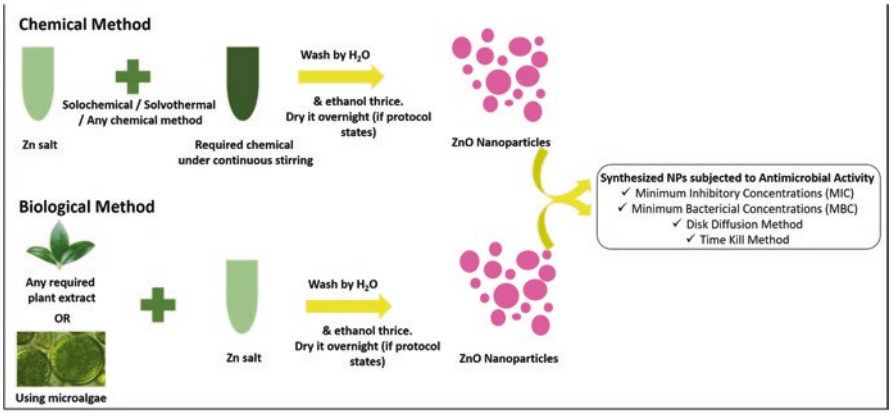

Fig. 10.1 Chemical and biological synthesis of ZnO NPs and its antimicrobial assay

biosynthesized ZnO NPs using *Artemisia aucheri* extract against *Staphylococcus aureus* (Gram-positive) and *E. coli* (Gram-negative) by agar well diffusion method. The results revealed good inhibition zones of ZnO NPs against both *S. aureus* and *E. coli* by 7 mm and 5 mm in diameter, respectively, due to release of Zn^{2+} ions which could potentially alter the inhibition of active transport and amino acid metabolism. This change may have a catastrophic effect on the enzyme system, which is a result of the size and solubility of Zn^{2+} dependent employed in culture reactive oxygen species (ROS) formation, which is tightly reliant in photocatalytic induction and antibacterial activity of ZnO nanoparticles enhanced during UV exposure (Nezamabadi et al. 2020). A study demonstrated antimicrobial activity of ZnO NPs synthesized using *Catharanthus roseus* extract by disk diffusion method against *S. aureus*, *S. pyogenes*, *B. cereus*, *P. aeruginosa*, *P. mirabilis*, and *E. coli*. The NPs showed antibacterial potential against all these pathogenic bacteria with the inhibition zones of about 11.09–11.74 mm diameter as compared to Streptomycin. These results indicated interaction of ZnO NPs with the bacterial cell and its better activity against Gram-positive as compared to Gram-negative bacteria. Also, effect of ZnO NPs at three different concentrations – 700, 900, and 1500 ug/ml – were determined on bacterial growth curve which showed inhibitory effect at higher concentration, and, therefore, it was concluded that at low concentration, zinc oxide is bacteriostatic, whereas at high concentration, it is bactericidal (Gupta et al. 2018). The microalgae synthesized ZnO NPs proved as antimicrobial agents against *S. aureus*, *K. pneumonia*, *E. coli*, *Micrococcus luteus*, and *Citrobacter freundii* which showed significant inhibition zone. Hence, it was concluded that these NPs could be used to treat infectious and non-infectious diseases (Jamil et al. 2020).

Elkady et al. synthesized ZnO NPs in nanotubes form and examined for its antimicrobial activity against four MDR bacteria: *Staphylococcus aureus* and *Bacillus subtilis* (Gram-positive) and *Escherichia coli* and *Pseudomonas aeruginosa* (Gram-negative) using disk diffusion method. Due to high surface area of nano-ZnO, equivalent to 17.8 m^2/g, results showed higher antimicrobial activity against all bacterial strains and confirmed by MIC that lower concentrations of nano-ZnO could be used as antimicrobial agents in a suitable formula (Elkady et al. 2015). ZnO NPs synthesized by solvothermal method elevated the antimicrobial action of ceftazidime and ciprofloxacin which improved the antibiotics usage by MDR *Acinetobacter baumannii* and changed the morphology of bacterial cells from rods to cocci shape. This study suggested antibiotic concentrations in cells are thought to be increased by ZnO NPs inhibiting antibiotic efflux or boosting antibiotic entrance into cells, and this rise in antibiotics seems to cause morphological alterations in the body (Ghasemi and Jalal 2016). A similar study on multi-resistant bacteria *A. baumannii* investigated ZnO NPs as an alternative antimicrobial agent because of its mode of action (Tiwari et al. 2018). In additional research, synergistic effects were tested by mixing ZnO NPs with antibiotics such as tigecycline, vancomycin, erythromycin, and ofloxacin to observe the effectiveness against multidrug-resistant bacteria which showed elevation in antibacterial activity among resistant bacteria, and an effective zone of inhibition was identified in *Enterococcus* sp., *S. aureus*, and *Proteus mirabilis* (Yusof et al. 2019).

Firstly, the antibacterial potential of ZnO NPs could be enhanced by combining it with other metal oxide nanoparticles. For instance, ZnO and CuO NPs showed effectiveness against *E. coli* and *S. aureus* at exponential growth phase, whereas Ag and ZnO NPs inhibited growth of *M. tuberculosis* strains without leading to bacterial death. Secondly, it could be enhanced by using ZnO NPs with spindle-shaped graphene oxide (GO) nanoparticles which inhibits the growth of *B. subtilis*, *Enterococcus faecalis*, *E. coli*, and *S. typhimurium*. Third method includes coating ZnO NPs with modifying agents; for example, gelatin-coated ZnO NPs inhibit the growth of Gram-negative bacteria more as compared to that of Gram-positive bacteria. Next method is to modify synthesis method which leads to geometric characteristic changes of nanoparticles – ZnO NPs synthesized by sonochemical method showed more inhibitory properties as compared to classical physiochemical methods against *S. aureus*, *P. aeruginosa*, *B. cereus*, and *S. typhimurium* (Gudkov et al. 2021).

The importance and significance of ZnO NPs in numerous fields has sparked a worldwide interest in investigating their antibacterial properties. When compared to organic-based disinfectants, ZnO NPs have unique features and have a long life, which has prompted its use as an antibacterial agent. Because of their enormous surface area-to-volume ratio, they can be used as novel antibacterial agents, which have recently become a source of concern for researchers (Sirelkhatim et al. 2015; Table 10.1).

10.4 Antimicrobial Potencies of Iron/Iron Oxide Nanoparticles (IONPs) Against Drug-Resistant Microbes (Fig. 10.2)

Iron oxide (III) is an extremely stable oxide that crystallizes in hexagonal shape in nature as hematite α-Fe_2O_3 (Tadic et al. 2017). Along with other metal oxide nanoparticles, IONP has grabbed attention of researchers due to its unique magnetic properties. Magnetic nanoparticles can range in size from a few nanometers to tens of nanometers. They are usually the same size as or smaller than the protein molecule, making it easier for the cells or viruses to interact with one other or attach to/infiltrate the biological matrix of interest (Shah et al. 2019). Different sorts of biological entities, such as enzymes, proteins, nucleotides, and antibodies, can interact and bind with the functionalized IONPs. Based on functionalization procedures, it can even interact with pharmaceuticals. As a result, an external magnetic field can be employed to release it into the targeted tissues, organs, or tumors (Chorny et al. 2010; Gao et al. 2009). IONPs' inherent antibacterial properties encourage their investigation as potential treatment agents for infectious illnesses. A study has reported bactericidal effect of Fe_2O_3 NPs against *E. coli* and *S. aureus*, and the effect was found to be elevated with the increase in concentration of NPs (Rufus et al. 2016). Yan et al. (2020) evaluated antimicrobial potential of IONPs synthesized

Table 10.1 Few reports represent antimicrobial potential of ZnO NPs synthesized by different methods of varying sizes against Gram-positive and Gram-negative bacteria

Synthesis method	Size	Target bacteria and microbial effects	References
Green synthesis using *Cassia auriculata* leaf extract	20–30 nm	Strong bacterial activity against *B. subtilis*, *K. pneumonia*, *P. aeruginosa*, and *P. mirabilis*	Ramesh et al. (2021)
Synthesis of ZnO NPs using aqueous extract of ash derived from *Musa balbisiana Colla pseudostem*	45–65 nm	Excellent antibacterial activity against *E. coli*, *S. aureus*, *B. subtilis*, and *P. aeruginosa*	Basumatari et al. (2021)
Nosaka method	3 nm	Effective inhibition against *S. aureus*	Emami-Karvani and Chehrazi (2011)
Wet chemical method (modified using 3-aminopropyltriethoxysilane (APTES) and dimethyl sulfoxide (DMSO))	28 nm & 39 nm	Inhibition against *E. aerogenes*, *E. coli*, *K. oxytoca*, *P. aeruginosa*, *S. aureus*, and *S. pyogenes*. Induced more antimicrobial effect against Gram-negative bacteria as compared to Gram-positive bacteria	Esparza-González et al. (2016)
Synthesis by microalgae	Ranging from 1 nm to 100 nm	Proved as antimicrobial agent for *E. coli*, *S. aureus*, *Micrococcus luteus*, *K. pneumoniae, Citrobacter freundii*	Jamil et al. (2020)
Biological synthesis using *Catharanthus roseus*	Ranging between 62 nm and 94 nm	Inhibition zone against *S. aureus*, *S. pyogenes*, *B. cereus*, *P. aeruginosa*, *P. mirabilis*, and *E. coli*	Gupta et al. (2018)
Solochemical method	37 nm	Antimicrobial activity against *S. aureus*	Sornalatha et al. (2015)
Solvothermal synthesis	12 nm	Growth inhibition of *S. typhimurium*	Raghupathi et al. (2011)
Biosynthesis by *lactobacillus plantarum* TA4	Ranging from 49.2 nm to 369.5 nm	Inhibitory and bactericidal efficacy against *E. coli*, Salmonella sp., *S. aureus*, and *S. epidermidis*	Mohd Yusof et al. (2021)
Biosynthesis using *Bauhinia tomentosa* leaf extract	22–94 nm	Higher inhibition zone against *P. aeruginosa* and *E. coli* (Gram-negative bacteria) than *B. subtilis* and *S. aureus* (Gram-positive bacteria)	Sharmila et al. (2018)
Green synthesis using *Trifolium pratense* flower extract	Ranging from below 100 to 190 nm	Zone of inhibitions against *S. aureus*, *E. coli*, and *P. aeruginosa*	Dobrucka and Długaszewska (2016)

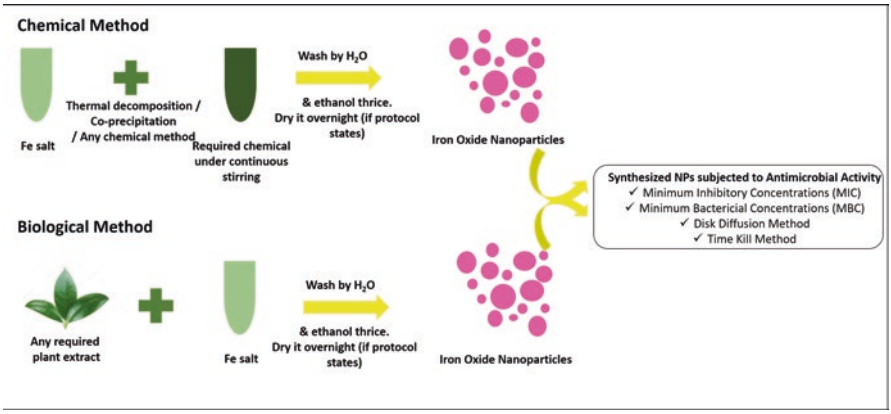

Fig. 10.2 Chemical and biological synthesis of IONPs and its antimicrobial assay

using durian rind extract and durian rind extract by disk diffusion assay which showed zone of inhibition against *E. coli* (Gram-negative) and *S. aureus* (Gram-positive) bacteria. The extent of inhibition was found to be lower for durian rind extract as compared to IONPs maybe due to the presence of anti-infection effects and lesser penetrating ability to harm bacterial cells. The mechanism for IONPs for effective antimicrobial properties might be because of formation of free radicals and stronger penetrating properties that ultimately damage the bacterial cells and make the surface porous to act on it (Yan et al. 2020). A study demonstrated antibacterial activity of *Cinnamomum verum*-functionalized Fe_3O_4 nanoparticles (9.4 nm size) using matrix-assisted pulsed laser evaporation (MAPLE) technique onto gastrostomy tubes (G-tubes) towards Gram-negative and Gram-positive bacterial colonization. It was concluded improved anti-adherent properties of modified NPs surfaces reduced Gram-negative and Gram-positive bacterial colonization. Moreover, the phenotypical data showed significant differences on *S. aureus* biofilms where inhibition rates for incipient biofilms were found to be more than 4-fold which ranges up to 3-fold for mature biofilms, whereas for *E. coli* it ranged from 2.5-fold to 2-fold grown onto the bioactive Fe_3O_4@CVmodified G-tube surface. According to this study, they can be utilized safely for antibacterial protection of medical surfaces and gadgets that will be employed for a variety of patients with severe conditions (Anghel et al. 2014). It was observed that functionalized IONPs which enhanced its free radical scavenging properties three times than unfunctionalized IONPs. The MIC confirmed the antimicrobial activities of functionalized IONPs against bacterial strains (*S. aureus*, *B. subtilis*, and *E. coli*) and fungal strains (*C. albicans*, *S. cerevisiae*, *A. niger*, and *Trichoderma* sp.) which concluded to be used as antimicrobial agent for the development of biomedicines (Shah et al. 2019). A study demonstrated positively charged magnetic nanoparticles as an antimicrobial agent against multidrug-resistant *E. coli* and *S. aureus* which has been observed to be trapped by electrostatic interaction. Due to exposure to radiofrequency, there was loss of membrane potential, and membrane-associated complexes were found to be

dysfunctional which ultimately killed bacteria and blocked formation of biofilm without creating antimicrobial resistance (Chaurasia et al. 2016; Table 10.2).

10.5 Mode of Action of ZnO NPs and IONPs Against Drug-Resistant Microbes

Due to increasing drug-resistant infections, metal oxide nanoparticles have gained an utmost importance for treatment of antimicrobial-resistant diseases. To function as antimicrobial agent, there should be contact between NPs and bacterial cells through either of the interactions – receptor ligand (Gao et al. 2014), electrostatic attraction (Li et al. 2015), hydrophobic interactions (Luan et al. 2016), or Van der Waals forces (Armentano et al. 2014). The nano-size of particles enables them to

Table 10.2 Few reports represent antimicrobial potential of IONPs synthesized by different methods of varying sizes against Gram-positive and Gram-negative bacteria

Synthesis method	Size	Target bacteria and microbial effects	References
Biosynthesis using *Phoenix dactylifera* extract	950–3000 d.nm	Antimicrobial activity against *K. pneumonia* and *E. coli*	Batool et al. (2021)
Green synthesis using spinach leaf and banana peel extracts	10–70 nm (spinach leaf extract) and 20–50 nm (banana peel extract)	Both showed effective antibacterial potential against *B. subtilis* and *E. coli*	Tyagi et al. (2021)
Thermal decomposition	Avg 97 nm	Effective bactericidal propensity against *S. aureus* by destruction of mitochondrial membrane	Das et al. (2020)
Co-precipitation method	10–20 nm	Antimicrobial propensity against *B. subtilis* and *E. coli*, due to enhanced ROS production	Arakha et al. (2015)
Co-precipitation method	8 nm	Microbiostatic effect against *E. coli* due to generation of ROS, superoxide radicals (O_2-), hydroxide radical (OH^-) and singlet oxygen ($_1O^2$)	Chatterjee et al. (2020)
Biological synthesis using *Azadirachta indica* leaf extract	Ranging between 50 nm and 100 nm	Inhibition zone against *S. aureus*, *K. pneumonia*, *P. aeruginosa*, *L. sphaericus*, and *B. safensis*	Muthukumar et al. (2017)
Chemical method	Approx. 70 nm	Zone of inhibition against *E. faecalis*, *S. aureus*, *E. coli*, *P. aeruginosa*, *S. epidermidis*, *K. pneumonia*, and *C. albicans*	Prucek et al. (2011)
Biosynthesis by durian rind extract	10 nm	Effective antibacterial properties against *E. coli* and *B. subtilis*	Yan et al. (2020)

enter the bacterial membrane easily and interacts with the basic cellular components by impacting shape and functional of cell membrane (Wang et al. 2017; Fig. 10.3).

The following are the mechanisms responsible for antimicrobial potential of metal oxide nanoparticles:

(i) *Generation of Reactive Oxygen Species (ROS)*

One of the crucial antibacterial mechanism of NPs is ROS-induced oxidative stress where oxygen species are produced during basic metabolism which are highly reactive (Slavin et al. 2017). To prevent damage to vital biomolecules in the cell, universal intracellular defense mechanisms have evolved to deal with this unwanted chemical. However, when people are under a lot of stress, their levels of ROS will skyrocket, and it's thought that this is one of the main NP mechanisms of action that stops bacteria from growing (Padmavathy and Vijayaraghavan 2008; Kumar et al. 2011). The superoxide radical (O_2-), hydroxyl radical (OH), hydrogen peroxide (H_2O_2), and singlet oxygen (O_2) are the four forms of reactive oxygen species (ROS) that have varying levels of activity and dynamics. For instance, calcium oxide and magnesium oxide NPs produce O_2, zinc oxide NPs produce H_2O_2 and OH, whereas copper oxide NPs contain all four forms of reactive oxygen (Wang et al. 2017). When oxygen enters unwanted reduction states and transforms into free radicals, superoxide, and peroxides instead of water, ROS are formed. A stress on the cell, such as UV light, DNA damage, or NPs, may cause ROS output to rise to a toxic level for the cell, resulting in cell damage or death (Madl et al. 2014). The toxicity of nanomaterials has been linked to the development of reactive oxygen species

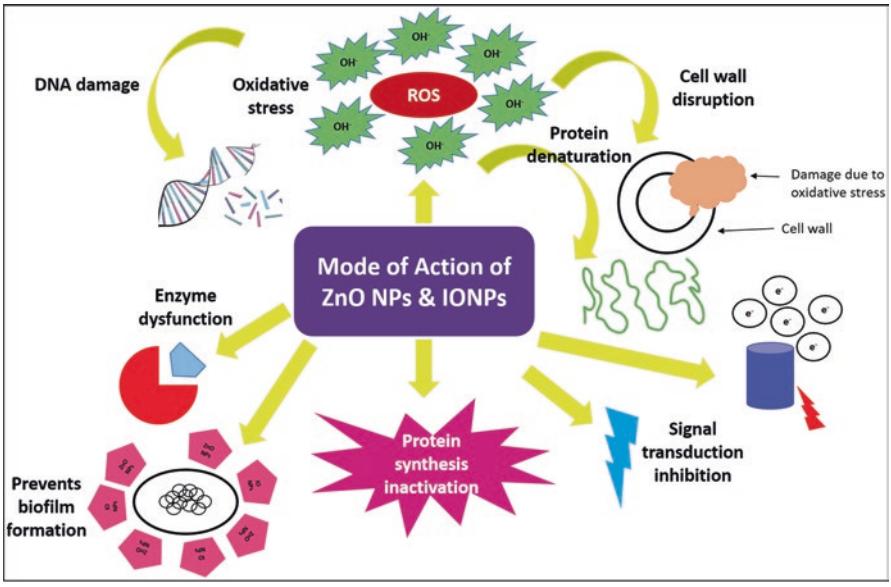

Fig. 10.3 Represents mechanisms responsible for antimicrobial activity of ZnO NPs and IONPs against drug-resistant microbes (Wang et al. 2017; Vega-Jiménez et al. 2019; Slomberg et al. 2013)

(ROS) such as hydroxyl radicals, superoxide anions, and hydrogen peroxide, which inhibit DNA replication and amino acid synthesis, and through lipid peroxidation, it damages the bacterial cell membrane which compromises membrane semipermeability and suppresses oxidative phosphorylation (Hemeg 2017). Studies revealed that ROS plays a vital role in interaction between DNA and bacterial cells and elevates the levels of gene expression of oxidative proteins which ultimately leads to bacterial cell apoptosis. Moreover, proteins could be attacked by ROS, and activity of periplasmic enzymes could be suppressed which are responsible to maintain the bacterial cell's morphology and physiological processes (Wu et al. 2011; Padmavathy and Vijayaraghavan 2008).

(ii) *Adsorption of Nanoparticles to Bacterial Cell*

Biosorption is a process in which metal ions of NPs bind with negatively charged functional groups cell membrane such as phosphate and carboxyl groups. The charge difference between bacterial membranes and metal oxide NPs leads to electrostatic attraction, which causes metal oxide NPs to accumulate on the bacteria surface, modifying the structure and permeability of the cell membrane. Gram-negative bacteria have a higher negative charge as compared to Gram-positive bacteria, so the electrostatic interaction between them would be greater. As the membrane pores are of nanometers size, they exhibit larger surface area, and, hence, metal oxide nanoparticles are more efficient. Each metal ion has different active sites; for instance, zinc ions can bind to the –SH groups of proteins with a high affinity. The structured and closely spaced cell membranes become disorganized and confused, destroying their intrinsic function and causing bacterial death. Similarly, the extracted cations from metal oxide NPs accumulate in the cell wall creating pits in it which leads to change in permeability due to continuous release of lipopolysaccharides, membrane proteins, and intracellular factors. Furthermore, this process has been related to the bacterium's death due to the disruption of replication of adenosine triphosphate (ATP) and deoxyribonucleic acid (DNA) leading to its death (Wang et al. 2017; Padmavathy and Vijayaraghavan 2008; Lemire et al. 2013; Stankic et al. 2016).

(iii) *Protein and Enzyme Dysfunction*

Interference of NPs with bacterial protein synthesis has grabbed the attention of researchers in upcoming years; ultimately it has been proved as another mode of antimicrobial activity by metal oxide nanoparticles. The oxidation of side chains of amino acids gets catalyzed by metal ions resulting in formation of carbonyls bound to proteins, and this carboxylation levels serve as a marker for protein's oxidative damage. This ultimately results in loss of catalytic activity in enzymes leading to protein degradation (Wang et al. 2017; Vega-Jiménez et al. 2019).

(iv) *Signal Transduction Inhibition*

Signal transduction of bacteria is inhibited by metal oxide nanoparticles where its electrical properties interact with nucleic acid which suppresses cell division due to modification of replication of chromosomal DNA and plasmid in microorganisms. The vital component of signal transduction mechanism in bacteria is

phosphotyrosine which gets dephosphorylated by NPs and hampers signal transduction which ultimately inhibits bacterial growth (Vega-Jiménez et al. 2019).

(v) *Expression of Efflux Pump*

During antimicrobial mechanism, metal oxide nanoparticle carriers elevate the antibiotic serum levels and protect the drugs from detrimental reactions by target bacteria and thus maintain the potency of drugs acting as antimicrobial agent (Huh and Kwon 2011). Resistance to standard antibiotics is caused by increased efflux and decreased absorption of drugs in bacterial cells. However, there are studies which showed impact of NPs on this mechanism, inhibiting drug resistance (Wang et al. 2017).

(vi) *Biofilm Inhibition*

Formation of biofilms is an important mechanism in the development of bacterial resistance as they protect embedded microorganisms by escaping from antibiotic effect. Furthermore, bacterial biofilms serve as a "breeding habitat" for frequent resistance mutations, as well as the exchange and modification of these mutations among bacteria (Khameneh et al. 2016). Few studies revealed ZnO NPs and IONPs could prevent or inhibit biofilm formation due to their smaller size, higher surface-to-volume ratio, and morphology; for instance; rod-shaped NPs are more effective than spherical-shaped NPs (Slomberg et al. 2013). Carmen et al. revealed inhibition of biofilm development of *C. albicans* and *C. tropicalis* strains due to *R. officinalis* essential oil coated with Fe_3O_4 NPs to obtain nanobiosystem on the catheter surface analyzed using viable cell counts and confocal laser scanning microscopy (CLSM) examination (Chifiriuc et al. 2012). As compared to gold and silver nanoparticles, IONPs with different surface coatings show capacity to use external magnetic field and penetrate into biofilms inhibiting their development (Mahmoudi and Serpooshan 2012). ZnO NPs also act against biofilm formation by coating glass surfaces and producing ROS that interferes with biofilm formation of *E. coli* and *S. aureus* (Applerot et al. 2012).

10.6 Conclusion and Future Outlook

ZnO NPs and IONPs have unique physicochemical properties which contribute them to highlight their potentials as antimicrobial agent in various industries affected by MDR strains such as food, water, textiles, oil, and gas. Thus, because of their biocidal and immune-potentiating capabilities, ZnO NPs and IONPs are currently being explored as a possible alternative to antibiotics. Due to increased disorders caused by microorganisms which are resistant to antibiotics, it is imperative to have constant track on emerging antimicrobial nanoparticles for future development in medicine and healthcare. Understanding of mechanisms, biodistribution, and pharmacokinetics approach of nanoparticles to combat against microorganisms has become a key to find the potencies of antimicrobial nanomaterials. Even though in vitro methods showed multiple alternatives to achieve this goal, some of them

have limitations, and, therefore, it is essential to initiate certain guidelines and quality standards for research in nano-antimicrobials. Moreover, very few in vivo studies have been researched regarding nanoparticles as antimicrobial agents which should also be given a priority to understand the mode of action in depth.

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Chapter 11
Carbon Nanostructures for Fighting Antimicrobial Resistant Bacteria

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Abstract Various diseases have existed among the humans for a very long time. Humans, since ancient times, have devised different methods/medicines to prevent and cure various ailments from different diseases by bacteria. Nowadays due to the advancement in science and healthcare, various antibiotics have been developed to tackle these diseases and the disease-causing microbes. Various antimicrobials are coming at a rapid pace; they have brought new challenges to the human race. Many bacteria have developed resistance to the antimicrobials which have caused mortality in many individuals and also resulted in serious ailments like *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus*, *Enterobacteriaceae*, *Pseudomonas aeruginosa*, etc. These microbes have developed resistance to many medicines by various antimicrobial resistance mechanisms like biofilm formation, modifying the active agent of the medicine, etc. Antimicrobial resistance bacteria are a global concern, and to counter effect this issue, nanotechnology has been given consideration.

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Carbon-based nanostructures have proven to be effective in tackling antimicrobial resistance. Due to their various advantages like small size, modifiable properties by engineering methods, etc., they have been utilized widely as drug delivery vectors, therapeutics, etc. Moreover, the effectivity and cost-effectiveness of in vitro and in vivo studies have been proven. Thus, the introduction of nanotechnology has given a new perspective for tackling antimicrobial resistance.

Keywords Diseases · Healthcare · Nanotechnology · Antimicrobial resistance · Bacteria · Microbes · Carbon nanostructures

11.1 Introduction

Pharmaceutical firms and researchers are looking for novel antimicrobial medicines as a result of the rise of infectious illnesses caused by various pathogenic microorganisms. Humans are highly irritated and poisoned by several antibacterial agents (Varghese et al. 2013). The development of novel antimicrobial agents that are effective, resistant-free, low-cost, and of natural origin is of great importance (Miethke et al. 2021). Antibiotics work by restricting or eliminating microorganisms in a bacteriostatic or bactericidal manner (Kohanski et al. 2010). These medicines work by forming a connection with any important microbial metabolic components and by stopping pathogens from forming functioning biological molecules (Belkaid and Hand 2014). In the current situation, these microorganisms are acquiring resistance to antibiotics, due to which there is a decrease in their efficiency and increased chances of therapeutic failure (Tanwar et al. 2014). Inherent infections connected with MDR bacteria are intimately linked to the worrisome global rises in morbidity and death caused by medication resistance that arises via natural selection (Mocan et al. 2017). Patients are more likely to contract hospital-acquired bacterial infections, which can extend hospital stays and increase mortality rates (Cornejo-Juárez et al. 2015). It was found in the study conducted by Aliberti S. et al. that the patients that were infected by antimicrobial-resistant organisms were found with double hospital stays and an increase in death rates (Aliberti and Kaye 2013). MDR bacteria are a worldwide health concern as they increase the diseased people's morbidity and death rates and affect the clinical outcomes of a wide variety of people in intensive care units, having surgery, transplantation, or cancer therapy (Van Duin and Paterson 2016).

Nanomaterials (NMs) have piqued researchers' interest as a way to overcome the antimicrobial resistance pattern (Munir et al. 2020). These provide an excellent basis for updating the materials' physiochemical characteristics (Fig. 11.1), leading in much more potential antibacterial agents (Hajipour et al. 2012; Verma et al. 2021). Various researchers and scholars are increasingly doing the study on finding the solution for the problem of antimicrobial agents. Some examples of polymeric

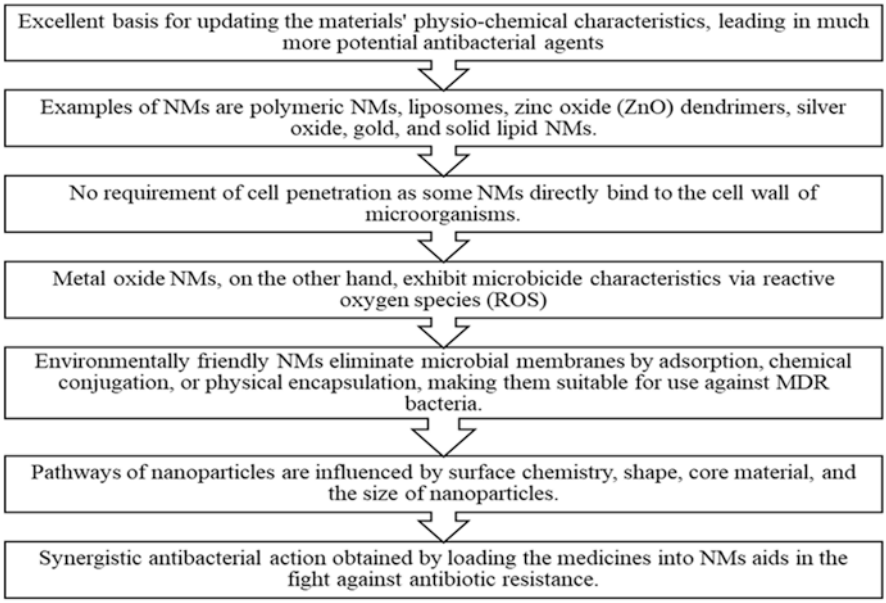

Fig. 11.1 Properties of nanoparticles

NMs are liposomes, zinc oxide (ZnO) dendrimers, silver oxide, gold, and solid lipid NMs (Munir et al. 2020). Some NMs directly bind to the cell wall of microorganisms, thereby eliminating the requirement for cell penetration. Metal oxide NMs, on the other hand, exhibit microbicide characteristics via reactive oxygen species (ROS) (Munir et al. 2020).

NMs have the potential to eliminate the microbial membranes by the process of adsorption, chemical conjugation, or physical encapsulation which makes NMs environmentally friendly for useful against MDR bacteria (Zhang et al. 2010). The pathways of nanoparticles are influenced by their surface chemistry, shape, core material, and size (Gupta et al. 2019; Verma et al. 2018, 2019). Furthermore, the synergistic antibacterial action obtained by loading the medicines into NMs aids in the fight against antibiotic resistance. With these factors, NM-based products play an important role in improving treatment accuracy by interfacing with bacteria‘s cellular system and serving as an antibiotic replacement (Munir et al. 2020). It is a new and promising way to deal with the use NMs in antibacterial treatment to defeat the bacterial obstruction arrangement (Fig. 11.1).

Table 11.1 Highlights the bacterial species causing harm in humans

Species	Damage	References
Salmonella spp., *Campylobacter* spp.	Expanded number of hospitalizations and expanded dreadfulness and mortality	Kaye et al. (2004)
Superbugs having super-resistance gene (NDM-1)	β-Lactam anti-microbials to be enzymatically corrupted render tiny organisms resistant to a wide range of anti-microbials	
MDR *Mycobacterium tuberculosis* (MDR-TB)	Impervious to current antibiotics	

11.2 Antimicrobial Resistant Bacteria: A Global Concern

MDR (Multidrug-resistant) microorganisms are the organisms which are resistant to more than one antibiotic (superbugs). A same microbe can receive varied medication obstruction properties from multiple living forms, leading to the formation of MDR "superbugs." They are posing a worldwide threat and danger to the whole population (Ssekatawa et al. 2020; Makabenta and Nabawy 2021; Munir et al. 2020; Willyard 2017).

Unreasonable utilization of antitoxins has drawn out for cure against MDR microscopic organisms and has been utilized as a prophylactic treatment for different diseases which are the driving reason for opposition (Laxminarayan et al. 2013). These microbes develop resistance to microorganisms by modulating the DNA, RNA and protein combination, biofilm arrangement and restraint of cell divider union to overpower the antimicrobial dangers. Bacteria also possess Mec-A quality that makes the bacteria resistant towards anti-infection agents such as penicillin or penicillin-like anti-microbials and methicillin. There are numerous other ways possessed by the microorganisms that make them impervious to the antimicrobials (Baptista et al. 2018).

For the past 20 years, new kinds of antitoxins are declining at a rapid phase rendering no alternative to treat MDR microorganisms. This has led to crisis circumstances and colossal financial effect. The cases of methicillin-resistant *Staphylococcus aureus* (MRSA) infections have decreased in the United States, Europe, Canada, and South Africa in recent years, whereas MRSA infections have increased in Sub-Saharan Africa, Australia, Latin America (90%), and India (47%) (Chaudhary 2016) (Tables 11.1 and 11.2).

11.3 Antimicrobial Resistance Mechanism

Classification of antimicrobial agents can be done on the basis of antimicrobial activity mechanism. Prominent groups are substance that hinders the synthesis of cell wall, causes depolarization of cell membrane, hinders synthesis of protein, hinders synthesis of genetic material, and hinders pathways adapted by bacteria for

Table 11.2 Indicates the specific bacteria resistant to antimicrobial agents

Infectious agents	Developed antibiotic resistance against	References
Vancomycin-resistant MRSA clinical isolates vanA, vanB, or vanC	Resistant to vancomycin, teicoplanin cross-protection	Begum et al. (2020), Gupta et al. (2019), and Chaudhary (2016)
Staphylococcus aureus strains	Methicillin resistant and resiatant to lactam antibiotics Protection from penicillin	
Enterococcus, *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and *Acinetobacter*	Enhanced antibiotic resistance	
Gram-negative bacteria, Enterobacteriaceae Escherichia coli (CREE. coli)	Carbapenem-resistant bacteria which are capable of restricting antibiotic penetration into the outer membrane Third-generation cephalosporins, ESBLs, and fluoroquinolones	
Gram-positive bacteria, such as *Klebsiella pneumoniae* and *Neisseria gonorrhoeae*	Develop resistance against extended-spectrum beta-lactamases (ESBL) Carbapenems, vancomycin, and third-generation cephalosporins	
Staphylococcus pneumoniae	Penicillin-resistant or non-susceptible (or both)	
Non-typhoidal *salmonella* (NTS) and *Shigella* species	Fluoroquinolone-resistant	
Campylobacter spp.	Quinolones, macrolides, and lincosamides, chloramphenicol, aminoglycosides, tetracycline, ampicillin and other -lactams, cotrimoxazole, and tylosin	
Salmonella	Antibiotic medications, sulfonamides, streptomycin, kanamycin, chloramphenicol, and a portion of the β-lactam anti-microbials (penicillins and cephalosporins)	
Enterococcus faecalis	Penicillin-resistant	

metabolism (Reygaert 2018). Such wide range of mechanisms has given us chance to get better control over the microorganisms, but their improper management has caused resistance issue. Responsible factors are overconsumption of antimicrobial drugs and wrong prescription of antimicrobial therapy (Von Baum and Marre 2005) (Table 11.3).

Categories of resistance mechanism are inactivation or changes in drug, modification of the active site or receptor, alteration in permeability of cell which leads to decrease in drug deposition within cell, and biofilm formation (Santajit and Indrawattana 2016; Reygaert 2018) (Fig. 11.2).

Table 11.3 Mechanisms of antimicrobial class

Mechanism of action	Antimicrobial class
Hinder synthesis of cell wall	β-Lactams Glycopeptides
Depolarization of cell membrane	Lipopeptides
Hinder synthesis of protein	Aminoglycosides, Tetracycline } Binds with 30S ribosomal subunit Chloramphenicol, Lincosamides, Macrolides } Binds with 50S ribosomal subunit
Hinder genetic material synthesis	Quinolones
Hinder Pathways of metabolism	Sulfonamides Trimethoprim

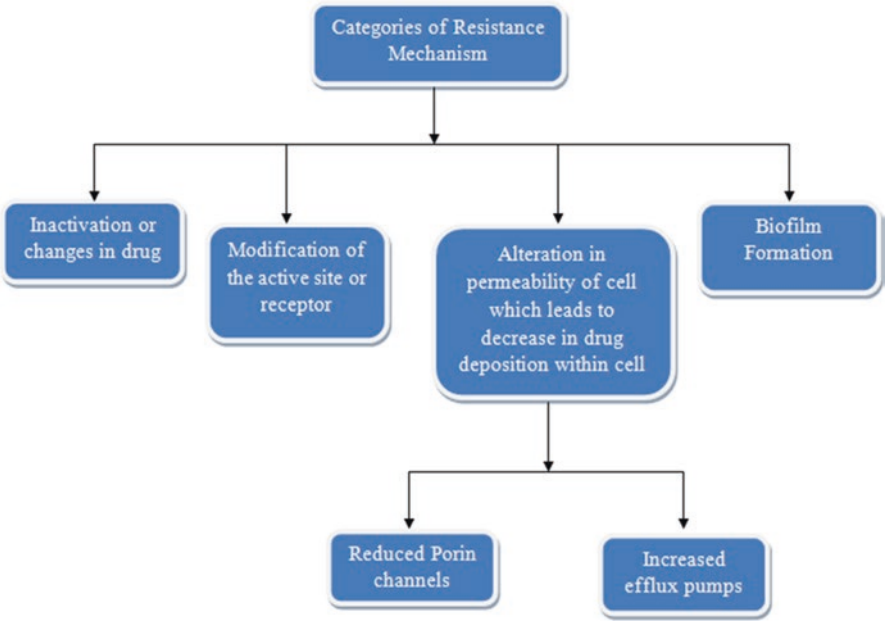

Fig. 11.2 Various categories of resistance mechanism of microbes

11.4 Inactivation or Changes in Drug

Enzymes are produced by bacteria which have the ability to permanently change and deactivate the antibiotics such as β-lactamases, aminoglycoside-modifying enzymes, or chloramphenicol acetyltransferase (Santajit and Indrawattana 2016). Basically there are two pathways for the deactivation of a drug, firstly by the

debasement of the drug and secondly by the shift in the functional group of the drug (Reygaert 2018).

β-lactamases enzymes include penicillinase, cephalosporinase, broad-spectrum β-lactamases, carbapenemases, etc. which hydrolyze the β-lactam ring that is essential for the activity of penicillin, cephalosporin, carbapenems, etc. resulting into their deactivation (Santajit and Indrawattana 2016). Tetracycline is another class of drug which can be deactivated by hydrolyzation.

Most common functional groups that are used for deactivation of drug are acetyl, phosphoryl, and adenyl groups. Most commonly used process is acetylation, i.e., transfer of acetyl group. This is used against aminoglycosides, chloramphenicol, streptogramins, and quinolones. Additionally, phosphorylation and adenylation are also implied against aminoglycoside.

11.5 Modification of the Active Site or the Receptor

There are many target sites in the bacteria where the antimicrobial drug can bind. Similarly, all these sites can be modified to achieve resistance against the drug. This is achieved by gene mutation (Santajit and Indrawattana 2016). For example, β-lactam drugs are mostly used against gram-positive bacteria, and they can achieve resistance by changing the chemical structure of the β-lactam (e.g., PBP2a site in *Staphylococcus aureus* through addition of mecA gene) or by changing the number of PBPs (penicillin-binding proteins). Increase in number of PBPS causes decrement in binding of the drug, whereas decrease in PBPs results in normal drug binding (Reygaert 2018).

11.6 Alteration in Permeability of Cell Which Results in Reduced Deposition of Drug Within Cell

Equilibrium should be maintained between intake and excretion of antibiotic to understand the sensitivity of bacteria to a drug. Decrease in passage of the drug through bacterial cell membrane results in antimicrobial resistance. Mechanisms involved in this are reduced porin channels on the cell membrane or increased efflux pumps (Santajit and Indrawattana 2016).

(i) *Reduced Porin Channels*

Proteins that are present on the cell membrane of the gram-negative bacteria are called porins. These porins serves as channel for the movement of many lipophobic substances such as antimicrobial agents. Decrement in the amount of *P. aeruginosa* porin protein channel OprD leads to reduced entry of drug making bacteria resistance to imipenem drug.

K. pneumonia strains also achieve resistance to β-lactams by loss of porins called OmpK35 and OmpK36 simultaneously along with the generation of certain β-lactamases enzymes.

(ii) *Increased Efflux Pumps*

Bacterial cells have encoded genes for efflux pumps. They are functional either integrally or induced under certain external stimulus. Multidrug efflux pumps exchange high variables of compounds. Efflux pumps have 5 families: ABC family (ATP-binding cascade), MATE family (multidrug and toxic compound extrusion), SMR family (small multidrug resistance), MFS family (major facilitator super family), and RND family (resistance-nodulation-cell division family).

RND works in association with membrane fusion protein and OMP porin to extrude substance throughout the cell membrane. MacB (ABC member) and EmrB (MFS member) act as tripartite pumps and cause efflux of macrolide drugs and nalidixic acid, respectively, resulting into resistance against them in bacteria.

Basically, increased amount of efflux pumps leads to elevated extrusion of the drugs from the cell leading from lesser interaction between drug and receptor causing resistance against antimicrobial agent (Reygaert 2018).

11.7 Biofilm Formation

Biofilms are network-like formation of microbial population as a layer on the extracellular polymeric substances formed by the biofilm on their own. Microbes present in the biofilm can interact with each other as well as with the surrounding. Extracellular matrix consists of polysaccharides, proteins, lipids, and extracellular microbial DNA. Three steps for biofilm generation are adhesion, growth and maturation, and detachment, which can be active or passive.

Biofilm serves as a mechanical and biochemical protection layer which gives a condition required for the activity of a drug. Hence, when the required condition is not achieved, antibiotic cannot enter the bacteria resulting into resistance (Santajit and Indrawattana 2016) (Table 11.4).

11.8 Carbon Nanotubes and Its Antimicrobial Properties

A hollow tube-like structure, having carbon as a structural unit and a diameter less than 1 nm to 50 nm, is known as carbon nanotube. It has a peculiar mixture of rigidity, toughness, and persistence (Mohapatra 1959; Dizaj et al. 2015). CNTs are cheaper and are more potent than the conventional medicine system. For example, transport of amphotericin B to the target site using covalently bonded CNT is inexpensive than the utilization of traditional liposomal amphotericin B (Mocan et al.

Table 11.4 Indicating the various mechanisms of antimicrobial resistance in microbes

Microbe/bacteria	Resistance mechanism	Antimicrobial agent	Reference
Gram-positive bacteria	Enzyme hydrolyzation of β-lactam ring	β-lactam	Reygaert (2018)
Staphylococcus aureus	Enzyme hydrolyzation of β-lactam nucleus	β-lactam	Lowy and Lowy (2003)
	Reduced affinity for PBP	Vancomycin (glycopeptide)	
	Affinity of enzymeDNA complex is reduced by causing mutations in QRDR region	Fluoroquinolone (quinolones)	
	Acetylation or phosphorylation of the drug	TMP-SMZ	
Campylobacter	C257T alteration in the gyrA gene resulting into Thr86Ile substitution in gyrase enzyme	Quinolones	Wieczorek and Osek (2013)
	Alteration in tet(O) gene which is responsible for the formation of ribosomal protection protein	Tetracyclines	
	Changes in 23S rRNA causes mutation in ribosomal target binding site or efflux pump (ABC family)	Azithromycin (macrolide)	
P. aeruginosa, *A. baumannii*, and *K. pneumonia*	Biofilm formation or gene mutation	Penicillin, cephalosporins (β-lactam)	Santajit and Indrawattana (2016)
Mycobacterium tuberculosis	Mutation in rpoB and gyrA genes	Rifampin	Gillespie (2002)
	Mutation in katG, inhA gene	Isoniazid	Dookie et al. (2018)
E. coli	Multidrug efflux systems (RND family) and change in AcrAB-tolC and NorM encoding for porins	Fluoroquinolone	Poole and Poole (2009) and Poirel et al. (2018)
	Alteration in Mef (MF family) or Msr (A) (ABC family)	Macrolides	
	Mutation in Tet gene	Tetracycline	

2017). They have drug transporting property in an effective manner (Azizi-lalabadi et al. 2020).

On the basis of structural layer of nanotubes, they are classified into three forms:

1. SWCNTs – Single-walled carbon nanotubes
2. DWCNTs – Double-walled carbon nanotubes

3. MWCNTs – Multi-walled carbon nanotubes (Azizi-lalabadi et al. 2020; Mohapatra 1959)

These nanotubes have one, two, or multi-layers of carbon cylinders, respectively. The antimicrobial activity of each form varies depending on their shape and surface area. SWNTs can be chair-like, snaky, and chiral dependent. MWNTs are constructed by combining some SWNTs of varying diameter (Mohapatra 1959).

11.9 Synthesis of CNTs

There are three techniques used for the production of carbon nanotubes: arc discharge, laser ablation, and chemical vapor deposition (CVP). In CVP, gases having carbon as composition are decomposed on the catalyst at the temperature less than 1000 °C, whereas arc discharge and laser ablation techniques are dependent condensation process. Solid carbon materials are heated (3000–4000 °C) to vaporize which generates carbon atoms which later gets condensed to form CNTs. Arc discharge technique is utilized for the formation of high-quality MWNTs and SWNTs (Taylor and Shenderova 2012). In this process, CNTs are entrapped along with helium gas in the middle of cathode and anode placed very close to each other. Then DC current is allowed to pass resulting in generation of heat that vaporizes the area of tube and generates small tubes (Azizi-lalabadi et al. 2020) (Table 11.5).

SWCNTs and MWCNTs have effective antagonistic effects for microorganisms irrespective of acute exposure. This explains that CNTs have therapeutic effect. As a toxicity parameter, SWNTs have more toxic effect than MWCNTs towards bacteria.

CNT toxicity level depends on its breadth, area, configuration, surface chemical group, number of coating, etc. The shorter the length of the tube, the higher antibacterial effect it will have as it interacts with microorganism by their open ends leading to additional plasma membrane injury. At solid surface, longer CNTs have less effect than the shorter CNTs. According to research, when MWCNTs extend up to 50 micrometer, the CNT wraps itself across the microorganism and causes osmotic breakdown of it (Al-jumaili et al. 2017).

Table 11.5 Discusses the types of carbon nanotubes and their effects on the various species

Carbon nanotubes	Target organism	Mechanistic action	References
SWCNT	*E.coli*, *S. aureus*	Adhere to bacterial cell wall, cause osmolytic stress on it, efflux of material of cytoplasm	Azizi-lalabadi et al. (2020) and Al-jumaili et al. (2017)
	B. subtilis	Damage to membrane, escape of cellular material, reduced volume of cell, elevated roughness of bacterial surface	
	S. epidermis	Loss of viability of cell causing its deactivation	Al-jumaili et al. (2017)
	S. typhimurium	Aggregation of cells in the form of needles	
	Gram-positive and gram-negative bacteria	Generation of reactive oxygen species	Mocan et al. (2017)
SWCNT-Ag	*E.coli*, *S. aureus*	Interactivity of SWCNTs-Ag with cells, alteration in structure of cell	Azizi-lalabadi et al. (2020) and Al-jumaili et al. (2017)
	Salmonella typhimurium	Mutation of genes responsible for metabolism and integrity of cell membrane	
MWCNT	*E.coli*, *S. aureus*	Adhere to bacterial cell, biofilm	Azizi-lalabadi et al. (2020) and Al-jumaili et al. (2017)
SWCNT and MWCNT	*Lactobacillus acidophilus*, *Bifido7bacterium adolescentis*	Diameter-dependent piercing, length-dependent wrapping	Mocan et al. (2017)
MWCNT-Ag	*E.coli*, *S. aureus*	Adsorption on the bacterial cell wall by producing electrical charges leading to loss of integrity of cell	Azizi-lalabadi et al. (2020) and Al-jumaili et al. (2017)
MWCNT-lysine	Gram-positive and gram-negative bacteria	Positive charge appears on cell membrane because of lysine causing adsorption	Mocan et al. (2017)

11.10 Antimicrobial Properties of CNT and CNT Composites

Antimicrobial property of CNT is because of damage caused to the bacterial membrane when CNT comes in direct contact to it. SWCNTS have powerful antimicrobial mechanism towards *Escherichia coli* as it causes intense membrane damage to bacteria and causes cell death. Decrease in CNT size increases surface to volume

ratio, leading to tight bonding between cell membrane of the bacteria (Azizi-lalabadi et al. 2020). Direct attachment of CNT with the plasma membrane influences its cohesion, breakdown process, and structure of *E. coli*. SWCNTs could penetrate the cell wall at greater degree compared to MWCNTs (Dizaj et al. 2015).

SWCNTs having functional groups –OH and –COOH show better antimicrobial activity towards Gram +ve and Gram –ve bacteria, whereas MWCNTs with –OH and –COOH show negligible antimicrobial activity. Longer SWCNTs cause bacterial cells to aggregate and cause stress on cell wall and also inhibit DNA reproduction (Azizi-lalabadi et al. 2020; Dizaj et al. 2015).

The charges present at the surface of the CNTs are also responsible for the bactericidal effect because it causes oxidative stress in microorganisms leading to interruption in its growth. Diameter is another factor which counts for the antimicrobial activity. CNT with small diameter is more effective as it acts as needle which sticks its one point to the microorganism and coming out to the other end. With larger diameters, CNTs connect to bacteria through side walls. This factor also causes disruption of cell wall as well as DNA and RNA production (Azizi-lalabadi et al. 2020).

CNT composites are formed by the combination of carbon nanomaterial along with biological polymers and nanoparticles like oxides of copper, zinc, titanium, elemental silver, etc. CNM has synergetic behavior with NP like carbon nanotubes-chitosan, carbon nanotubes-Ag, etc. (Table 11.6).

11.11 Conclusions and Future Aspects

Microbes have always been around us; they perform variety of activities and one of them antimicrobial resistance, that is, a global concern. This property has caused many treatments to go ineffective, thus contributing to increases in mortality rates of patients. Carbon nanotubes have revolutionized the scenario of antimicrobial resistance and are giving a new hope to prevent disease and deaths of humans due to this area of concern. The carbon nanotubes have reshaped the antimicrobial issues and have inhibited the growth of various microbes due to their various qualities by formation of ROS, chemical conjugation, high absorption rate, and retarding the respiration functions of microbes, in turn destroying them.

Table 11.6 Briefs about the two main types of carbon nanotubes with their properties and antimicrobial activities

Type of carbon nanotube	Properties	Derivatives	Method of synthesis	Uses	Target species	Antimicrobial effect and mechanism of action	Reference
Fullerene	C60 structure with 20 hexagonal and 12 pentagons with carbon atoms having one π & two σ bonds. Other features include low solubility	Organophosphorus compounds, diphosphates, and phosphonates	Arc discharge method, laser ablation, polyaromatic hydrocarbons irradiated with lasers, carbon laser vaporization	As photovoltaics, antioxidants, biopharmaceuticals, gas storage, for water purification and as catalysts	*E. coli*, *Salmonella* species, *Streptococcus* species, *Pseudomonas putida*, *S. aureus*	Inhibits bacterial growth and metabolism by impairing oxygen uptake by increasing the cyclopropane fatty acids in bacterial cell walls or by reducing the fatty acids in bacterial cell walls causing its destruction	Azizi-lalabadi et al. (2020) and Dizaj et al. (2015)
Graphene	Occurs naturally, 2D structure, crystalline material, that has a high surface to volume ratio with low specific gravity	Expandable graphite (EPG), exfoliated graphite (EFG)	Thermal baking, photoreduction, microwave-assisted reduction and CVD (chemical vapor deposition method)	Drug and gene delivery, cancer remedy, bio-imaging, tissue cell culture procedures	*E. coli*, *Salmonella typhimurium*, *B. subtilis*, *Enterococcus faecalis*	Cause destruction of cell wall and cell membrane by producing ROS by physical destruction and chemical oxidation	Dizaj et al. (2015)

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Chapter 12
Nanoformulations Against Multidrug-Resistant Members of ESKAPE Pathogens

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Abstract The rise and spread of antimicrobial resistance (AMR) and drug-resistant nosocomial infections have become a significant global threat for human health and well-being. Injudicious and persistent antibiotic usages have resulted in the creation of drug-resistant microorganisms. Multidrug-resistant (MDR) ESKAPE pathogens consisting of *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp. have been reported to raise the mortality and the expense of long-term therapy, significantly. With the drying pipeline of novel-efficient drugs declining, urgent need for novel therapies is required. Nanotechnology is a rapidly growing field of research with tremendous applications in medicine owing to their tiny size and extensive surface area. Recent reports on nanoformulations against MDR ESKAPE pathogens have revealed their enhanced therapeutic efficiency, bioavailability, target specificity, and antimicrobial activity confirming their potential role in nanoformulation strategies to combat ESKAPE pathogens. In this chapter, we discuss about the evolution of the resistance mechanisms in ESKAPE pathogens and how these pathogens are posing a serious threat for human health and environment. The chapter further discusses on the potential exploration of nanoformulations as emerging combating tool against ESKAPE with their drug delivery applications to these drug

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resistance determinants. Finally, we discuss about the various challenges faced for implementing ESKAPE nanoformulations in clinical settings.

Keywords Antimicrobial resistance · Drug resistance determinants · Biofilm · ESKAPE · Multidrug resistance · Nanoformulations

12.1 Introduction

In 1928, the miraculous medication penicillin ushered in the age of infections and had a huge impact on contemporary medicine since then. Injudicious antibiotic usage and continuous infection exposures have resulted in the overall rise of multidrug resistance (MDR) bacteria in nosocomial-related areas/regions. Recent reports on hospital-acquired infections (HAI) have identified ESKAPE pathogens as one of the major microorganisms resisting in these areas (Avershina et al. 2021). The ESKAPE microorganisms comprise of six major drug-resistant pathogens, i.e., ***E****nterococcus faecium*, ***S****taphylococcus aureus*, ***K****lebsiella pneumoniae*, ***A****cinetobacter baumannii*, ***P****seudomonas aeruginosa*, and ***E****nterobacter* spp. making them a major group of microorganisms involved in life-threatening nosocomial infections (Santajit and Indrawattana 2016). In 2017, the World Health Organization (WHO) produced a list of pathogens causing MDR infections for which new antimicrobials/antibiotics are urgently required to concentrate and steer research and developments (De Oliveira et al. 2020) enlisting ESKAPE pathogens as a critical priority 1 pathogens revealing their looming threat to humanity in upcoming years (Asokan et al. 2019). Similar reports were reviewed by the Department of Biotechnology (DBT) in India with *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa* as critical priority pathogens, *Staphylococcus aureus* and *Enterobacter* spp. as high-priority pathogens, while others in medium-priority pathogens list (DBT 2021).

Antimicrobial resistance (AMR) or antibiotic resistance (ABR) has become a global health threat where these microorganisms acquire new resistance mechanisms becoming "superbugs" and causing non-treatable MDR infections (Morrison and Zembower 2020). The rise of MDR bacteria has coincided with the drying up of the antibiotic research pipeline. To overcome these AMR situations, several attempts to find effective and innovative antibacterial drugs have been made in recent years. However, delivering powerful antimicrobial drugs in a safe and efficacious manner has proven to be a huge challenge. The use of nanotechnology has emerged as a proven and efficient tool for eradicating MDR and AMR. Recent advancements in the nanoformulations of drugs and other antimicrobials for targeting MDR ESKAPE pathogens have been proven to be advantageous concerning bioavailability, cost-effectiveness, efficiency, target specificity, and antimicrobial activity (Mba and Nweze 2021) that target antimicrobial resistance determinants such as biofilms, efflux pumps, cell membrane, and other enzyme production

mechanisms (Peterson and Kaur 2018). In this chapter, we highlight the evolution of ESKAPE pathogens and their resistance mechanisms in the environment. Nanoformulation is the newer technology to combat ESKAPE; further, we discuss various nanoformulation-based drug delivery to drug resistance determinants. At last, we discuss the challenges in implementing these nanoformulations in clinical trials and clinical settings.

12.2 ESKAPE Pathogens and Evolution of Their Resistance Mechanisms

The abbreviation "ESKAPE" refers to a collection of life-threatening nosocomial pathogens, viz., ***E****nterococcus faecium*, ***S****taphylococcus aureus*, ***K****lebsiella pneumoniae*, ***A****cinetobacter baumannii*, ***P****seudomonas aeruginosa*, and ***E****nterobacter* spp. (Pandey et al. 2021). The development of various antibiotics against these pathogens has led to the development of different resistance mechanisms for them to escape the antibiotics and survive in the environment. The development and marketing of new antibiotics and antimicrobials have slowed down since the 1990s (Conly and Johnston 2005). In the twentieth century, discoveries of antibiotic combinations such as imipenem/cilastatin/relebactam (Mansour et al. 2021), niclosamide-tobramycin (Berry et al. 2021), meropenem-vaborbactam (Patel et al. 2018), imipenem-relebactam (Zhanel et al. 2018), eravacycline-colistin (Ozger et al. 2019), and other combinations enhanced the targeting of pathogens.

12.2.1 Vancomycin-Resistant Enterococcus faecium *(VRE*fm*)*

In the 1980s, *E. faecium* and *E. faecalis*, well-known for gut commensal bacteria, became a prominent source of MDR hospital-acquired illness (Lebreton et al. 2013). *E. faecium* therapeutic importance stems from its inherent poor sensitivity to a wide range of antimicrobial drugs, including third-generation cephalosporins, vancomycin, ampicillin, and other antibiotics (Table 12.1) (Kolář 2018). Antibiotic exposure usually precedes VRE*fm* entry into the bloodstream of hospitalized patients, allowing VRE*fm* to become the dominant species in the gastrointestinal tract (De Oliveira et al. 2020; Carvalhaes et al. 2021). Other strains such as glycopeptide-resistant *Enterococcus faecium* ST80 and ST117 are also found to be residing in a healthcare facility (Rodríguez-Lucas et al. 2021). Apart from various antibiotics, small RNAs present in VRE*fm* are recently been found to be involved in daptomycin resistance (Sinel et al. 2017). Exposure to multiple drugs, VRE*fm* has developed various resistance mechanisms to survive the drug exposures in nosocomial areas. Virulence factors such as *asa1*, *gel E*, *cylA*, *esp*, *hyl*, and *Van*; resistance genetic determinants such as *vanA*, *vanB*, *vanM*, *vanN*, and *vanD*; and *D1*, *D2*, *D3*, *D4*, and *D5* resistant

Table 12.1 Clinical manifestation and drug resistance determinants of ESKAPE pathogens

ESKAPE pathogens	Antibiotic resistance	Clinical manifestations	Drug resistance determinants					References
			Cell membrane	Efflux pumps	Biofilm formation	Quorum sensing	Other mechanisms	
Enterococcus faecium	Vancomycin, ampicillin, linezolid, teicoplanin, piperacillin, cephalosporins	Bacteraemia, infective endocarditis, intra-abdominal and pelvic infections, urinary tract infections, central nervous system infections, skin and skin structure infections	Phosphatidylglycerol (PG), cardiolipin, lysyl-phosphatidylglycerol (LPG), and glycerolphospho-diglycodiacylglycerol (GP-DGDAG) rearrangement	EfrAB, EfmA	Hemolytic exotoxin (encoded by *cyl*), gelatinase (encoded by *gelE*), serine protease (encoded by *sprE*), hyaluronidase (encoded by *hyl*), endocarditis- and biofilm-associated pili genetic locus (*ebp*ABC), sortase-encoding gene (*srt*), pili (*pil*), aggregation substance (*agg* and *asa1*), collagen-binding protein (*ace*), enterococcal surface protein (*esp*), enterococcal endocarditis antigen (efaAfm for *E. faecium*)	Fecal streptococci regulator (*fsrA, fsrB, fsrC*)	Transposon genes (*inf*Tn916, tndX), plasmid rep genes, mutations in GyrA, ParC	Nishioka et al. (2009), Mishra et al. (2012), Lavilla Lerma et al. (2014), Miller et al. (2014), Sadowy and Luczkiewicz (2014), O'Driscoll and Crank 2015, Stępień-Pyśniak et al. (2019)

Staphylococcus aureus	Penicillin, methicillin, oxacillin, vancomycin, daptomycin, tetracyclines, linezolid	Vascular catheter-related infections, skin and soft tissue infections (SSTIs), pleuropulmonary infections, osteoarticular infections, infective endocarditis, impetigo, osteomyelitis, septic arthritis	Lower cell permeability led to defect in energy metabolism resulting in reduced drug intake	QacA, NorA, Nor B, NorC, MepA, MdeA, QacA/B, QacG, QacH, QacJ, LmrS, and Smr	crtN, hemolysin genes (*hld, hlb, hla*), *cap*8H, *tst, sea, eta, etb*, *cap*5H, *seb, see, sec*, and *sed*	Accessory gene regulator (Agr) system, *LuxS*	Transposon Tn552 or Tn552-like elements, e SCC mec elements, mecA gene, mutation in e multiple peptide resistance factor (mrpF) gene	Costa et al. (2013), Le and Otto (2015), Tong et al. (2015), Foster (2017), Derakhshan et al. (2021)
Klebsiella pneumoniae	Carbapenem, cephalosporin, piperacillin-tazobactam, ciprofloxacin, levofloxacin, and amikacin	Pneumonia, pyogenic liver Abscess, urinary tract, respiratory tract, lung, wound sites and bloodstream infections	Outer membrane protein A (ompA), lipid A modification	AcrAB, tolC, mdtK, OmpK35, OmpK36	Capsular polysaccharides, type 1 and type 3 fimbriae, virulence-associated genes (*mrkD, fimH-1, entB, iutA, ybtS*)	*LuxS*, autoinducers of signal system I (AI-1)	Extended-spectrum β-lactamase (ESBLs) and carbapenemase enzyme	Balestrino et al. (2005), Chung (2016), Ferreira et al. (2019), Nirwati et al. (2019)
Acinetobacter baumannii	Piperacillin, ceftazidime, amikacin, tetracycline, ampicillin-sulbactam, meropenem, ciprofloxacin, imipenem, and gentamicin	Ventilator-associated pneumonia, central line-associated bloodstream infection, catheter-associated urinary tract infection, surgical site infection	OmpA, K1 capsule, CarO, modification of LPS	AdeABC, AdeFGH, AdeIJK, AbeM, MacAB-TolC, EmrAB-TolC, A1S_1535, A1S_2795, and ABAYE_0913	Fimbriae, *plc*N and *las*B genes, *bap* gene	Auto inducers (AHLs),	β-Lactamase (TEM, SHV, GES, CTX-M, SCO, PER, VEB, KPC, OXAs, IMP, VIM, CARB, and AmpC), insertion sequence element (ISAba1-like sequence)	Kanafani and Kanj (2016), Lee et al. (2017), Aliramezani et al. (2019), Basatian-Tashkan et al. (2020)

(continued)

Table 12.1 (continued)

ESKAPE pathogens	Antibiotic resistance	Clinical manifestations	Drug resistance determinants					References
			Cell membrane	Efflux pumps	Biofilm formation	Quorum sensing	Other mechanisms	
Pseudomonas aeruginosa	Aminoglycosides, quinolones and β-lactams	Central venous catheter infections, pneumonia, soft tissue infections, urinary tract infections, and sinusitis	Bacterial cell wall biosynthesis blockage, OprF, OprB, OprD, OprE, OprO, OprP, OprC, OprH, OprM, OprN, and OprJ	MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM,	GacS/GacA system, extracellular DNA (eDNA)	LasI-LasR, RhlI-RhlR, and PQS-MvfR QS system	Extended-spectrum-β-lactamases (ESBLs); gene mutations of transcriptional regulators, *mexR, nalB, nalC,* or *nalD*, DNA gyrase (gyrA and gyrB); topoisomerase IV (parC and parE)	Dropulic et al. (1995), Pang et al. (2019)
Enterobacter spp.	Carbapenem, chloramphenicol, tetracycline, tigecycline, fluoroquinolones, trimethoprim	Endophthalmitis, brain abscess, meningitis, spondylodiscitis, endocarditis, urinary tract infections (UTIs)	Alteration of outer membrane (OM) permeability, lipopolysaccharide (LPS) modifications, porins (Omp35, Omp36, Omp37, LamB and PhoE)	AcrAB-tolC, AcrZ-AcrAB-TolC	Curli fimbriae, virulence-encoding (ter and sea) and resistance-encoding (blaCTX-M-9, qnrA1, aadB, aadA2, sukK, and sat) genes	Type 6 secretion system (T6SS2), C4 and C6-HSLs quorum sensing molecules, AHL signaling molecules	Mutations in phoQ, ampR, and pmrB, deletion of mcr-1 and mcr-2	Davin-Regli et al. (2019), Lazar et al. (2021)

to vancomycin and teicoplanin have been discovered in VRE*fm*-associated infections (Kiruthiga et al. 2020) (Ahmed and Baptiste 2018). Recent reports on the spread of VRE*fm* strain ST133 into the aquatic environment have been reported with vancomycin resistance-conferring *van*A gene cluster on transposon Tn*1546* (Biggel et al. 2021).

12.2.2 Methicillin-Resistant Staphylococcus aureus *(MRSA)*

The introduction and overuse of penicillin in the nineteenth century accelerated the emergence and spread of penicillinase-producing methicillin-resistant *Staphylococcus aureus* (MRSA). However, the first report of MRSA with reduced susceptibility to drug vancomycin came from Thailand (Trakulsomboon et al. 2001). Reports have confirmed the resistance of MRSA organisms to trimethoprim, ß-lactamase, chloramphenicol, tetracycline, and aminoglycosides (De Oliveira et al. 2020). Current economic considerations have steered biopharmaceutical firms away from new antibiotic research and approvals, leaving drug-resistant *S. aureus*-infected patients with little choice (Fukunaga et al. 2016). With a tendency to colonize and form biofilms, certain strains of MRSA have contributed to the spread of hospital-acquired MRSA (HA-MRSA) (Turner et al. 2019). However, the growing prevalence of community-acquired MRSA (CA-MRSA) has significantly become the major risk factor for their colonization in India (Mehta et al. 2020). MRSA has developed numerous resistant mechanisms to thrive in the environment. They express virulence factors such as hemolysin and leukocidin toxins and capsule and protein A immune-evasive surface factors as the line of defense (Turner et al. 2019). Apart from virulence factors, mobile genetic elements (MGEs) such as *blaZ*, *dfrA*, *dfrK*, *ermC*, *tetK*, and *tetL* have been identified to play a major role in providing resistance to penicillin, trimethoprim, erythromycin, clindamycin, and tetracycline antibiotics, respectively (Turner et al. 2019). The continuous exposure of bacteria to antibiotics has led to genetic changes and the production of other resistant strains such as vancomycin-resistant *S. aureus* (McGuinness et al. 2017).

12.2.3 Klebsiella pneumoniae

Klebsiella pneumoniae, gram-negative and clinically significant microorganisms, has sparked widespread public concern becoming a major albatross around the infection control professionals with majorly causing urinary tract infections (UTIs), pneumonia, surgical wound infections, cystitis, endocarditis, and septicemia (Effah et al. 2020). Third generation Cephalosporins (beta-lactam antimicrobials) and carbapenems are used for treating severe infections caused by Klebsiella pneumonia (Karaiskos et al. 2019). For the past few years, drug resistance rates of *K. pneumoniae* strains obtained from hospitals and other healthcare systems have increased dramatically leading to the emergence of extensively drug-resistant (XDR) *K.*

Table 12.2 Nanoformulations against major drug resistance determinants with their antimicrobial agents

Nanoformulation	Antimicrobial agent included in nanoformulation	Bacterial pathogens	Nanoformulation concentration	Targeted drug resistance determinant	Advantages provided by nanoformulation	References
Lipid-polymer hybrid nanoparticle loading the antibiotic linezolid (LIN-LPN)	Linezolid antibiotics	Methicillin-resistant *Staphylococcus aureus* (MRSA)	2–8 μg/ml	Bacterial biofilm	High linezolid payload (12% by weight of nanoparticles) and controlled released characteristic of antibiotics with MRSA biofilm growth Suppressed by 35–60% of the values achieved with free linezolid	Guo et al. (2020)
PEG-PLGA NPs that synergistically carried benzamide and rutin (RB-PEG-PLGA NPs)	Rutin and benzamide	MDR bacterial strains (*S. aureus* (MTCC 96) and *P. aeruginosa* (MTCC 2488))	180 μg/ml (for *S. aureus*) and 160 μg/ml (for *P. aeruginosa*)	Disruption of bacterial membrane and biofilm surface	Controlled release of antibacterial agents and 2 times lowered MIC as compared to free drugs with no toxicity against tested using human erythrocytes and human cell lines	Deepika et al. (2020)
Solid lipid nanoparticles of silver sulfadiazine (SSD-SLNs) laden chitosan gel supplemented with DNase-I	Silver sulfadiazine	*Pseudomonas aeruginosa* PA01 (ATCC 15692)	18.75 μg/mL	Bacterial biofilm	Improved cell viability of SSD-SLNs (90.3 ± 3.8%) as compared to SSD alone (76.9 ± 4.2%) and controlled release (83%) for up to 24 h further, the combination of SSD SLNs with DNase-I, inhibited around 96.8% of biofilm of *P. aeruginosa* as compared to SSD with DNase-I (82.9%), complete wound healing by SSD-SLN with DNase-I as compared to SSD and SSD-SLNs after 21 days	Patel et al. (2019)

Imipenem-loaded poly Ɛ-c aprolactone (PCL) nanoformulation (IMP/PCL)	Imipenem antibiotics	Imipenem-resistant *P. aeruginosa* and *Klebsiella pneumonia* clinical isolates	0.6–20 µg/mL	Bacterial biofilm	Faster microbial killing with 2–3 hours by (IMP/PCL), compared to the imipenem-loaded polylactide-co-glycoside (PLGA) and free drug, protection of imipenem from enzymatic degradation by resistant isolates, lowered the mutation prevention concentration of free imipenem by twofold, thereby preventing the emergence of resistance colonies, and eliminated bacterial attachment and biofilm assembly by 74 and 78.4%	Shaaban et al. (2017)
Quorum sensing inhibitor (ajoene) loaded nanoparticles (QSINPs) using the biopolymers, chitosan (CS), and dextran sulfate (DS) polymer	Ajoene	*Pseudomonas aeruginosa* PAO1	600 mg/kg body weight of infected mice	Quorum sensing regulated virulence and bacterial biofilm	Developed nanoformulation QSINPs exhibited double-fold anti-virulence activity than solo QSI agent against *P. aeruginosa*, further exhibited around 73–97% reductions ($p < 0.5$) in virulence factors when nanoformulation combines with ciprofloxacin. In addition, QSINP nanoformulation also showed around 1.75 log reductions in biofilm alone and 4.3 log reduction when combined with CIP	Vadekeetil et al. (2019)

(continued)

Table 12.2 (continued)

Nanoformulation	Antimicrobial agent included in nanoformulation	Bacterial pathogens	Nanoformulation concentration	Targeted drug resistance determinant	Advantages provided by nanoformulation	References
Antimicrobial silver nanoparticles (AgNPs) decorated in a layer-by-layer fashion with the oppositely charged aminocellulose (AM) and acylase (LbL ag@ AM_AC NP)	Aminocellulose (AM) and acylase (AC)	*P. aeruginosa* (ATCC 10145)	(6.25×10^7 NPs mL^{-1})	Bacterial biofilm and QS-related virulence factor	Eightfold lower MBIC (6.25×10^7 NPs mL^{-1}) and MBEC (1.25×108 NPs $mL - 1$) of the NPs decorated with AM and the anti-QS enzyme was observed compared to stand-alone AgNPs template demonstrating the effectiveness of nanoformulation in inhibiting QS-regulated pathological processes and at the same time eliminating the biofilm-forming bacteria at a lower dosage of the bactericidal agent, thus exerting less evolutionary pressure on bacteria for resistance development	Ivanova et al. (2020)

pneumoniae (resistant to carbapenem and cephalosporin) (CRPK) (Bi et al. 2017). A rise in CRPK bacteria-producing severe diseases was documented between 2005 and 2010 (Paczosa and Mecsas 2016). Several mechanisms such as extended-spectrum beta-lactamase (ESBLs), serine carbapenems, acquisition of MGEs, 16 s rRNA methyltransferase, cephalosporinases, topoisomerase, gyrase, LPS and PmrA-PmrB two-component genetic modification, plasmid-mediated quinolone resistance (PMQR), aminoglycoside-modifying enzyme (AME), and Mcr1 gene mutations are the prevalent resistance mechanisms among the XDR *K. pneumoniae* (Karaiskos et al. 2019). $bla_{CTX\text{-}M}$ and bla_{SHV} genes are the major ESBL virulence genes isolated from the clinical and healthcare systems (Carvalho et al. 2021). Recent investigations have revealed the involvement of efflux pumps (AcrAB-TolC), insertion elements (IS1, IS3), and integrons (Intl1) in the clinically isolated pan-resistant *K. pneumoniae* strains with overexpression of *acrB*, *ramA*, *phoQ*, and *phoP* virulence genes (Lv et al. 2021).

12.2.4 Acinetobacter baumannii

These microorganisms are typically found in hospital-acquired infections with high incidences in immunocompromised individuals referring to them as "red alert" microorganisms (Howard et al. 2012). *Acinetobacter* is commonly implicated in infections that are hospital-acquired or community-acquired and infect bloodstream, meningitis, wounds, and pneumonia (Morris et al. 2019). Various antimicrobials and therapies such as bacteriophage, gene transfer, radioimmunotherapy, photodynamic therapy, nanoparticles, and cathelicidins have been used to eradicate drug-resistant *Acinetobacter* (Howard et al. 2012). Reports have described the outbreak of *A. baumannii* in the neonatal intensive care units (NICUs) in Latvia with increased risk to newborns as HAIs (Gramatniece et al. 2019). Such infection outbreaks are certainly linked to the multidrug resistance acquired by the bacteria via injudicious or continuous exposure to antibiotics. In 2000, endemic carbapenem-resistant *A. baumannii* (resistant to carbapenems and other antibiotics) was reported in Brooklyn, New York, involving the strategies and practices to control the spread of MDR (Manikal et al. 2000). Further outbreak of *A. baumannii* in 2012–2013, accumulation of carbapenem resistance genes (*oxa23* and *oxa24*), tetracycline resistance genes (*tet39*), *sul2* gene (encoding sulfamethoxazole resistance), and *aadB* gene cassette (encoding gentamicin, kanamycin, and tobramycin resistance) in bacterial isolates from Tehran burns hospital were reported (Douraghi et al. 2020). Other virulence factors such as porins (OmpA), trimeric autotransporters, FhaBC secretion system, RecA, PmrAbB, and biofilm-associated proteins (BAPs) are also found to be produced by bacteria in biofilms and other environmental conditions (Mea et al. 2021).

12.2.5 Pseudomonas aeruginosa

Pseudomonas aeruginosa is a gram-negative bacterium that belongs to the family *Pseudomonadaceae.* It is the most opportunistic bacterium and is mostly associated with nosocomial infections and ventilator-associated pneumonia (Barbier et al. 2013). *P. aeruginosa* infections have become a great challenge because of its resistance to currently available antibiotics. (Lister et al. 2009). The World Health Organization (WHO) listed this carbapenem-resistant *P. aeruginosa* in the critical priority list to which there is an urgent need of developing new antibiotics. Studies have found *P. aeruginosa* mainly resistant to the aminoglycosides, beta-lactams, and quinolones. Antibiotic resistance in *P. aeruginosa* can be classified as intrinsic and acquired/adaptive resistance where production of antibiotic resistance enzymes, expression of efflux pumps, and low outer membrane permeability are seen with the acquired resistance of *P. aeruginosa* achieved by the horizontal gene transfer (HGT) or mutational changes. It also involves biofilm formation in the lungs of infected patients. In *P. aeruginosa* outer membrane acts as a selective barrier to prevent antibiotic penetrations, with the porins classified as specific (OprB, OprD, OprE, OprO, and OprP), non-specific (OprF), gated (OprC and OprH), and efflux (MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM) porins (Hancock and Brinkman 2002) contributing to antibiotic resistance (Dreier and Ruggerone 2015). MexAB-OprM is responsible for efflux of β-lactams and quinolones (Masuda et al. 2000; Dupont et al. 2005). MexCD-OprJ is able to pump out β-lactams (Okamoto et al. 2002). MexEF-OprN is capable of extruding quinolones (Llanes et al. 2011), while MexXY-OprM expels aminoglycosides (Masuda et al. 2000; Hocquet et al. 2003). *P. aeruginosa* possesses an inducible *ampC* gene, encoding the hydrolytic enzyme β-lactamase responsible for breaking the amide bond of β-lactam ring, leading to the inactivation of β- lactam antibiotics (Wright 2005). Further, mutational changes can also cause modification of antibiotic targets, reduced antibiotic uptakes, and antibiotic-inactivating enzymes.

12.2.6 Enterobacter spp.

Enterobacter, another gram-negative bacillus, is the microorganisms mostly involved in the nosocomial infections belonging to the family *Enterobacteriaceae*. To date, almost 22 species of *Enterobacter* have been identified that confer many drugs resistance genes such as cephalosporins in *Enterobacter cancerogenus*, carbenicillin to *Enterobacter asburiae*, and β-lactams to *Enterobacter cloacae* (Davin-Regli et al. 2019). *Enterobacter* is found to be highly resistant to carbapenems polymyxins, tigecycline, fosfomycin, and carbapenems (used in a double carbapenem regimen) leading to UTI infections (Ramirez and Giron 2020). In the early 1990s, the most common cause of *Enterobacter* nosocomial infections was *E. aerogenes* that led to the spread of pandemic clones in Western Europe (De Oliveira

et al. 2020). However, the spread and persistence of these microorganisms in the twenty-first century led to the production of carbapenem-resistant *Enterobacter* (Codjoe and Donkor 2017). Various virulence factors/genes involved are enlisted in Table 12.1.

12.3 Nanoformulations as an Emerging Combating Tool Against ESKAPE Pathogens

The incredible potential of nanoformulations in the pharmaceutical area to enhance healthcare has piqued scientists' interest, promoting substantial study throughout the world to gain a competitive advantage. Several nanoformulated products are studied in human research with approval by US Food and Drug Administration (FDA) for treating drug-resistant infections and other diseases. The rapid advancement of nanotechnology has dominated the drug delivery sector resulting in the development of drug-formulated deliveries with several clinical testing (Khiev et al.

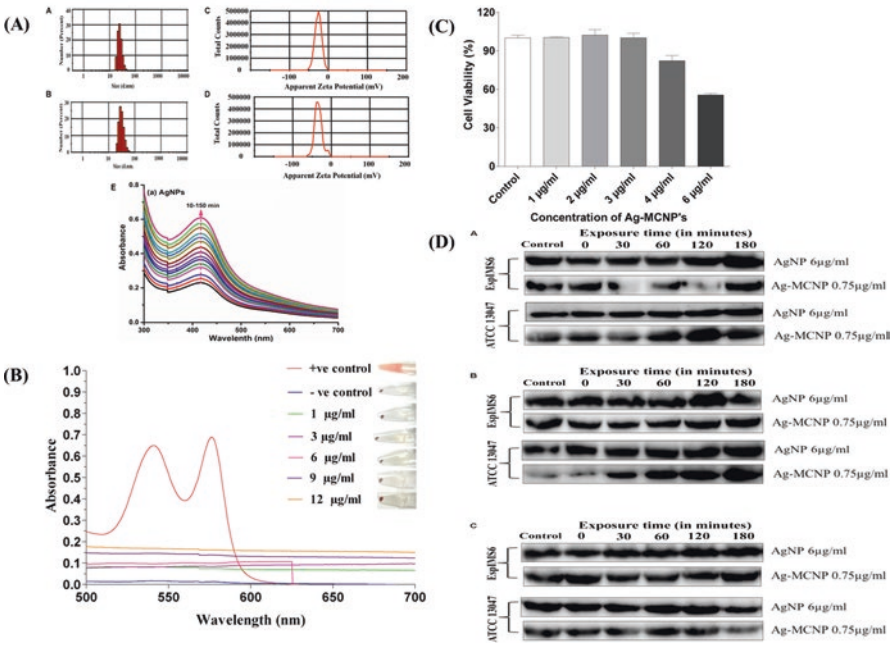

Fig. 12.1 Antibacterial efficacy of polysaccharide-capped silver nanoparticles against MDR *Enterobacter cloacae* clinical isolate (EspIMS6) harboring multidrug efflux system AcrAB-TolC; (**a**) characterization of Ag-NPs; (**b**) hemolysis activity of Ag-NPs; (**c**) cytotoxicity assay of Ag-MCNPs on macrophage RAW 264.7 cell line; (**d**) effect of silver and silver-metal composite nanoparticles on AcrAB-TolC expression. (Reproduced from Mishra et al. (2018) https://doi.org/10.3389/fmicb.2018.00823 under Creative Commons; Copyright Frontiers Media, Switzerland)

2021). ESKAPE pathogens being on the priority list of several countries, various nanoformulations have been developed and are under development for treating infection caused by MDR organisms (Lee et al. 2019). Nanoformulations involve formulation in surface chemistry, reactivity, and other properties of nanosized materials making them useful in other applications of environmental science, engineering science, cosmetology, etc. (Siddiqui et al. 2020). Nanocrystals, nanoemulsions, micellar encapsulation, nanodendrimers, and nanoliposomes are some examples of nanoformulations that enhance drug solubility, bioavailability, drug efficiency, and targeting (Patra et al. 2018).

Nanoemulsions, also known as mini-emulsions, are the dispersing systems with kinetic stability that have emerged as the potential tool for addressing the bioavailability difficulties lined with weakly water-soluble medicinal compounds (Pandey and Kohli 2020). Besides bioavailability, nanoemulsions exhibit multifunctionalities for carrying numerous antimicrobials with dual targeting capabilities (Chime et al. 2014). Khan and Ramalingam (2019) investigated ten nanoemulsions against eight ESKAPE pathogen strains showing their antimicrobial efficiencies as antibiofilm agents. Besides nanoemulsions, erythromycin-conjugated nanodendrimers against *S. aureus*, *S. epidermidis*, *S. saprophyticus*, and P. aeruginosa have shown great antimicrobial, bacteriostatic, and bactericidal activities with sustainable delivery of drug to the target site (Fallah et al. 2018). Recent technology of combining nanoformulations with antimicrobial peptides (AMP) has attracted researchers as natural host defense peptides against AMR (Mukhopadhyay et al. 2020). AMP dendrimers against MDR ESKAPE pathogens have improved the drug/antimicrobials targeting, pharmacokinetics, and efficiency (Kawano et al. 2020; Song et al. 2021). Patrulea et al. (2021) studied the synergistic effects of antimicrobial peptide dendrimer-chitosan polymer conjugates against *P. aeruginosa* via damaging cell membrane with the absence of toxicity to mammalian cells. Recently, nanoformulation of colistin-loaded human albumin nanoparticles (Col/haNPs) against MDR *Acinetobacter* and *Klebsiella* resulted in the decline of bacterial growth over time and inhibition of biofilm formation representing Col/haNPs as a promising tool with greater antimicrobial activity (Scutera et al. 2021).

12.4 Nanoformulation-Based Drug Delivery to Drug Resistance Determinant in ESKAPE

Ineffectiveness of existing drugs and the emergence of multidrug resistance in ESKAPE led to the development of novel strategies that can efficiently reverse multidrug resistance. Recent leads showed that nanoformulation-based drug delivery of antimicrobial agents against drug resistance determinants is an effective strategy to tackle multidrug resistance in ESKAPE as they effectively restore the efficacy of old unresponsive antibiotics and reduce toxic side effects associated with higher drug doses by reducing minimum inhibitory concentration without contributing to

resistance emergence; for example, ampicillin silver nanoformulation showed MIC in range of 3–28 μg/ml (lower than the MIC of ampicillin alone (12–720 μg/ml)) against ampicillin-resistant *E. coli* and *S. aureus* and multidrug-resistant *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (Khatoon et al. 2019). Further studies on bacterial strain did not show any resistance development even after exposure to ampicillin silver nanoformulation up to 15 successive cycles demonstrating the emergence of resistance against ampicillin silver nanoformulation (Khatoon et al. 2019). Another study reported enhanced antibacterial effect nanoformulation of biogenic cefotaxime conjugated-silver nanoparticles with the highest reduction in MIC [26–96%] against cefotaxime-resistant MDR *E. coli* and *MRSA* and no cytotoxic effect on normal cell lines (human RPE-1), restoring the efficacy of otherwise unresponsive cefotaxime (Halawani et al. 2020) highlighting the need to incorporate nanoformulation strategies into the development of next-generation antimicrobial therapeutics (Table 12.2).

12.4.1 Cell Wall, Cell Membrane, and Membrane Permeabilization

Bacterial cell wall/membrane makes up the first and most powerful line of bacterial defense preventing interaction of an antimicrobial agent with its target molecule. Membrane permeability plays an important role in providing a protective layer for regulating the inflow and intracellular concentration of antimicrobial agents; hence, the nanoformulation damaging bacterial cell becomes the prime focus of research for combating ESKAPE; for example, graphene (Gr)-based nanoformulation containing curcumin (C.C.M.) and zinc oxide nanoparticles (ZnO-NPs) displayed a wide range of anti-microbial activity against MRSA biofilm and also showed >five-fold improved inhibitory effect when GrZnO nanocomposites combined with curcumin (31.25 μg/ml M.I.C. of nanoformulation contrasting with GrZnO-NCs or C.C.M. alone having M.I.C. value of 125 μg/ml) with bacterial cell wall damage and cytoplasmic spillage as a major mechanism of inhibitory action, thereby diminishing their metabolism (Oves et al. 2020). In another study novel chitosan-mastoparan nanoconstruct (Mast-Cs NC) was designed and assessed for its therapeutic potential against clinical multidrug-resistant (MDR) *A. baumannii* and reported significantly lowered MIC nanoformulation compared to chitosan alone, with loss of cell membrane integrity (Hassan et al. 2021). Further, Thorat et al. (2021) synthesized gold nanorods (GNRs) coated pegylated thiol, mPEG-SH, further modified by adding curcumin, and a cell-targeting deoxyribonucleic acid (DNA) aptamer, displaying bacterial cell wall disruption and block in biofilm formation through photothermal action mechanism, and killing of MRSA due to the combination of photothermal effect, ROS generation, and transmembrane potential loss.

12.4.2 Biofilm Formation

Bacterial biofilm emerges as a severe health concern due to its multidrug resistance ability. Biofilm is defined as an intricate three-dimensional aggregation of bacteria attached to a surface and buried inflexibly in an extracellular polymeric substance matrix (Srinivasan et al. 2021) further helping bacteria to withstand the harsh environmental/physiological conditions or factors such as dehydration, antibiotic, biocides stress (Kaur et al. 2021) and played a major role emergence of multidrug resistance (MDR)/ pan-drug resistance (PDR)/ extensive drug resistance (XDR) by preventing the penetration of antibiotic inside the biofilm via EPS; increasing the chance for the genetic exchange among the bacterial species due to high population density and proximity of cells in biofilm; accumulation of antibiotic degrading enzymes; the presence of either non-growing cell (dormant or persister cells) /cells which triggered stress response under unfavorable chemical condition within the biofilm (Jolivet-Gougeon and Bonnaure-Mallet 2014; Balcázar et al. 2015; Srinivasan et al. 2021). Therefore, discovering novel strategies that can treat and prevent biofilm becomes the prime focus in combating AMR.

Nanoformulations such as chitosan oligosaccharide-capped gold nanoparticles (COSAuNPs) are shown to inhibit biofilm formation as well as eradication of preexisting mature biofilm, in addition to reduced virulence factor in *P. aeruginosa* (Khan et al. 2019). Similarly, curcumin-loaded poly(lactic-co-glycolic) acid nanoformulation with a drug loading of ~98 μg of curcumin/mg and release of ~45% of cargo displayed biofilm disruption and strong antibacterial activity compared to pure curcumin against *E. coli* and *S. aureus* (Kumari et al. 2020). Hydrophilic antibiotics such as gentamicin commonly used for treating *Pseudomonas* infection face problems such as relative short half-life limiting their application in clinical settings; therefore Abdelghany et al. (2012) developed a controlled-release gentamicin formulation using poly(lactide-co-glycolide) (PLGA) nanoparticles that enhance in vitro and in vivo antimicrobial effects off gentamicin on both planktonic and biofilm-based infection through controlled drug release from PLGA nanoparticles and optimized encapsulation. Further, this optimized formulation, when incorporated in murine peritoneal-infected mice model, resulted in both free and nanoparticle-encapsulated gentamicin effectively clearing the infection (both serum and peritoneal lavage) by the 96 hours suggesting nanoformulation could act as a potential agent exhibiting inhibitory properties against the ESKAPE pathogenesis arisen from biofilm formation (Abdelghany et al. 2012).

12.4.3 Quorum Sensing

The chemical communication process involved in the regulation of cooperative and communal activities in bacteria such as biofilm formation, virulence production, and bioluminescence is defined as quorum sensing (QS) (Qin et al. 2018). Hence

inhibition of quorum sensing has been emerged as a promising alternative to deal with MDR/XDR/PDR bacterial pathogens. Sharma et al. (2020) developed zingerone-loaded chitosan nanoparticles (Z-NPs) nanoformulation with 67% drug entrapment efficiency and pH-dependent controlled release of zingerone, when evaluated against *P. aeruginosa*, depicting significant downregulation of quorum sensing-related genes (*rhlR*, *rhlI*, *lasR*, and *lasI*), the complete absence of quorum sensing signaling molecules with the eradication of biofilm, and reduction of motility phenotypes (swimming, swarming, and twitching motilities). Similarly, nanostructured lipid carriers (NLCs) containing α-terpineol (αT) when evaluated against *P. aeruginosa* resulted in a significant reduction of gene expression of key QS-related genes (*lasI*, *lasR*, *rhlI*, and *rhlR*) and QS-associated genes (*rhlAB*, *toxA*, *lasB*, and *plcH*) with suppression of QS-related virulence factor production and biofilm formation compared to conventional antibiotics (Bose et al. 2020).

12.4.4 Efflux Pump Inhibition

In recent years, multidrug efflux pumps (EPs) are established as major determinants of AMR in both gram-negative and gram-positive bacteria, extruding multiple antibiotics, toxic substances, and metabolite out of cell mostly in a non-specific manner, playing a vital role in the process such as virulence, biofilm formation, stress adaptation, pathogenicity, and transportation of essential nutrient, hence emerging as a potential drug target for combating AMR (Shriram et al. 2018). Khan et al. (2020) synthesized dextran-capped gold nanoparticles (GNPDEX) with attached concanavalin-A (ConA) and methylene blue (MB) photosensitizer (MB@GNPDEX-ConA formulation) that showed the multitargeted killing of MDR *Klebsiella pneumoniae*, targeting major determinants of pathogenicity such as efflux pump, cell wall, and bacterial biofilm by the combined effect of both photodynamic therapy (PDT) and efflux pump inhibitor (carbonyl cyanide m-chlorophenylhydrazone). Further, they also reported 96.2, 92.9, 80.8, and 70% biofilm reduction in the presence of MB@GNPDEX-ConA nanoconjugate with varied concentrations of MB such as 20, 10, 5, and 2.5 μg/ml in the presence of EPI as compared to 80.8, 71.5, 53.9, and 38% reduction in control biofilm (absence of CCCP), further reporting bacterial killing by more than 3 log10 via PDT and EPI combinations, confirming EPI-based enhanced killing of MDR pathogens. In another study nanoliposome formulation co-loaded with piperine and gentamicin was investigated with remarkable inhibition and killing of MRSA pathogen via piperine-mediated inhibition of efflux pump and increased intracellular concentration of gentamicin (Khameneh et al. 2015), hence highlighting the importance of efflux pump inhibition in tackling multidrug resistance in ESKAPE. Figure 12.1 depicts the antibacterial efficacy of polysaccharide-capped silver nanoparticles against MDR *Enterobacter* species.

12.5 Challenges in Clinical Applications of ESKAPE-Combating Nanoformulations

Apart from the several advantages of nanoformulation such as protection of biomolecules from degradation, improved pharmacokinetics, enhanced solubility and bioavailability, reduced toxicity, and enhanced therapeutic efficacy (Agrahari and Hiremath 2017), implementation of nanoformulation in clinical setting still faces challenges that include biological understanding, large-scale manufacturing, biocompatibility and safety, government regulation, and cost-effectiveness as compared to conventional formulations (Hua et al. 2018).

12.5.1 Large-Scale Manufacturing/Scale-Up and Reproducibility

One of the most important factors slowing the pace of nanoformulations in clinical settings is the physiological complexity of nanoformulation. A formulation that required laborious or complex procedures and costly materials for synthesis generally is not compatible with large-scale production and, therefore, has a limited clinical translation potential. It is easier to maintain the size, composition, and complexity of nanomaterials at a smaller laboratory scale than at a large scale. Challenges arise when nanoformulation becomes more complex by the addition of multiple components in single nanocarriers/coating of formulation with multiple ligands, targeting molecules, or encapsulation of more than one antibacterial agent; therefore, the effective clinical translation, nanoformulation, must be prepared by a method that allows large-scale production with same high level of quality and reproducibility during scale-up (Muthu and Wilson 2012; Paliwal et al. 2014; Tinkle et al. 2014; Hua et al. 2018).

12.5.2 Biological Understanding

Considerable fewer research efforts in understanding the relationship between nanomedicine behavior (intracellular uptake, trafficking nanomaterial distribution, and retention in complex biological network), patient's biology and disease heterogeneity in patients are likely the major reasons for failure seen in the implementation of nanoformulation in clinical settings. Employing patient pre-selection strategies (preselecting patients likely to respond to nanomedicine-based therapy) and adopting a disease-driven approach to develop new nanoformulations and understanding between disease pathophysiology and nanomedicine behavior are the factors needed to be improved to access nanoformulation translatability and applicability (Hare et al. 2017). Lack of specific regulatory guidelines for

characterization and preclinical development of the nanoformulation-based product at the biophysiological level has hampered their potential in clinical practice (Agrahari and Hiremath 2017). The approval process for nanodrugs is essentially the same as that for any medicines and, therefore, is no longer appropriate to confirm clinical safety, efficacy, and quality of nanomedicines (Ventola 2017) due to nanomedicine properties such as the complex structure, unclear interactions with cell, tissue within the human body, and multifunctional nature of some formulation; hence, regulatory standard protocol specifically validated for nanomedicines which should take into account nanoformulation complexity, pharmacokinetics, safety, and toxicity profile and also provide information on patient selection and clinical trials is a must.

12.5.3 The Economic and Financial Barrier

Despite several patents of nanodrug delivery technologies, commercialization is still in its early stage, because of the high developmental costs of nanodrugs and medical devices (Zhang et al. 2016); in addition, the success of nanodrugs is also hampered by the fact that expenses involved in development and regulatory approval may not be compensated by limited sales for drugs especially in cases of increasingly complex nanodrugs that are associated with higher cost (Ventola 2017). Hence economical and financial barriers are also regarded as the biggest limitations in the successful implementation of nanoformulation-based drugs in clinical settings.

12.5.4 Nanoformulated Drug Characterization and Quality Control Challenges

Nanoformulated drug characterizations include analysis of stability, toxicity, size, morphology, surface functionality, charge, distribution, drug loading, solubility, entrapment efficiency, drug release, and retention that required advanced approaches and instruments such as small-angle X-ray scattering (SAXS), wide-angle X-ray scattering (WAXS), transmission electron microscopy (TEM), liquid chromatography-mass spectrometry (LC/MS), high-performance liquid chromatography (HPLC), atomic force microscope (AFM), the micropositron emission tomography (PET)/CT imaging system, and FRET imaging together with spectroscopy methods that are not only expensive but also require a team of expert to perform data analysis and interpretation increasing the cost of nanoformulation drug manufacturing and testing (Landesman-Milo and Peer 2016). Further low therapeutic efficiency of nanoformulation by self-aggregation at low drug concentration and the swelling mechanism that leads to increase in the size of nanoformulated drug further add to limited translation of nanoformulation in clinical settings (Jeevanandam et al. 2016).

12.5.5 Biocompatibility and Safety

Despite several pharmacokinetic advantages of nanodrugs, there is increasing concern over their safety and biocompatibility. Several in vitro and in vivo studies have shown that some nanoparticles used in nanoformulation demonstrated toxicity in the biological system causing cytotoxicity, inflammation, allergic response oxidative stress (generating ROS and free radicle), and DNA damage (genotoxicity). Nanoparticle toxicity is very complex and multifactorial depending on various physiological factors such as size, shape, composition, charge, and reactivity with biological system; hence a better understanding of pharmacodynamics, safety, and toxicity profile of nanodrugs and limitation of each nanoformulation-based drug delivery system is very crucial for the development of efficacious nanodrugs (Onoue et al. 2014).

12.6 Conclusion

Several approaches for nanoformulations have been developed so far. Among all these nanoformulations, nanoemulsions, nanoliposomes, nanodendrimers, etc. are the most promising models to combat and deliver drugs/antimicrobials. Nanopharmaceuticals and nanomedicines such as Emend, Ostim, Rapamune, Vitoss, Ritalin, TriCor, Doxil, DaunoXome, Onivyde, DepoCyt, Marqibo, AmBisome, Adagen, Oncaspar, Copaxone, Eligard, etc. are currently available in the market (Farjadian et al. 2019). Controlling the particle size, shape, controlled manufacturing, production, modifications, nucleation, pharmacokinetics, growth kinetics, and functionalization can lead to various nanoformulations that can target various drug-resistant determinants. The controlled release of drugs/antimicrobials/combinations to the target site will increase the antimicrobial efficiency and effectiveness via deep penetrations (Kumar et al. 2020). Biofilm formations and quorum sensing being interlined can be inhibited by the exposure of the nanoformulations (Jegel et al. 2022). However, to fully comprehend the biological effectiveness of nanoformulations, toxicity and biological activities must be properly investigated prior to clinical trials with the challenges of implementing these ESKAPE nanoformulations in clinical settings. Henceforth, these nanoformulated medications can be a promising tool in the future for combating and delivering drugs to the MDR ESKAPE pathogens.

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