Chapter 4

# Current Approaches for Exploration of Nanoparticles as Antibacterial Agents

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Additional information is available at the end of the chapter

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**Abstract**

The ascending anxiety regarding antimicrobial resistance as well as the recalcitrant nature of biofilm-associated infections call for the development of alternative strategies to treat bacterial diseases. Nanoparticles have been considered as one of the emerging and promising platforms in this respect. Their unique physical and chemical properties may lead to fine-tuned interactions between them and bacteria. In this chapter, we aim to provide an overview on the use of nanoparticles as antimicrobial agents. Both antibacterial and anti-biofilm activities of nanoparticles and their current approaches will be reviewed. The *in vitro* methods that are used to evaluate the potency of nanoparticles as antimicrobial agents will be discussed in detail.

**Keywords:** antibacterial agents, nanoparticles, antibacterial resistance (AMR), anti-biofilm agents, *in vitro* methods

## 1. Introduction

The term antimicrobial was derived from the Greek words anti (against), micro (little), and bios (life), and it refers to all agents that act against microorganisms. Thus, antimicrobials include agents that act against bacteria (antibacterial), viruses (antiviral), fungi (antifungal), and protozoa (antiprotozoal). Among these, antibacterial agents are by far the most widely known and studied class of antimicrobials. Nowadays, the emergence of antimicrobial resistance (AMR) among the microbial pathogens greatly increases the threat generated by bacterial infections. Drug-resistant bacteria lead to poor clinical outcomes increasing health care costs and mortality. In the US, the estimated health care costs associated with the treatment of

infectious diseases are annually more than 120 billion dollars, and further, treatment of infections caused by resistant pathogens costs 5 billion dollars per year [1]. According to the US Center for Disease Control and Prevention (CDC), more than two million antibiotic-resistant infections occur every year in the US and they lead to 23,000 deaths [2]. In the European Union, antibiotic-resistant infections are responsible for 25,000 deaths every year [3]. Both Gram-positive, especially methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecium* (VRE), and drug-resistant *Streptococcus pneumoniae*, and Gram-negative bacteria, namely multidrug-resistant *Acinetobacter baumannii* (MRAB), carbapenem-resistant *Enterobacteriaceae* (CRE), and *Pseudomonas aeruginosa*, display resistance to multiple drugs and are of serious concern [4]. In addition, biofilm formation complicates treatment of various infections. Biofilm-related infections, such as chronic wounds and urinary tract infections, pneumonia in cystic fibrosis patients, and infections related to the use of medical devices, comprise up to 80% of all human bacterial infections, and affect millions of people resulting in up to 550,000 deaths every year [5–7]. The emergence of AMR has been mainly attributed to the inappropriate and excessive use of antimicrobials in humans and animals. In many countries, unregulated availability of antibiotics without prescription results in promotion of overuse [8]. Further, inappropriate prescription of antibiotics also contributes to the promotion of resistant bacteria. The reports have shown that the diagnosis, choice of treatment agent, or duration of antibiotic therapy were incorrect in 30–50% of the cases [9], and 30–60% of antibiotics prescribed in intensive care units have been inappropriate or unnecessary [10]. Extensive agricultural usage of antibiotics in livestock as growth supplement is another reason of excessive consumption of antibiotics by humans, through the intake of resistant bacteria in the food supplies.

It is estimated that more than 70% of all pathogenic bacteria are resistant to at least one of the conventional antibiotics [11]. Antimicrobial resistance is acquired on both cellular and community levels [12]. Acquirement and dissemination of resistance genes is a process that occurs over time. Nevertheless, the evolution of bacterial resistance is substantially accelerated by the dispensable use of antibiotics [13]. Further dissemination of resistance genes between bacterial species has led to the emergence of multidrug-resistant (MDR) bacteria [14]. Community level of resistance is caused by biofilm formation [15]. However, when it comes to biofilms, the genetically transferable, conventional resistance mechanisms are not the leading cause of decreased antimicrobial susceptibility [7]. Bacterial biofilms are structured communities of bacteria embedded in a matrix of extracellular polymeric substances (EPS) that can be formed on variety of surfaces, such as tissues and medical devices [16]. Biofilm is a transient phenotype that makes even sensitive bacteria without known genetic basis for resistance to display remarkably reduced susceptibility to antimicrobials and host immune responses [17]. Many factors contribute to the antimicrobial tolerance of biofilms. First, biofilm matrix can restrict penetration of antibiotics and protects the cells from detrimental insults [18]. Secondly, biofilms comprise a heterogeneous population of cells that are in different physiological states due to decreasing oxygen and nutrient gradients existing between the surface and deeper layers of biofilms. For example, cells located in the deepest part of the biofilm tend to display a slower growth rate and, therefore, are less

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susceptible to antibiotics that are developed against dividing cells [19, 20]. Further, the non-dividing, dormant population of bacteria, referred also as persister cells, is in well-protected mode and highly tolerant antibiotics. These cells survive even from prolonged antibiotic treatment and serve as reservoirs for infections [21]. Consequently, biofilm bacteria can be up to 1000 times more tolerant to antimicrobial agents than planktonic cells of the same species [22].

Despite this, pharmaceutical companies have substantially declined investments in antimicrobial drug discovery during the past few decades [23]. Antimicrobial drug discovery is not economically attractive, and regulatory requirements have become very challenging [3, 24]. The need of novel bactericidal agents has increased due to the emergence of multi-drug resistant bacterial strains and biofilm-associated infections. Consequently, attention has been especially devoted to emerging nanoparticle-based materials in the field of antimicrobial therapies. In this chapter, the existing nanoparticles as antimicrobial means and the current *in vitro* test methods that will ease clinical translation of nanomaterials by establishing *in vivo* relevant data will be described and discussed.

## 2. Nanotechnology-based antibacterial therapies

Antibacterial applications of nanotechnology are gaining importance to prevent the catastrophic consequences of antibiotic resistance. Nanotechnology can be implemented as preventives, diagnostics, drug carriers, and synergetics in the antibacterial therapies.

The unique properties of nanomaterials compared to its bulk form make them favourable for antibacterial therapies. Many inorganic and organic nanomaterials represent inherent antibacterial properties that are not expressed in their bulk form. Fast and sensitive bacterial detection can be provided with nanoparticle-based approaches. Furthermore, nanoparticles offer discrete advantages as antibacterial drug delivery systems. They can be designed as targeted, environmentally responsive, combinatorial delivery systems [25]. Another approach of nanomaterials for the antibacterial therapy is as vaccine that contains nanoparticles as adjuvants or delivery vehicles, which provoke immune responses against bacterial infection. In the following parts in section 2.1 and 2.2, the existing nanotechnologies for the antibacterial delivery systems and inherently antibacterial nanoparticles will be discussed in detail.

### 2.1. Nanomaterials as antibacterial delivery systems

The existing disadvantages of conventional antibiotics can be solved to some extent by using nanomaterial-based antimicrobial delivery systems. In such approaches, the conventional antibiotics can be loaded into the nanoparticles through physical encapsulation, adsorption, or chemical conjugation. By this way, the pharmacokinetics and therapeutic index of the drug can ideally be improved compared to the free form of the drug. The aimed-for advantages are

provided by the improved serum solubility, prolonged systemic circulation lifetime of the drug, targeted delivery of the drug to the site of infection, sustained and controlled release of the drug, and also combinatorial drug delivery to the site of interest that could be reached by virtue of the nanoscopic delivery system [26–28]. This rationale of nanotherapeutics in this case aimed to enhance the therapeutic effect and minimize the side effects of antibiotics, starts with the appropriate design of nanoparticles. In nanoparticles design, the particle size, surface properties, and the release profile of the therapeutic agent have vital impact on the success of the therapeutic approach. Various nanoparticles-based drug delivery systems have been designed and investigated for improving the efficacy of antibiotics of the administered drugs, the most common of which shall be outlined in the following.

Lipid-based nanoparticles are widely used for the delivery of antibacterial agents. They can be designed as liposomes, solid lipid nanoparticles (SLN), and nanostructured lipid carriers (NLC). Liposomes are one of the most studied nanosystems for antimicrobial therapy in various diseases. Liposomes are spherical lipid vesicles with bilayered membrane structure, consisting of amphiphilic lipid molecules. Since their structure is similar to the bacterial cell membrane, efficacious interaction between liposomes and cells can be obtained. These interactions may create adsorption, endocytosis, lipid exchange, and fusion of the liposomes. Especially, the design of liposomes that cause fusion and is known as fusogenic is the most attractive one in the sense of efficiency. Fusogenic liposomes are able to destabilize the bacterial membrane and release their therapeutic content inside the cells [29, 30]. The structure of liposomes, where an aqueous cavity is surrounded by lipid membranes, empowers them to transport both lipophilic and hydrophilic drugs (in lipid bilayers and aqueous compartments, respectively) without chemical modification, protecting them from degradation [31]. SLNs are composed of a solid lipid core stabilized by surfactants and are moderately amorphous structures in which bilayers are not distinguished. They can provide long-term stability and better incorporation efficacy for hydrophobic drugs and can be easily scaled-up in production. NLCs were developed in order to overcome the limitations of SLNs regarding low-loading capacity for nonhydrophobic drugs and their stability issues. In the NLC structure, liquid lipids are used to stabilize the construct, which allows a biphasic drug release profile with initial burst release continued with sustained drug release. Liposomes have shown to be successful in combating resistant pathogens. Especially, their modified designs are used to improve the potency of formulations in bacterial resistance and clearance [32]. Additionally, researchers have confirmed the feasibility of SLN and NCL as drug carriers, however, their advantages over liposomes have not been confirmed with human data [33]. Most of the research on SLN and NCL as antimicrobial carriers are still in the preclinical stage.

To date, a significant number of reports on the activity of antibiotic-conjugated polymeric nanoparticles against various infections, including those caused by drug-resistant pathogens, have been published [34]. Notably, high biocompatibility of these structures, additional to improved pharmacokinetic properties, supports the potential of these nanosystems as new tools to treat infections. Polymeric nanoparticles can be prepared from natural and synthetic polymers with the prerequisite of biocompatibility and biodegradability. In the polymeric antibacterial drug delivery systems, drug molecules can be incorporated in the internal

part of the particles, on the surface of polymeric nanocarriers with covalent or non-covalent bonds, imprinted in the polymeric nanoparticles or encapsulated in the stimuli-responsive shell of polymeric nanoparticles [34]. The encapsulation route of the drug into the polymeric nanoparticle drug delivery system plays a key role in the nanocarriers' pharmacokinetic profile. The action mechanism of the polymeric nanoparticles is defined by the physicochemical properties and the composition of the particles. Polymeric nanoparticles may interact with the bacterial cell wall via passive or active targeting. Passive targeting is based on particle size and the ability of particles to disturb the structure of bacterial membrane leading to pore formation in the membrane. For active targeting of polymeric nanoparticles, the surface of polymeric nanoparticles is usually functionalized with specific antibodies and aptamer bacteriophage proteins providing specific identification for the detection of pathogens and interaction between the particles and pathogens. The reported studies reveal that both the active and passive targeting strategies to deliver antimicrobial agents with polymeric nanoparticles improve their activities compared to their free forms [35–37].

Dendrimers are highly branched macromolecules employed as antibacterial drug delivery systems. The unique properties of dendrimers, such as well-defined 3D structures, available functional groups, and their ability to mimic cell membranes, make them potential drug carriers. Both hydrophobic and hydrophilic drug molecules can be incorporated separately or at the same time into dendrimer structures. Lipophilic molecules can be incorporated inside the cavity of dendrimers, and hydrophilic agents can be covalently or physically attached to the surfaces of dendrimers. The antibacterial can be used in the building of dendrimer blocks, whereby the synthesized dendrimers themselves become potent antimicrobials. Dendrimers aid to improve the solubility, penetration, and controlled release of the drug molecules. Currently, the existing research in the design of dendrimers as antibacterial drug delivery systems also focuses on species-selective dendrimer biocide formulations. For instance, peptide, glycol, and glycopeptide dendrimer designs provide effective therapy for the bacterial infections.

An inorganic nanomaterial, in contrast to the organic materials listed above, which has also shown promise for antibacterial therapies is mesoporous silica nanoparticles (MSNs). In the design of MSN-based drug delivery systems, their advantageous characteristics (biocompatibility, high surface area, tunable particle diameter, mesoporous structure, and ease of functionalization) have been exploited. The designs with targeted and sustained release mechanisms make them powerful candidates also for antibacterial therapies. In the use of MSNs as drug delivery vehicles for antibacterial therapeutics, their surface functionality along with the size and shape are crucial parameters to improve and optimize the efficacy [38]. Their surface functionalities can be modified to target both planktonic bacteria and biofilms [39]. In recent studies, the utility of MSN for efficient antibiotic delivery [36, 40, 41] and hybrid antibacterial materials preparation by incorporating antibacterial enzymes [42], peptides [43], metal ions/particles [44], and polymers (surface modifiers) [45] to MSNs has been reported.

For rational and efficient utilization of these nanomaterial-based drug delivery systems, systematic investigation of pharmacokinetics and biodistribution should be carried out. The pharmacokinetics and biodistribution of nanoparticles are defined by their physicochemical

properties [38]. Apart from their physicochemical properties, the administration routes and their elimination from the body need to be systematically evaluated. Hence, thorough evaluation of the current nanoparticle-based drug delivery systems in antibacterial therapies is important for their translation into the clinic. To date, four liposomal/lipid complex drug delivery systems for antibiotic delivery have been approved for use in human patients, including Abelcet, AmBisome, Amphotec, and Fungisome [46]. This should come as no surprise with regard that a liposomal formulation was the first nanodrug to hit the market in 1995 (Doxil®), and they have been studied since the early 1980s.

### 2.2. Nanomaterials as active antibacterial agents

Various types of inorganic and organic nanoparticles have been utilized as antibacterial agents. The inherent antibacterial properties of some metals and metal oxides have been known for centuries. An important advantage of antibacterial metal and metal oxide nanoparticles is that they have multiple modes of action, which is why microbes can scarcely develop resistance to them.

Among the inorganic antibacterial particles, silver nanoparticles are the most intensively investigated ones and capable to kill both Gram-positive and Gram-negative bacteria, having even shown to be effective against drug-resistant species [46]. Besides silver nanoparticles, other metal nanomaterials have also been studied for antimicrobial treatment, including gold [47], copper [48, 49], tellurium [50, 51], and bismuth [52]. Moreover, many studies have revealed the antibacterial activity of metal oxide nanomaterials, such as zinc oxide (ZnO) [53], copper oxide (CuO) [54, 55], magnesium oxide (MgO), nitric oxide (NO) [56], titanium dioxide ($TiO_2$) [57], aluminum oxide ($Al_2O_3$) [58], magnetic iron oxide ($\alpha$-$Fe_2O_3$) [59], and cerium oxide ($CeO_2$) [60] nanoparticles. The toxic mode of metal and metal oxide nanoparticles against bacterial cells has been associated with ROS generation and membrane disruption [61]. According to literature findings, the release of ions is designated as the driving force behind the antimicrobial properties of antibacterial nanoparticles.

Cationic polymeric nanoparticles have been considered as promising organic antibacterial nanoparticles. They can be composed of natural or synthetic cationic polymers. The antibacterial polymeric nanoparticles kill microorganisms upon their contact with bacterial cells due to the strong interaction of their cationic surfaces with the bacterial cells [62]. The mechanisms of action have been proposed for how these cationic groups are able to disrupt the bacterial cell membrane, with some requiring hydrophobic chains of certain lengths to penetrate and burst the bacterial membrane. Moreover, different polymeric nanosized antibacterials with long-term antibacterial activity, chemically stable, and ability to bind to surfaces of interest have been reported. These include lipid nanoparticles, quaternary ammonium polyethyleneimine-incorporated polymeric nanoparticles [63, 64], chitosan [65], and self-assembled peptide nanoparticles [66]. In addition to the above-mentioned metallic and polymeric nanoparticles, carbon-based nanostructures have shown antibacterial effects. For instance, the antibacterial activity of fullerene [67] and carbon nanotubes [68, 69] (single-walled or multi-walled) derivatives have been observed. However, the antibacterial mechanism of carbon-based nanostructures is still under debate and has not received particular attention, possibly due to the difficulties of their dispersion in water, especially in case of the carbon nanotubes [70].

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## 3. Advantages and challenges of nanotechnology-based antibacterial treatments

Promising approaches for the effective delivery of therapeutic compounds can be provided by the use of nanoparticles as drug carriers. Literature findings and clinical results have surely presented several clinical advantages of antimicrobial nanoparticles and their utilization as drug carrier systems. Antimicrobial nanoparticles are of great interest as they provide a number of benefits over free antimicrobial agents. In detail, nanocarriers can conquer the solubility and stability issues and reduce side effects [62]. With the use of nanocarriers in the delivery of drugs, combination drug therapy can be achieved by incorporating two or more drugs or different therapeutic modalities into the carrier matrix. The surface modifications can be carried out by conjugating targeting ligands on the nanocarriers that are not known by the immune system and specifically targeted to special microorganisms. Administration of antimicrobial agents using nanoparticles can increase the overall pharmacokinetics by progressing therapeutic index, extending drug circulation, and providing controlled drug release. Multiple mechanisms of action can be provided by the antibacterial nanoparticles, which prevent the development of antibacterial resistance by many pathogenic bacteria. Several routes of administration, including oral, nasal, parenteral, intraocular, and so on, can be employed with the nanotechnology-based antibacterial treatments.

The significant advantages of nanomaterials as antimicrobial agents are their modularity in design, enabling a multimodal approach that makes it especially difficult for bacteria to develop resistance mechanisms against these. Namely, a nanotechnology-based antibacterial agent can be constructed out of several components that possess antimicrobial activities in themselves, such as, for instant, be composed of an antibacterial core material (e.g. metal or metal oxide) surrounded with an antibacterial polymeric shell or coating, in which antibiotic drugs could be incorporated [71]. The core material could further be "prickly," which physically can destroy the bacterial cell wall by a "nano-piercing" process once the polymeric shell has been dissolved, leading to the disruption of bacterial integrity and lysis, as presented in a recent study by Wu et al. where zinc-doped copper oxide prickly nanoparticles exhibited high bacterial killing efficiency owing to the provided core particle nanostructure [72]. Furthermore, varying possibilities for combination therapy together with existing (commercial) antibiotics to reach synergistic effects are evident [14, 73, 74].

Although nanoparticle-based antibacterial treatments promise significant benefits and advances in addressing the key hurdles in treating infectious diseases, there are challenges in translating this exciting technology for clinical use. These include thoroughly evaluating the interactions of nanoparticles with cells, tissues, and organs, which accordingly recalibrates doses and identifies proper administration routes to obtain desired therapeutic effects. Hence, to provide a clinical translation of nanomaterials, standardized *in vitro* experimentations that will provide *in vivo* relevant data should be established [75]. In section 4, we describe existing *in vitro* methods for testing antimicrobial activity. In addition, the current methods commonly employed in testing the antibacterial and anti-biofilm activity of nanoparticles are discussed with the relevancy and pitfalls.

## 4. Methods to study antimicrobial activity *in vitro*

Traditionally, antimicrobial research has focused on planktonic bacteria, and there is a variety of test methods available for evaluation of antimicrobial activity against planktonic cells [76]. The Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) are the major contributors to harmonized antimicrobial susceptibility testing (AST) providing uniform procedures for testing and analysis of antibacterial activity. CLSI standards clearly define the specific and essential requirements for materials, methods, and methodologies that need to be followed without any modifications because deviations from the approved procedures might affect the experimental outcome [77]. All the critical elements for the testing, such as the culture medium, inoculum density, and incubation conditions, are listed. Unlike the antibacterial assays, standardized methods for anti-biofilm studies are scarce. No standard methods have been approved by CLSI or EUCAST for evaluation of antimicrobial activity against biofilms. Altogether, five standards (ASTM E2196, ASTM E2647, ASTM E2562, ASTM E2799, and ASTM E2871) set by the American Society for Testing and Materials (ASTM) exist, and they all are applicable as such only for *Pseudomonas aeruginosa* biofilms [78]. Moreover, only one standard is intended for susceptibility testing. Further, due to distinct phenotype and heterogeneity of biofilm bacteria, conventional *in vitro* methods used for assessment of bacterial susceptibility to antimicrobials are not appropriate for biofilm-growing bacteria [79]. Lack of standardization makes a comparison of the test results difficult, and further, can lead to the generation of conflicting data between studies since the experimental outcome is strongly dependent on the assay conditions and materials employed in the testing.

### 4.1. Antimicrobial susceptibility testing

*In vitro* susceptibility assays are performed to assess the antimicrobial susceptibility of microorganisms in order to provide efficient treatment for infections [80]. Moreover, they are used for resistance surveillance and in research laboratories to study antimicrobial activity of antimicrobial agents. Determination of the minimum inhibitory concentration (MIC) is the most widely used measure of the antimicrobial susceptibility of microorganisms. The MIC is defined as the lowest concentration of an antimicrobial (expressed as mg/L or μg/μL) required to inhibit the visible growth after overnight incubation. In addition, the minimum bactericidal concentration (MBC) is frequently used. The MBC refers to the lowest concentration of an antimicrobial that kills 99.9% of the original inoculum after a certain incubation period (**Figure 1**). These measures can be easily achieved and compared with each other when standardized methodologies and protocols are followed. A number of guidelines and standardized protocols for MIC and MBC determinations exist that include information on each step of the testing, ranging from storage and preparation of antibiotic stocks to interpretation of the results against particular microorganisms [81].

#### *4.1.1. Dilution methods*

Dilution methods are used to determine the MIC values of the antimicrobial agents. Moreover, they serve as reference methods for antimicrobial susceptibility testing. The minimum inhibitory

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concentration can be determined both on agar (agar dilution) and in broth (broth dilution). Standards for agar and broth dilution techniques used to assess the *in vitro* susceptibility of aerobically grown bacteria are described in CLSI document M07-A9. The document covers the performance, experimental conditions, reporting of the minimal inhibitory concentration (MIC), quality control procedures, and limitations of the recommended methods.

When conducted on agar, a two-fold diluted series of the antimicrobial agent is incorporated into agar medium followed by inoculation of standardized suspension of the given organism onto the agar plate. Broth dilution can be performed in tubes (macrodilution, volume 2 ml) or in microtiter well plates (microdilution, volume ≤500 μl) containing a two-fold diluted series of antimicrobial agent prepared in the liquid growth medium that is inoculated with a standard inoculum of bacteria followed by a defined incubation period under particular conditions. After the incubation, the outcome is read based on turbidity or growth zones, and the MIC is defined. The MIC value can also be utilized to distinguish between bactericidal and bacteriostatic activities. Alternatively, when using microdilution, the MIC can be determined spectrophotometrically according to the EUCAST protocol [81]. In addition to CLSI and EUCAST standards, ISO-20776-1 standard proposes acceptable performance criteria for microdilution method. After broth dilutions, the MBC can be determined by sub-culturing the samples from tubes or wells and plating on agar to determine the number of cells (CFU/ml) after incubation for 24 h. Then, MBC is defined as the lowest concentration at which 99.9% of the final inoculum is killed (**Figure 1**). The main advantage of the dilution method is a gen-

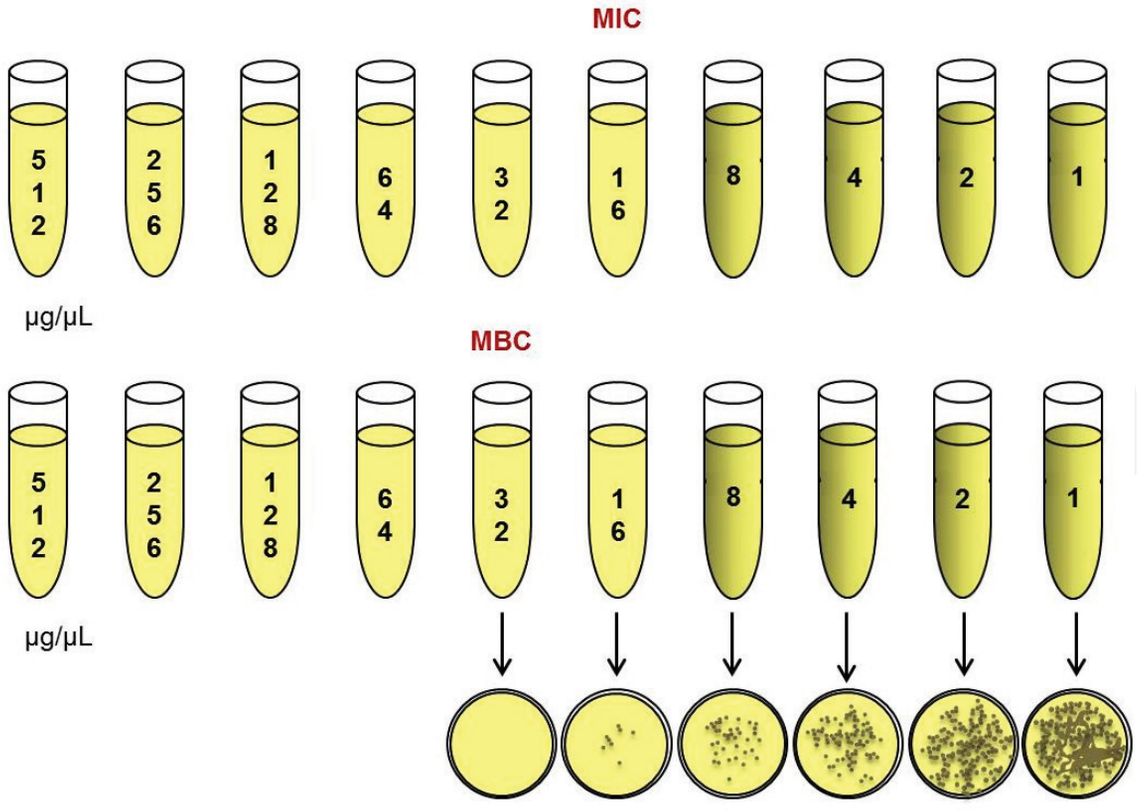


**Figure 1.** Minimum inhibitory concentration (MIC) versus minimum bactericidal concentration (MBC).

eration of quantitative data, the MIC value. Moreover, the assay is overall reproducible, and small amounts of antimicrobials are needed when the microdilution method is utilized. By contrast, large amounts of antimicrobial agents are needed in macrodilution testing. The main disadvantage of the method is several steps in sample preparation, which in turn, increases the possibility of errors.

#### *4.1.2. Diffusion methods*

Standards for antimicrobial disk susceptibility test are proposed in the document M02-A12 by the CLSI. Specifications for the agar (type, depth), concentration range of the test antimicrobial, concentration of the microorganism and incubation conditions (time, temperature and atmosphere) are included. Also, interpretation of the results, quality control procedures, and limitations of the methods when used for susceptibility testing of aerobically growing bacteria are described. Agar disk diffusion method is routinely applied to the *in vitro* susceptibility testing of bacteria. The Kirby-Bauer test is the most thoroughly described disk diffusion method for which interpretive standards exist (**Figure 2**). A filter paper disc impregnated with antimicrobials at different concentrations is placed on an agar plate, and the antimicrobial diffuses from the disc into the agar around the disc. Thereafter, the plate is inoculated with a standardized suspension of a microorganism followed by incubation. After the incubation period, the growth inhibition zones around the discs are measured. The diameter of the zone is depending on the antimicrobial susceptibility of microorganism. The disc diffusion test is simple to perform, but it only provides qualitative data and categorizes microorganisms as susceptible, intermediate, and resistant based on the susceptibility. Thus, it cannot be used to distinguish between bactericidal and bacteriostatic effects. Commercially available zone reader systems can be utilized to calculate an approximate MIC value by comparing the zone size and standard curve of the bacteria and antibiotic stored in an algorithm [82].

#### *4.1.3. Combined dilution and diffusion method*

The antimicrobial gradient diffusion method is based on the establishment of an antimicrobial concentration gradient in the agar medium to measure the antimicrobial susceptibility. Thin plastic test strips marked with concentration scale and impregnated with antibiotic concentration gradient are placed on agar plates that have been inoculated with a standardized

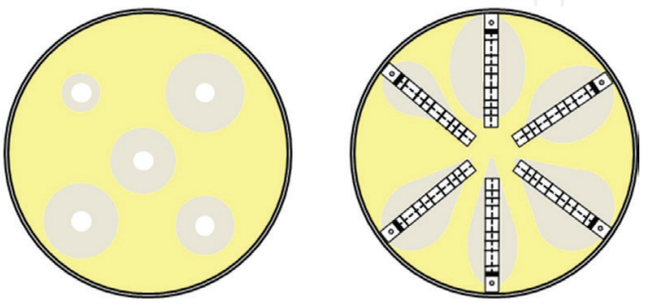

**Figure 2.** Disk diffusion test and antimicrobial gradient diffusion method. On the left, agar plate showing zone of inhibition by different antimicrobials of diameter of zones of inhibition refers to the susceptibility of a microorganism.

inoculum (**Figure 2**). After incubation overnight, the experimental outcome is read, and the MIC can be determined by the intersection of the lowest part of the ellipse-shaped growth inhibition area with the test strip. E-Test is the commercially available test for this purpose.

#### *4.1.4. Time-kill test*

Time-kill assay is complementary to MIC and MBC determinations. It provides information on the dynamic interaction between the antimicrobial and microorganism, thus revealing whether the antimicrobial effect is time or concentration dependent. Such activities can be investigated utilizing the standard protocol M26-A by CLSI and ASTM2315. These protocols are frequently modified. Time-kill assay is usually conducted at a concentration twice or four times the MIC. Standardized inoculum is added to a nutrient broth containing the antimicrobial at various concentrations. A sample is taken from each concentration at the inoculation time and after selected time points. Samples are serially diluted and viable plate counts are performed. The kill curves are constructed by plotting the log CFU against time. A 3-log reduction in cell counts corresponding the killing of 99.9% is considered as significant antimicrobial activity [83]. Alternatively, measurement of luminescence can be utilized to determine the time-kill relationship. Luminescence is detected by the ATP assay, in which adenosine triphosphate (ATP), an indicator for bacterial viability is quantified, and the number of viable cells is determined based on the amount of ATP. The assay uses luciferase reaction, in which luciferin is converted to oxyluciferin in the presence of molecular oxygen and ATP, and generates light by luminescence. Luminescent signal is proportional to the number of viable cells [84].

#### *4.1.5. Methods to study antimicrobial susceptibility of biofilms*

Since conventional susceptibility testing methods are not applicable for biofilms, and the MIC values do not provide a valid estimation of the antibiotic concentration needed to treat biofilm-related infections, the minimum biofilm inhibitory concentration (MBIC) has been determined instead. The MBIC determines the susceptibility of bacteria when the biofilm is forming and refers to the lowest concentration of an antimicrobial, in which no visible growth occurs after exposure to antimicrobial after the incubation period, and it can be recorded by optical reading [85]. Based upon the viable plate counts, the MBIC is defined as the lowest antimicrobial concentration in which there is no time-dependent increase in the mean number of viable cells between two exposure times [86]. Moreover, the Calgary Biofilm Device (CBD) can be used for determination of MBIC, as well as the minimum biofilm eradication concentration (MBEC), which is defined as the lowest concentration of an antimicrobial required to eradicate the established biofilms [87] along with susceptibility of planktonic bacteria (MIC) [22]. The commercially available CBD consists of 96 pegs mounted on the lid of a 96-microtiter well plate. Biofilms are first formed on the pegs for a defined time period. After the incubation period, the lid is transferred to another 96-well plate containing antimicrobials in fresh culture media at various concentrations. The MBEC is defined as the concentration of antimicrobial in which no visible growth can be detected [88]. ASTM 2799 standard describes the operational parameters required to grow, treat, sample, and analyze *Pseudomonas aeruginosa* biofilms using MBEC assay. In this assay, the experimental outcome is reported as log10 colony form-

ing units (CFUs) per surface area, and the antimicrobial efficacy is assessed as the log10 reduction of viable cells. The experimental outcome can be evaluated using the CLSI guidelines for interpretation. However, breakpoints for resistance are not available, and those for planktonic bacteria are not applicable for biofilm bacteria [86]. Even though the ASTM protocol describes the specific experimental conditions only for *P. aeruginosa* biofilms, it can be used for other species with some modifications.

### 4.2. Methods to assess the anti-biofilm activity

Several assays with distinct endpoints are essential for the determination of the antimicrobial activity against biofilms. These assays rely on quantification of (i) viable cells in the biofilm, (ii) total biomass and (iii) biofilm matrix. An ideal anti-biofilm agent would target biofilm viability, biomass, and the matrix. Most of the assays are based on various staining methods. Several models have been proposed for evaluation of antimicrobial activity on biofilms. Depending on the flow of nutrients and bypass the waste products, biofilm models can be classified as closed and open systems [89]. Microtiter well plate-based assays are the most commonly used, while the Calgary biofilm device, substratum suspending reactors, and the flow cell system are the most widely used biofilm models for *in vitro* susceptibility testing [79]. Because the experimental outcome is affected by the choice of the model system, it is utmost important to select a model, in which biofilms can be formed in conditions close to *in vivo* settings.

#### *4.2.1. Crystal violet staining*

Crystal violet (Hexamethyl pararosaniline chloride, CV) assay is not only one of the oldest but also most widely used staining methods applied to biofilm quantification [90, 91]. Crystal violet is an inexpensive and basic dye that is used to measure the effects on total biomass of biofilms. Crystal violet binds indifferently to both negatively charged bacteria and polysaccharides present in the EPS. After staining, adsorbed dye is eluted in a solvent, such as acetic acid or ethanol. The amount of the dye solubilized by the solvent is measured spectrophotometrically, and it is directly proportional to biofilm biomass [92, 93]. Disadvantages of the method are shortcomings in its dynamic range, laboriousness, and low reproducibility [92]. Experimental conditions, bacterial species, concentration, and nature of the solvent used, as well as incubation time are crucial steps that affect the experimental outcome. Furthermore, the assay does not sort out living or dead cells or biofilm matrix, thus not providing any information on the number of living bacterial cells [93] and, more importantly, imprecise information on the antimicrobial activity. However, the method can be used for both Gram-negative and Gram-positive bacteria and fungi, but the optimal assay conditions, such as temperature, incubation time, and solvent, vary between species [94, 95].

#### *4.2.2. Resazurin staining*

Resazurin, also known as alamar blue (7-hydroxy-3H-phenoxazin-3-one-10-oxide), is a non-invasive, non-fluorescent dye, which is reduced to resorufin, a pink, fluorescent dye as a result of metabolically active cells and bacterial viability. Resorufin is detected spectrophotometrically

to determine the viable cells [96]. Resazurin staining assay can be used to assess the antimicrobial activity based on the effects on viability of various microorganisms grown in suspensions or as biofilms [97]. However, time of resazurin reduction is species and strain specific. Consequently, the experimental conditions, such as incubation time and resazurin concentration, need to be optimized to obtain reproducible data [93, 98].

#### *4.2.3. Fluorescein diacetate (FDA) assay*

The assay is used to measure nonspecific esterase activity of viable microbial cells that converts colorless, nonfluorescent FDA to fluorescein, which is a green fluorescent compound that can be quantified fluorometrically [88]. The assay is not widely used because the dye rapidly leaks from the cells and is unstable. Moreover, hydrolysis of FDA to fluorescein in the absence of live cells and quenching of fluorescence by assay solutions may also occur under certain conditions, thus making the reliability of the assay questionable. However, the assay enables biofilm quantification without removing biofilm from the place where it has been formed, allowing the quantification of entire biofilm [84, 99].

#### *4.2.4. Other viability assays*

Biofilm viability can be assessed using tetrazolium salt reduction assay, in which tetrazolium salts, such as MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), XTT (2,3-bis-[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide), and MTS (3-[4, 5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium), are reduced to formazan dyes. The color change can be quantified spectrophotometrically. The amount of formazan dye produced is proportional to the number of viable cells. However, the amount of reductase enzyme produced by cells is dependent on metabolic activity [100, 101]. In other words, cells that are metabolically less active when encased in a biofilm produce also reduced amount of reductase enzyme that can lead to identification of artificially low quantities of cells when viable cells are detected using this assay. Moreover, high densities of bacteria may produce the maximum amount of formazan product leading to optical densities comparable with even higher cell densities [102].

#### *4.2.5. Viable plate counts (colony forming units)*

Viable plate counts (colony forming unit counting) are used to assess the biofilm viability based on cell counting. The assay can be used to evaluate the efficacy of antimicrobials to prevent biofilm formation or eradicate pre-formed biofilms, respectively [103]. Depending on the assay mode, bacteria and antimicrobials are added simultaneously to the microtiter well plates or biofilms are first allowed to form followed by the exposure to antimicrobial. After an incubation period, biofilms are dislodged and disaggregated. The resulting suspensions are carefully homogenized, for example, by vortexing or sonication [93]. Subsequently, suspensions are serially diluted and plated or spread on agar. Colony forming units (CFUs) per surface area or volume are counted after an incubation period. The experimental outcome can be evaluated as reduction in CFUs compared to untreated control biofilms. The method gives

accurate information on bacterial viability [94]. However, it is regarded as a time-consuming and laborious methodology because of serial dilutions and plating. Additionally, special attention needs to be addressed to the detachment and disaggregation steps to avoid false negative/positive results. The complete recovery and disaggregation of biofilm need to be ensured by applying methods that do not affect viability of the biofilm cells [93, 104].

### 4.3. Testing of antimicrobial activities of nanoparticles

With respect to the evaluation of antimicrobial activity of nanoparticles, only one specific standard is set by the International Organization for Standardization (ISO), and it is intended for determination of the antimicrobial potency of silver nanoparticles against *Staphylococcus aureus* by measuring the release of muramic acid using gas chromatography-mass spectrometry (GC-MS) [105]. Thus, conventional methodologies for antimicrobial susceptibility testing have been not only adapted but also modified for investigation of antimicrobial activity of various nanoparticles [102]. In antimicrobial testing, experimental outcome is affected by many factors, such as solvent, inoculum preparation, type of the culture media, and incubation conditions, and these factors have been also found to be influential when testing antimicrobial activities of nanoparticles [76, 106]. For example, choice of the culture media can have a substantial impact on antimicrobial activity of nanoparticles. Media composition and its pH may affect the experimental outcome because of the impact on physicochemical properties of nanoparticles, and further, media type (solid vs. liquid) has found to profoundly influence the antimicrobial activity of nanoparticles [1, 107, 108].

Both standardized and modified microdilution and macrodilution methods have been applied to the determination of MIC and MBC values to evaluate the antimicrobial susceptibility of several microorganisms to nanoparticles [76, 109, 110–116]. Additionally, resazurin staining assay has been employed to determine the MIC. The MIC was recorded as the lowest concentration at which color change from blue to pink occurred [117]. Even though standardized antimicrobial susceptibility testing protocols can be followed, no standards describing the synthesis of nanoparticles exist. Differences in the synthesis methodology are known to impact the particle size and chemical composition of the nanoparticles, which, in turn, can further affect the antimicrobial activity and cause variability in the experimental outcome [1, 118]. Hence, the impact of such factors has to be taken into consideration when results between studies are compared.

Diffusion methods can be applied alongside dilution assays to confirm the antimicrobial susceptibility of microorganisms [108, 119, 120]. Agar disk diffusion tests performed both according to the standardized protocols and with modifications are frequently used for susceptibility assessment and evaluation of antimicrobial activity of nanoparticles [109, 121–124]. Paper disc method has been employed as an alternative to standardized single disk method [112]. Further, agar well diffusion assays have been successfully utilized for the evaluation of antimicrobial effects of nanoparticles [120, 125, 126].

Antimicrobial effects can also be determined by reading the optical density [125]. Even though measurement of optical density is a straightforward method, it is not the most suitable for measuring the activity of nanoparticles because nanoparticles as such can also interfere with the optical density [46, 127]. Viable plate counts have been frequently performed according to

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the various protocols to assess the antimicrobial efficacy of nanoparticles against both planktonic and biofilm-growing bacteria [109, 128, 110–112, 116]. Samples can either be spread or pipetted on agar plates followed by overnight incubation and determination of the number of CFUs [116, 111, 113–115]. Especially, when quantifying the biofilm bacteria, efficient disaggregation of samples is of great importance to avoid false positive results.

Crystal violet staining is the most widely applied staining assay to investigate the antimicrobial activity of the various nanoparticles against biofilm-growing bacteria [129, 130, 121, 116, 131, 132]. By combining assays that quantify different features of biofilms, more relevant information on the activity of nanoparticles can be obtained. In that context, effects of nanoparticles on biofilm inhibition have been studied using viable plate counts and crystal violet staining in parallel [133, 134]. Additionally, LIVE/DEAD and crystal violet staining has been combined for the same purpose [133, 134]. Crystal violet staining has been also used together with resazurin staining assay to assess the impact of nanoparticles on total biomass, including the matrix components and biofilm viability, respectively [135]. Further, crystal violet along with phenol has been applied to quantify the effects on biomass and EPS [136]. Antimicrobial agents displaying bactericidal effects have usually an impact on both viability and biomass, while antimicrobials acting like detergents affect only the biomass [137]. Further, to distinguish between bactericidal and bacteriostatic activities, LIVE and DEAD staining of bacterial biofilms can be conducted using a combination of fluorescein diacetate (FDA) and propidium iodide (PI) dyes or by commercially available LIVE/DEAD kit containing propidium iodide (PI) and SYTO9 fluorescent dyes [136, 138]. Commonly used microtiter well plate-based assays are summarized in **Table 1**.

In order to gain information on the mechanistic action of nanoparticles, antimicrobial assays can be conducted in two modes, prior to and post biofilm formation. In the pre-exposure mode, nanoparticles and bacteria are simultaneously added, whereas in post-exposure mode, biofilms are first allowed to form, followed by the exposure to nanoparticles. Crystal violet staining has been used to evaluate the impact of nanoparticles on biofilm formation and eradication, respectively [139, 140, 127, 141], and viable plate counts have been utilized in the

| Assays | Endpoint | Read-out | Planktonic bacteria | Biofilm | Ref. |
|---|---|---|---|---|---|
| Measurement of optical density | Growth inhibition, MIC | Absorbance | X | X | [119, 125] |
| Resazurin staining assay | Viability, MIC | Fluorescence | X | X | [117, 136] |
| Tetrazolium salt reduction assay (MTT, XTT, MTS) | Viability | Fluorescence | X | X | [111, 132, 142, 143] |
| Crystal violet staining assay | Biofilm biomass, MBIC | Absorbance | – | X | [132] |
| Fluorescein diacetate (FDA) assay | Viability | Fluorescence | X | X | [143, 144] |

**Table 1.** Microtiter well plate–based assays used to investigate antimicrobial activity of various nanoparticles.

investigation of the antimicrobial efficacy of nanoparticles in the prevention of biofilm formation and eradication of pre-formed biofilms [111, 112].

## 5. Conclusion

There is a strong demand to develop novel antimicrobial materials, and the emergence of nano-technology is creating a variety of options in this respect. Numerous nanoparticles exhibit antibacterial activity against several bacterial species. Today, nanomaterials are a promising platform to control bacterial infections in a broad range of applications. However, the absence of standardizations in testing methods leads to inconsistency in results. The foremost requirement of the assays applied to the evaluation of antimicrobial activity is reproducibility. Antimicrobial activity should be tested against various microorganisms, preferably against representatives of both Gram-negative and Gram-positive species. Moreover, a combination of several assays is preferred to confirm the activity. Several standardized methodologies exist for testing the antibacterial activity of conventional agents against planktonic bacteria. These methods are not applicable for biofilms, and further, they do not allow the prediction of the *in vivo* activity against biofilm-growing bacteria. The majority of the bacterial infections are nowadays attributed to biofilm formation, standardized test methods are urgently needed for more accurate evaluation of antimicrobial activity against biofilms. Even though nanoparticles represent a prominent approach to combat both multi-drug resistant and biofilm-related infections, lack of standardization of synthesis and testing methodologies is a significant concern. Several assays have been reported so far to test the anti-biofilm activity of nanoparticles-containing formulations. However, since antimicrobial assays are sensitive to variation in assay conditions, only standardization of these methods enables comparative analysis between studies.

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