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Nanomaterial-based therapeutics for antibiotic-resistant bacterial infections

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Abstract

Antibiotic-resistant bacterial infections arising from acquired resistance and/or through biofilm formation necessitate the development of innovative ‘outside of the box’ therapeutics. Nanomaterial-based therapies are promising tools to combat difficult bacterial infections, featuring the capacity to evade existing mechanisms associated with acquired drug resistance. In addition, their unique size and physical properties give them the capability to target biofilms, overcoming refractory infections. In this review, we highlight the general mechanisms by which nanomaterials can target bacterial infections associated with acquired antibiotic resistance and biofilms. We emphasize design elements and properties of nanomaterials that can be engineered to enhance potency. Finally, we present recent progress and remaining challenges for widespread clinical implementation of nanomaterials as antimicrobial therapeutics.

Introduction

The emergence of antibiotic resistance in bacteria has resulted in the challenge of refractory infections[1,2]. Multidrug-resistant bacteria are a global crisis, increasing morbidity and mortality of infected patients and negatively impacting the outcome of a wide range of groups, including those in intensive care units, undergoing surgery, transplantation, or cancer treatment[2,3]. A 2017 report from WHO’s Global Antimicrobial Surveillance System highlighted antibiotic resistance as a world-wide challenge[4]. The estimated cost of treating a patient with an antibiotic-resistant infection is US $50,000, with an estimated US $20 billion

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Author Contributions

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societal costs annually[5]. The use, and in some situations misuse, of antibiotics, combined with the scarcity of new therapeutics entering the antibiotic pipeline, further exacerbate this public health threat[6].

Planktonic (free-floating) bacteria are central players in multiple health threats, including sepsis[3]. Infections associated with planktonic bacteria present acute threats and are rapidly becoming more challenging to treat due to rising rates of acquired antibiotic resistance. This challenge is amplified when bacteria form biofilms, which are associated with recurring and chronic bacterial infections[7]. The ability of bacteria to protect themselves within biofilms complicates treatment of numerous infection-types, including chronic wounds, osteomyelitis, and infective endocarditis[8]. Antibiotic resistance associated with the biofilm state is distinct from acquired resistance, but can compound and exacerbate therapeutic challenges[9]. Biofilms produce extracellular polymeric substance (EPS) that may serve as a barrier against host immune response and some conventional antimicrobial agents[7,9]. More importantly, biofilms exhibit a diversity of altered phenotypes, including slow growth rates, presence of persister cells, and creation of spatial and chemical heterogeneities that contribute to resistance to many available antibiotics[10,11].

Antibiotics are currently the main therapeutic strategy for treating both planktonic and biofilm infections[12]. They target processes necessary for growth and/or survival of bacteria, including cell wall/cell membrane synthesis/maintenance, or production of DNA, RNA or essential proteins. Many antibiotics are derived from products that have been deployed by microorganisms to combat one another for billions of years. The offensive tools generated by microbes in this warfare have generated defense responses; bacteria have developed the intrinsic ability to evolve and escape the killing mechanisms of many traditional antibiotics[13]. Eradicating multidrug resistant (MDR) bacteria may require multiple or high dosages of antibiotic agents or the use of ‘last resort’ antibiotics[12]. Adding to the therapeutic challenge, when bacteria are in biofilms, biofilm-associated resistance becomes a compounding factor, oftentimes requiring aggressive physical removal of the biofilm through aggressive debridement, for example, accompanied by high doses of antimicrobial chemotherapy[14,15]. These strategies can result in long and expensive treatments, with the possibility of adverse effects and uncertain outcomes.

Nanoparticles (NPs) access antimicrobial modalities that are novel to bacteria, and hence not in their natural defensive arsenal (BOX 1). Recent advances in nanomaterial-based systems provide new opportunities to address MDR planktonic alongside biofilm infections, acting either as inherent therapeutics or nanocarriers for antimicrobial agents[16]. The unique physico-chemical properties of nano-sized materials, such as size, shape, and surface chemistry, influence their therapeutic activity[17]. The sizes and shapes of different nanomaterials are analogous to bacterial biomolecular components, affording a variety of interactions that can be regulated through surface functionalization. High surface to volume ratios and multivalent interactions are important for creating antibacterial NPs[16,17]. Nanoparticles are able to evade existing resistance mechanisms and may be less prone to select for resistance than are conventional antibiotics (BOX 2)[47]. Moreover, nanomaterials have the ability to eradicate bacteria in biofilms[17]. Taken together, nanotechnology provides

a new toolkit for the creation of efficient treatment strategies against MDR planktonic and biofilm infections.

In this review, we illustrate how nanomaterials could be used to combat MDR bacterial infections. We discuss properties and design elements that result in therapeutic efficacy, providing insight into how nanomaterials might be tailored to optimize activity against planktonic and biofilm bacteria. Finally, we highlight the status of clinical development of antibacterial nanomaterials.

Mechanisms against planktonic bacteria

The array of sizes and shapes accessed by nanomaterials offers unique capabilities for targeting bacteria[21] (FIG. 1). Nanoparticles can employ multiple bactericidal mechanisms, including direct cell wall and/or cell membrane damage, generation of reactive oxygen species (ROS) and/or binding to intracellular components. Most antibiotics target cell walls/cell membranes or disrupt intracellular processes. Nanomaterials can access these pathways, albeit in different ways, and offer advantages in combating antibiotic-resistant pathogens relative to small molecule drugs (TABLE 1). Further, nanomaterials can be used as nanocarriers for delivery of therapeutic agents[19,21]. The mechanisms employed by nanomaterials arise from their unique physico-chemical properties, in particular multivalent interactions with bacterial cells. Van der Waals forces, receptor-ligand interactions, hydrophobic interactions and electrostatic attractions play a role in NP-bacteria interfaces[58].

Cell wall and membrane disruption

The cell envelope has evolved to serve as a physical barrier towards antimicrobials. Teichoic acids - present in the cell wall of Gram-positive bacteria - and lipopolysaccharide - found in the outer membrane of Gram-negative bacteria - have phosphate groups that render bacterial surfaces negatively charged. This highly polar environment limits penetration of hydrophobic antimicrobials across bacterial membranes, compromising their activity[58].

Bacterial cell surfaces are more negatively charged than are those of mammalian cells, facilitating preferential electrostatic interactions with positively charged materials[68]. The charge density and hydrophobicity of the NP surface are important factors in designing NPs to selectively disrupt bacterial membranes[36, 69 , 70]. Highly cationic nanomaterials can bind to the surface of mammalian cells, as can NPs with overly hydrophobic surfaces, reducing selectivity. Cationic nanomaterials with good amphiphilic balance can provide potent antimicrobial effects with low hemolysis and cytotoxicity[36].

A range of nanomaterial-based strategies focus on targeting the negatively charged surface of planktonic bacteria[33,36,55,69]. Yang et al. fabricated biodegradable cationic and amphiphilic polycarbonates that self-assemble into cationic micellar NPs, killing methicillin-resistant *S. aureus* (MRSA). These polymeric NPs interact with bacteria through electrostatic interactions, resulting in disintegration of the membrane and cell lysis[71]. ‘Nanoknifes”, materials with sharp-pointed edges, are particularly effective in compromising bacterial membrane integrity. In one study, single-walled carbon nanotubes and graphene oxide ruptured the cell surface of *Ralstonia solanacearum* leading to cytoplasmic leakage

and bacterial death[72]. The ability of bacteria to develop resistance against therapeutics that damage the cell envelope is likely to be limited, making these strategies promising for long-term use with minimal risk of emergence of bacterial resistance[55,73].

Generation of reactive oxygen species

Reactive oxygen species (ROS) are byproducts of cellular oxidative metabolic processes that affect cell differentiation, signaling, survival and death[22]. Accumulation of excessive ROS results in lethal oxidative stress. ROS can damage cells through multiple mechanisms, in particular through reaction of superoxide and hydroxyl radicals with thiols in proteins, deactivating membrane-located receptors[74]. There are several mechanisms by which NPs generate ROS: 1) direct ROS production from the NP surface or from leached ions; 2) interaction with intracellular organelles; and 3) oxidation through interaction with redox active biomolecules, including NADPH oxidase[23]. Some metal-based NPs employ ROS generation as their major antibacterial mechanism due to their inherent photocatalytic activity (i.e., photodynamic therapy)[22,30,75]; reviews discussing ROS activity of metal nanoparticles are available[22,23,76].

An example of ROS-based antibacterial activity is the release of free Cu^{+} from copper iodide (CuI) NPs, generating ROS and damaging bacterial DNA and intracellular proteins of *E. coli* and *B. subtilis*[77]. Silver-zinc oxide nanocomposites likewise exhibited antibacterial activity against *S. aureus* and antibiotic-resistant *E. coli* ascribed to potent ROS generation and release of silver (Ag^{+}) and zinc (Zn^{2+}) ions. These combined processes then generated a cascade of bactericidal effects, including damaged cell membranes, protein dysfunction, inhibition of DNA replication and leakage of intracellular materials[78]. Silver and other Fenton-inactive metals increase ROS in bacteria by their ability to disrupt cellular donor ligands coordinating with iron, such as cysteine, and to induce release of Fe from [4Fe-4S] clusters. This Fe release then increases ROS formation[22].

Gold nanoparticles (AuNPs) have also shown enzyme-like activities[79]. Mesoporous silica (MSN) can provide support and enhance the stability and catalytic-activity of the AuNPs[80]. AuNPs bound on the surface of bifunctionalized MSN (MSN-AuNPs) displayed peroxidase- and oxidase-like activities, killing both Gram-positive and Gram-negative bacteria. The dual enzyme-like activity of this system increases efficiency of ROS production increasing oxidative stress to bacteria[81].

Damage to intracellular components

Cellular homoeostasis and intracellular signaling pathways are central to the function and survival of bacteria. Nanomaterials can be engineered to interfere with these processes, ultimately leading to cell death. These disruptions include alteration in gene or protein expression or DNA damage[82,83]. As an example, AuNPs were functionalized with 4,6-diamino-2-pyrimidinethiol, an analogue of 2-pyrimidinethiol (found in *E. coli*), to generate pyrimidine-capped AuNPs (Au-DAPT)[84]. These NPs completely inhibited proliferation of MDR strains of *E. coli* and *P. aeruginosa*. Mechanisms of action of Au-DAPT were elucidated through the following: 1) gel electrophoresis showing the ability of NPs to bind bacterial DNA; 2) TEM images displaying leakage of nucleic acids and

binding to ribosomes and chromosomes; 3) an *E. coli*-free transcription/translation system demonstrating protein synthesis inhibition; and 4) colorimetric assays showing selective chelation of Mg^{2+}, destabilizing the cell membrane. Similarly, polymer-coated silver NPs killed *E. coli* cells by inhibiting both the Krebs cycle and amino acid metabolism[85]. Polymers were used to modify the surface of AgNPs to increase interactions with bacterial cells. The mechanism of action was confirmed by the downregulated expression of *aceF*, *frdB*, *gadB*, *metL* and *argC*, ultimately leading to cell death.

Delivery of therapeutic agents

Several nanodrugs - liposomal nanoformulations in particular - have been FDA-approved and made available for clinical use to treat different diseases, including cancer[86]. Similarly, NPs may be used as carriers for delivery of antimicrobial agents[87]. Therapeutics can be encapsulated inside NPs or bound to their surfaces[88,89]. NPs offer protection of these agents against enzymes and molecules that might otherwise degrade them. This protection can increase therapeutic efficiency of a drug, resulting in decreased dosage requirements to achieve desired effects and therefore reduced host toxicity[90]. The use of delivery systems can also enhance stability, solubility and biocompatibility of otherwise pharmacologically challenging antibiotics. Use of nanocarriers can minimize selection of resistance through delivery of therapeutics that elicit multiple mechanisms of action, and through targeted release of cargo which prevents exposure of bacteria to sub-inhibitory doses of the drug[42,44]. For instance, the antibiotic gentamicin loaded into poly(lactide-co-glycolide) NPs exhibited improved antimicrobial activity against *in vitro* and *in vivo P. aeruginosa* infection[88]. Subsequently, levofloxacin loaded into silver core-embedded mesoporous silica nanovehicles (Ag@MSNs@LEVO) afforded a synergistic treatment of MDR isolates of *E. coli*. The silver component of the system not only functions as a carrier but also imparts antimicrobial effects via silver ion generation. In an *in vivo* murine peritonitis model, treatment with Ag@MSNs@LEVO reduced bacterial burden by three orders of magnitude, with concomitant reduction of damage to the spleen and peritoneum. No toxic side effects were observed[91]. In a related approach, ampicillin was attached to the surface of AuNPs and AgNPs, yielding broad-spectrum bactericidal agents that evade resistance mechanisms of MDR strains of *P. aeruginosa* and *Enterobacter aerogenes* and of MRSA[89].

Therapeutic selectivity and enhancement of delivery efficiency can be achieved via release of drug in response to specific stimuli[44,92]. Bacterial infection sites are weakly acidic and that can be targeted[34,44,93]. For example, vancomycin was encapsulated in a pH-responsive, surface charge-switching triblock copolymer poly(D,L-lactic-co-glycolic acid)-b-poly(L-histidine)-b-poly-(ethylene glycol) (PLGA-PLH-PEG). Therapeutic cargo was released only upon interaction with the acidic infection site, providing a target for vancomycin delivery[93]. PLGA was chosen due to its low toxicity and ease of surface fine tuning; PEG reduced off-target interactions, prolonging circulation time; and PLH provided the charge-switchable characteristic of the polymer. The selective protonation of the imidazole groups of PLH at weakly acidic conditions allows for a stimuli-responsive effect. Biomaterials can also provide charge-switching behavior, with pH-triggered release of vancomycin achieved using chitosan NPs[34]. Furthermore, bacterial toxins can be used as a trigger for release of antimicrobials. Lecithin and DSPE-PEG3400 were used to coat a mixture of fatty acids,

forming liposome-based nanoreactors that release calcium peroxide and rifampin in the presence of alpha-toxin, a pore-forming toxin produced by *S. aureus*[94]. This strategy selectively targets pathogenic bacteria as demonstrated by the higher antimicrobial activity against MRSA and minimal effect on non-pathogenic *B. subtilis*.

Overall, nanomaterials provide multiple bactericidal pathways to combat bacteria and evade antibiotic resistance mechanisms. Appropriate engineering of size, shape and surface properties provides a broad design space for novel antimicrobial agents.

Combating planktonic bacterial infections

Drug resistant hospital-acquired (nosocomial) infections are challenging to treat. A group of pathogens comprised of *Enterococcus faecium, S. aureus, Klebsiella pneumoniae, Acinetobacter baumannii, P. aeruginosa,* and *Enterobacter* species - collectively termed as 'ESKAPE' pathogens - is responsible for the majority of nosocomial infections, complicating the conditions of patients that are often immunocompromised[2,3,6]. Methods for treating infections caused by these pathogens are becoming increasingly limited due to the rapid rate of resistance development even against 'last resort' antibiotics[6]. In this regard, nanomaterials can provide a lifeline for therapeutic design, as studies have shown that there is limited to no resistance development observed with nanomaterial-based strategies[47, 51, 71,73].

Numerous studies have explored the utility of nanomaterials against the 'ESKAPE' pathogens (FIG. 2)[100, 101, 102,103]. Qiao et al. reported the activity of star-shaped polymeric peptide NPs (SNAPPs) against MDR Gram-negative ESKAPE pathogens, *in vitro* and in an *in vivo* murine peritonitis model[63]. Researchers designed artificial antimicrobial peptide (AMP)-inspired peptide polymer NPs consisting of lysine and valine residues that self-assemble into star-shaped unimolecular structures, mimicking AMPs. SNAPPs elicit multiple proposed bactericidal mechanisms, including damage to outer and inner cell membranes, disruption of ion efflux/influx regulation and induction of an apoptotic-like death pathway. The proposed multimodal antimicrobial activity of SNAPPs renders the barrier to resistance high. Comparing the concentration that results in death of 50% mammalian cell population (IC_{50}) and concentration which kills half of bacterial isolates [minimum bactericidal concentration (MBC_{50})], SNAPPs had a therapeutic index higher than colistin, a drug of last resort for MDR Gram-negative bacillary infections. Furthermore, MDR *A. baumannii* did not acquire resistance towards SNAPPs after multiple passages in sub-inhibitory concentrations. Liposome-based NPs are another promising system, restoring potency of the antibiotics cefepime, imipenem and ceftazidime against MDR *P. aeruginosa*[104], chloramphenicol against MRSA[105] and amikacin against *K. pneumoniae*[106] through efficient drug delivery. Similarly, delivery of antimicrobial peptides was achieved with the use of PLGA NPs, providing a successful treatment strategy for *P. aeruginosa* lung infection in an *in vivo* murine model[95].

Combating intracellular bacterial infections

Bacteria can reside within mammalian cells, giving rise to recurring systemic infections[107]. For example, *Salmonella enterica* is a common facultatively intracellular pathogen that causes life-threatening food-borne infections in millions of people worldwide each year[108]. *Salmonella* can survive and replicate inside host cells, including macrophages. Intracellular localization of bacteria adds a level of complexity to treatment, because many antibiotics have limited ability to cross mammalian cell membranes and can also be actively exported out by the host cell[109, 110]. Nanomaterials can mitigate this challenge through their ability to penetrate inside eukaryotic cells, as well as via their high drug loading capacity (FIG. 2).

In one example of nanomaterial-based treatment of intracellular infections, enrofloxacin-loaded docosanoic acid solid lipid nanoparticles (SLNs) increased intracellular accumulation of enrofloxacin up to ~40-fold and enhanced *Salmonella* killing inside macrophages[111]. In another approach, colistin, a poorly permeable antibiotic, was formulated into liposomes functionalized with a bacterial-derived protein to promote internalization into eukaryotic cells to provide therapeutics with high oral bioavailability[112]. In yet another strategy, gentamicin was loaded into mesoporous silica nanoparticles with bacterial toxin-responsive lipid bilayer surface shells. Functionalized with bacteria-targeting peptide $UBI_{29–41}$, allowing targeted treatment of intracellular *S. aureus*[97].

Mycobacterium tuberculosis is another example of an intracellular pathogen that survives within host macrophages, invading the lungs and causing tuberculosis (TB)[113]. Several studies have demonstrated the activity of nanomaterials against intracellular *Mycobacterium* species. Yang et al. reported a library of cationic star-shaped polycarbonate nanostructures with excellent wide-spectrum antimicrobial activity and low rates of hemolysis[114]. Mannose-functionalized polycarbonate demonstrated enhanced intracellular antimycobacterial activity by targeting mannose receptors on the surface of macrophages. In another study, biodegradable multimetallic microparticles (MMPs), consisting of Ag NPs and ZnO NPs encapsulated within PLGA polymer, were utilized as a pulmonary delivery system to enable delivery of antituberculosis drug rifampicin within alveolar macrophages[115]. Further, the ability of AgNPs and ZnONPs to interact with and compromise bacterial membrane stability furthered the antimicrobial effects of the system.

Nanomaterial-based strategies to combat other intracellular pathogens have been developed. For example, AuNP-DNA aptamer conjugates loaded with antimicrobial peptides showed activity against intracellular *Salmonella enterica*[96] and *Vibrio vulnificus*[116] in *in vivo* murine infection models. Gentamicin-loaded AuNPs decorated with phosphatidylcholine eradicated intracellular *Listeria monocytogenes* and *P. aeruginosa* in infected macrophages[87].

Therapeutic strategies against biofilms

MDR biofilm infections present a particularly difficult therapeutic challenge[117]. The matrix provided by the EPS may provide a barrier to some cellular and small molecule (e.g., antibiotic) assaults. Bacteria embedded within EPS matrix are capable of synergistic interactions, cell-to-cell communications and transfer of resistance genes[10,11]. Furthermore,

the lower layers of the matrix have low oxygen and nutrient supply, inducing formation of dormant persister cells, which promote antimicrobial tolerance and resistance[117,118].

Overcoming the physical barrier presented by biofilms is needed to combat biofilms. The EPS is comprised of biopolymers including nucleic acids, proteins and polysaccharides that provide a three-dimensional protective scaffold for bacteria. The matrix is rich in negatively charged components and hydrophobic groups, with pores filled with water facilitating transport of nutrients[10]. Tuning surface functionality and design of NPs can facilitate biofilm penetration (BOX 3)[119, 120]. Size and electrostatic interactions are important factors influencing biofilm penetration profile of nanomaterials. Generally, uncharged NPs with sizes <350 nm have higher mobility across pores inside biofilms while cationic NPs have good distribution throughout the matrix[62,121,122,123].

Targeting resident pathogens

Upon biofilm penetration, nanomaterials can interact with bacteria and exert the therapeutic mechanisms discussed above for planktonic bacteria (FIG. 3a). For instance, the efficient biofilm penetration profile and bacteria membrane-damaging activity of poly(oxanorborneneimide)-based cationic polymeric NPs eradicated MDR biofilms of *P. aeruginosa*, *E. cloacae* complex and MRSA[62]. In another approach, the use of stimuli-responsive NPs provided activation of bactericidal effects in a spatio-temporally controlled manner. pH-responsive silver nanoantibiotics (rAgNAs) were developed using self-assembled silver nanoclusters and charge-switchable ligands poly(ethyleneglycol)-poly(aminopropyl imidazole-aspartate)-polyalanine (PEG-PSB-PALA)[130]. Protonation of the imidazole groups in the low-pH biofilm microenvironment induced disassembly of rAgNAs due to electrostatic repulsion with silver ions. Disassembly into smaller Ag nanoclusters allowed biofilm penetration, killing deeply embedded MRSA cells. Similarly, application of an external magnetic field facilitated biofilm penetration of silver nanoparticles[134]. Superparamagnetic iron oxide nanoparticles were coated with silver rings; the generated magnetic field allowed biofilm penetration, with silver conferring antibacterial activity.

Nanomaterials can also deliver therapeutics to bacterial cells embedded within the EPS matrix. For example, although the potent antimicrobial carvacrol, an essential oil found in oregano and thyme, poorly penetrates biofilms, Rotello et al. utilized carvacrol to eradicate biofilms using biodegradable oil-in-water crosslinked polymeric nanocomposites (X-BNCs). X-BNCs eliminated MDR biofilms of both Gram-negative and -positive bacteria while maintaining minimal cytotoxicity towards mammalian cells[73]. The polymer scaffold (PONI-GMT) contained guanidinium, maleimide and tetraethylene glycol monomethyl ether groups. The cationic property of the nanocomposite was attributed to guanidinium. The presence of maleimide groups provided crosslinking sites and an additional mode of degradation while the tetraethylene glycol monomethyl ether imparted hydrophilicity to the assembly. Careful design of the polymer increased solubility, stability, biodegradability, and antimicrobial potency of carvacrol oil while assisting its penetration across the matrix. Similarly, nanoscale liposomes delivered the antibiotic amikacin through size-dependent

biofilm penetration, treating chronic *P. aeruginosa* biofilm lung infections[135]. This system is currently at Phase III of clinical trials.

Disrupting the EPS matrix

Beyond killing bacteria, it is important to disrupt the EPS matrix for treatment of biofilms[136]. EPS scaffold remaining after treatment can be inhabited and populated by other microbes. Different NP-based approaches can be employed to disperse the EPS matrix, including mechanical disruption and delivery of matrix-degrading enzymes (e.g., DNAse, hydrolase, protease) (FIG. 3b). For example, poly(lactic-co-glycolic acid) NPs loaded with ciprofloxacin were functionalized with DNase I to eradicate *P. aeruginosa* biofilms[137]. DNase degraded eDNA which then rendered the 3D network fragile and susceptible to ciprofloxacin. Similarly, AuNPs functionalized with proteinase-K dispersed *Pseudomonas fluorescens* biofilm[138]. Alternatively, magnetic iron oxide nanoparticles (MNPs) disrupted MRSA biofilms with the application of direct current (DC) and alternating current (AC) magnetic fields[139]. Application of a rotating DC magnetic fields mechanically damaged the biofilm matrix. MNPs traversing across the 3D network acted as “shield breakers”, destroying the biofilm through static friction. Exposure of MNPs to AC magnetic field resulted in a localized increase in temperature that dispersed embedded cells. Since the mechanisms of action of these MNPs do not include killing of bacteria, this system offers a long-term anti-biofilm strategy that may escape resistance development.

A promising strategy for targeting biofilm growth is the interruption of bacterial communication systems essential for coordinated activities, including colonization and biofilm development. Bacteria communicate through quorum sensing (QS), a process that can be sabotaged to prevent formation of biofilms or induce their dispersion[48,124,140]. Decho et al. demonstrated that hampering QS can silence bacterial communication[141]. Silicon dioxide NPs (Si-NPs) decorated with β-cyclodextrin (β-CD) blocked communication between *Vibrio fischeri* cells. *V. fischeri* exhibits bioluminescent output controlled by population density, that can be monitored via the QS signaling molecule acylhomoserine lactone (HSL). The β-CD group of Si-NPs binds to HSL, quenching its activity. As a result, the luminous output of *V. fischeri* was reduced. Further, downregulation of luminescence genes, *luxA* and *luxR*, was observed. Other studies have demonstrated inhibition of biofilm formation and virulence factors by deactivating quorum sensors using liposome-based NPs[43], chitosan nanoparticles[35, 142] and metal-based nanoparticles[143,144].

Nanomaterial penetration profiles predict success of biofilm elimination. Size and amphiphilicity mainly influence NP distribution across the biofilm. The exact interactions of NPs with the EPS also depend on the type of biofilms which varies by species and in some cases strain of bacteria. The controllable parameters of nanomaterials provide a flexible toolkit to address the diversity of biofilm infections.

Combating biofilm infections

The number of biofilm-related infections continues to grow each year[145 , 146]. Bacteria can form biofilms in and on tissues and organs, including on skin, in the oral cavity, and on linings of gastrointestinal and respiratory tracts[8,117]. Biofilms largely contribute to

chronic and persistent infections. With advances in the understanding of medical biofilms, nanotherapeutic strategies have emerged to potentially address biofilm infections.

Oral biofilms

The oral cavity is a prevalent site for biofilms; *Streptococcus mutans* is a common oral biofilm pathogen. The acidic microenvironment of dental biofilms (i.e., plaque) results in destruction of tooth apatite, causing dental caries[147,148]. NP-based strategies have been used to address oral biofilm-associated infections, taking advantage of the highly acidic oral biofilm microenvironment. Liposomes coated with the quaternary ammonium-modified chitosan were used to deliver the antibiotic doxycycline to *Porphyromonas gingivalis* oral biofilms[149]. The residual amines of chitosan provided pH-responsive groups that were protonated under acidic conditions, providing pH-based activity. Similarly, nanocarriers fabricated with pH-responsive block copolymers that can bind to negatively charged hydroxyapatite were used to deliver farnesol[150] and chlorhexidine[151] for treatment of dental caries. NPs that induce ROS production and EPS matrix degradation are also being investigated for oral biofilm treatment. For instance, catalytic NP (CAT-NP) consisting of biocompatible Fe_3O_4 were utilized to catalyze *in situ* generation of free radicals from H_2O_2, resulting in a reduction of *S. mutans* biofilms[152]. Coating iron oxide NPs with FDA-approved polymers, such as dextran, increased its stability in aqueous formulation and enhanced biocompatibility with oral soft tissues[153]. The iron-supplying nanotherapeutic ferumoxytol was ‘reinvented’ from an iron deficiency drug into a topical oral biofilm therapeutic[99]. This FDA-approved iron-based nanoparticle possesses a pH-dependent peroxidase-like property that provides localized catalytic activity (FIG. 2e). This work demonstrated that ferumoxytol can bind within the biofilm matrix and generate free radicals from H_2O_2, resulting in *in situ* bacterial death and EPS degradation. Both a human-derived *ex vivo* model and an *in vivo* rodent dental caries model revealed efficacy in preventing acid damage of the enamel and suppression of dental caries without altering the oral microbiota and with safety towards gingival and mucosal tissues.

Wound biofilms

Wound infections affect ~300 million people worldwide, with treatment costs estimated as high as $25 billion in the US alone[154,155]. In these infections, necrotic tissue fosters attachment of bacteria and provides nutrients that enhance bacterial proliferation and biofilm formation, which impedes wound healing by inhibiting re-epithelialization and prolonging inflammation[15,145,156]. Silver NPs incorporated in hydrogels or in wound wraps are commonly used to treat wound infections[157]. Other types of nanoparticles have also been increasingly studied for the treatment of biofilm-infected wounds[158,159]. For example, copper particles incorporated into biodegradable nanofibers prevented formation of and eradicated preformed biofilms of *P. aeruginosa* and *S. aureus.* Further *in vitro* and *in vivo* studies are underway to demonstrate the applicability of this strategy for wound dressings[160]. Another strategy utilizes the amphiphilic core-shell polymeric NP, DA95B5, which removes preformed biofilms of MRSA via nanoscale bacterial ‘debridement’[98] (FIG. 2f). DA95B5 can diffuse through the EPS, disrupting biofilms by weakening attachment of bacteria to the matrix. An *in vivo* murine excisional wound biofilm model demonstrated effective dispersal of MRSA biofilms. DA95B5-soaked hydrogel pad dressings reduced

bacterial counts in mice up to ~4 log CFU. Notably, the NP exhibited minimal *in vitro* eukaryotic cell lysis and low *in vivo* toxicity. Combination of these NPs with molecules that accelerate the wound healing process, including growth factors, anti-inflammatory molecules and extracellular (ECM) mimics, can further NP-based strategies. As an example, a pH-responsive antimicrobial nanofiber network, formed by the self-assembly of octapeptide IKFQFHFD, was incorporated into a hydrogel and loaded with cypate and proline[161]. The octapeptide possessed an inherent antimicrobial property via cell wall and membrane disruption; cypate is a photothermal drug that is anticipated to disrupt EPS matrix; and procollagen component proline is added to aid in collagen and ECM matrix reformation. The hydrogel eradicated MRSA biofilms and facilitated healing in chronic wounds as demonstrated in an *in vivo* diabetic mice model.

Towards clinical translation

There has been a rapid increase in the exploration of antimicrobial nanomaterials for treatment of MDR planktonic bacteria and biofilm infections. Most studies have been conducted *in vitro*, with fewer proceeding to animal models, and still fewer proceeding to human testing[86,162]. Developing appropriate *in vitro* and *in vivo* models that demonstrate efficacy and safety of NPs will provide clinical feasibility for their use. Several reviews have summarized appropriate *in vitro* and *in vivo* models to explore depending on the type of infection being targeted[7,145,163].

Successful clinical translation will require standardized guidelines for evaluating biocompatibility and nanotoxicology. Most formulations undergoing clinical testing are nanocarriers for antibiotic delivery or antimicrobial silver nanoparticles (TABLE 2). Two liposomal nanoformulations for controlled delivery of antibiotics are currently at Phase III clinical trials. Arikace was designed to improve the therapeutic efficiency of amikacin as well as alleviate its renal and neurological toxicity[164]. Pulmaquin is nanoliposome-based formulation for the rapid and delayed release of ciprofloxacin[165]. Many challenges still hamper nanodrug translation into clinical settings such as safety concerns, however, it is likely only a matter of time until these novel therapeutics provide solutions for currently unmet clinical demands[86,162].

Conclusions and perspectives

Nanomaterials present an emerging ‘outside of the box’ toolkit for treatment of resilient MDR planktonic bacteria and biofilm infections. Their tunable properties, particularly their surface functionalities, provide design spaces that can be fine-tuned to maximize therapeutic effect while minimizing host toxicity. In this review, we provided examples of how NPs can combat bacteria in both planktonic and biofilm forms, using a wide range of mechanisms. Nanomaterials can access multi-modal antibacterial mechanisms that are novel, slowing or stopping the generation of drug resistance. NPs have potential as topical treatments for oral and wound biofilm-associated infections. Strategies combining bactericidal effects and biofilm dispersion, however, are required to assure complete eradication of biofilms. Stimuli-responsive NPs that take advantage of unique microenvironments at infection sites, such as pH and pathogen-derived metabolites, provide one of the many pathways to target MDR bacteria using nanomaterials. Systemic safety and long-term effects of NPs on the

body are still among the major barriers to clinical use. Current studies are determining the pharmacokinetic profile of NPs to better understand their fate in the body.

The generation of effective antimicrobial nanomaterials requires interdisciplinary collaborations among chemists, biomedical researchers (including microbiologists), and engineers. Likewise, partnership between fundamental, translational and industrial agencies will be instrumental in moving antimicrobial nanomaterials to the clinic. Overall, nanomaterial-based treatment strategies offer a promising alternative to antibiotics for difficult-to-treat infections, alleviating challenges faced in the post-antibiotic era.

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Glossary

Osteomyelitis
Bone infection

Infective endocarditis
Infection of endocardium, typically of heart valves

Persister cells
Subpopulation of dormant, antibiotic-tolerant bacterial cells that is able to resume growth after antimicrobial stress is relieved

Debridement
Surgical removal of damaged or dead tissue from an infected wound

Nanocarriers
A drug delivery platform in the nanoscale range (1–1000 nm). Common nanocarriers include liposomes, polymers and micelles

Peritonitis
Inflammation of the peritoneum, the tissue layer lining the inner wall of the abdomen, often as a result of bacterial infection

Therapeutic index
A quantitative measure of the relative safety of a drug determined by the dosage that produces a therapeutic effect without host toxicity and the concentration that results in dangerous side effects

Quorum sensing
A process whereby bacteria communicate and perform coordinated activities in response to a particular cell population density determined by specific signaling molecules

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Box 1|

What are nanomaterials?

Nanomaterials

Nanomaterials are organic, inorganic or hybrid particles, with some defining their size as ≤100 nm, and others including particles ≤ 500 nm[18]. They have an almost unlimited range of structures and morphologies, from rods to pyramids to fibrous networks to spheres with hollow or solid interiors bearing rough or smooth surfaces[19]. Materials in the nanoscale realm possess distinctive physico-chemical characteristics, including size, shape and surface, compared to their bulk counterparts[20]. The unique properties of nanomaterials have revolutionized many technologies and industries, including medicine. Being comparable in size to biomolecules and bacterial intracellular structures, nanomaterials can be engineered to exhibit new therapeutic modalities[21]. Representative classes of nanomaterials for antimicrobial application include metal-based NPs, carbon-based NPs, polymeric NPs, nanocomposites, liposomes and smart nanomaterials.

Metal-based NPs

Metal-based NPs are comprised of either pure metals (e.g., gold, silver, iron) or their compounds, e.g., oxides. Their primary mechanisms of toxicity involve reactive oxygen species production and impairment of membrane function[22,23]. This type of NPs has been demonstrated to be effective in treating several MDR bacterial infections[24,25,26,27]. Silver-based nanomaterials are the most established metal antimicrobials; although the exact mechanism of action for silver NPs is unknown, two widely proposed modes of actions include disruption of membranes by leached silver ions and ion-mediated killing[28,29].

Carbon-based NPs

Carbon-based NPs include carbon quantum dots[30], nanotubes[31] and 2-D materials, including graphene[32]. Their bactericidal action involves physical and chemical damage, however, specific mechanisms are yet to be understood. In one study, multi-walled carbon nanotubes prevented formation of *Klebsiella oxytoca, Pseudomonas aeruginosa* and *Staphylococcus epidermidis* biofilms by blocking bacterial settlement[33].

Polymeric NPs

Polymeric NPs can either be natural or synthetic. Natural polymers are used to fabricate cationic and pH-switchable antimicrobial NPs[34,35]. Synthetic polymeric NPs can mimic the activity of antimicrobial peptides[36,37]. Moreover, polymeric micelles are used as nanocarriers to improve the solubility, stability, efficacy and pharmacokinetic profiles of drugs[38]. Dendrimers are regular polymeric molecules comprised of a central core, branch-like structures radiating from the core, and outer surface bearing functional groups. Glycopeptide dendrimers have been shown to inhibit biofilms of *P. aeruginosa*[39].

Nanocomposites

Nanocomposites are hybrids of inorganic and organic NPs. For example, incorporation of silver NPs into the cationic polymer, poly(2-dimethylamino)ethyl methacrylate,

resulted in a synergistic antimicrobial activity against *P. aeruginosa* and *Staphylococcus aureus*[40].

Liposomes

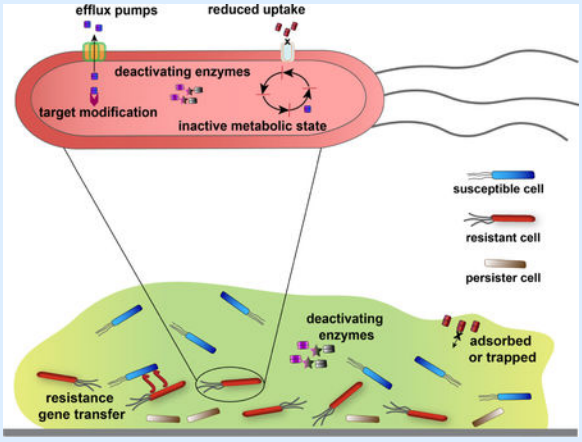
Liposomes are vesicles composed of one or more phospholipid bilayers with an aqueous inner core. Being membrane-based structures, they have good biocompatibility and are useful antimicrobial delivery vehicles[41]. They can encapsulate hydrophilic drugs in their aqueous interior or hydrophobic drugs in their phospholipid membrane[42,43].

Smart nanomaterials

Smart nanomaterials can respond to stimuli, such as pH and bacterial toxins (endogenous), or light, temperature and ultrasound (external), to produce changes in their characteristics that allows them to exert their antimicrobial action[44,45,46]. For instance, hybrid micelles composed of poly(ethylene)glycol, poly(aspartamide), 2-(diisopropylamonio)ethylamine, azithromycin and *cis*-aconityl-D-tyrosine can shrink in size, reverse surface charge and release drug cargo in response to the acidic environment of *P. aeruginosa* biofilms[45].

Box 2|

Antibiotic resistance mechanisms



Bacteria have acquired multiple survival mechanisms that enable them to evade killing by antibiotics. The biofilm state itself confers resistance against antibiotics, separate from genetically acquired antibiotic resistance[48,49]. Nanomaterials are novel to bacteria and can circumvent resistance mechanisms that affect traditional antibiotics; and nanomaterials can target bacterial biofilms.

Resistance gene transfer and target modification.

Bacteria communicate and share genetic information with one another, resulting in spread of resistance genes across bacterial populations[10, 50]. Expression of resistance genes allows modification of antibiotics and protection from them as well. Unlike traditional antibiotics that have specific targets, nanomaterials can have multiple killing mechanisms because they access multiple targets, making emergence of resistance less likely than with traditional antibiotics[47].

Deactivating enzymes.

Resistant bacteria can harbor extracellular and/or intracellular enzymes that degrade antibiotics. The multiple mechanisms of action of NPs and their abiological structure allow them to escape deactivation by these enzymes[47].

Reduced uptake.

Gram-negative bacteria have evolved to limit entry of antibiotics through porin mutations[22]. Nanomaterials, however, enter bacterial cells through other mechanisms, such as endocytosis and membrane fusion[47]. As an example, liposomal NPs loaded with antimicrobial agents enter via membrane fusion and rapidly release high concentrations of antimicrobial agents to membranes or into the cytoplasm[51].

Efflux pumps.

Efflux pumps, which are often upregulated in antibiotic-resistant bacterial cells, actively transport antimicrobial agents outside of bacterial cells. Nanoparticles can block these efflux pumps, increasing accumulation of antibiotics inside bacterial cells[52,53].

Inactive metabolic state.

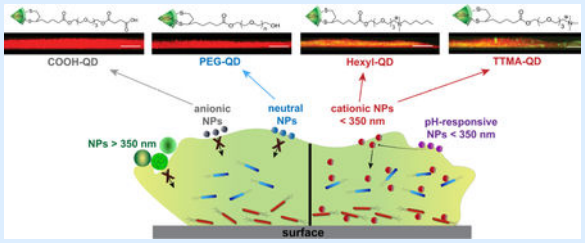
Persister cells are subpopulations of metabolically inactive bacteria with reduced susceptibility to antimicrobials[54]. The killing mechanisms of many nanomaterials, membrane damage in particular, do not require bacteria to be in a state of active growth, rendering these agents active against persisters[55].

EPS limits penetration.

The protective nature of the EPS of bacterial biofilms restricts penetration of some antibiotics, such as aminoglycosides, due to electrostatic repulsion[50,56]. While other antibiotic groups can diffuse into the inner layers of biofilms, the complex gradient of nutrients and waste can diminish their antimicrobial effects[57]. The unique surface chemistry of nanomaterials allows facile penetration into biofilms, and interaction with deeply embedded bacterial cells. The amphiphilic balance of many nanomaterials helps them exert multiple interactions with EPS, including hydrophobic and electrostatic interactions, maximizing adsorption by and diffusion across biofilms.[17,47]

Box 3|

Nanomaterial properties and design elements



Thoughtful engineering of nanomaterial surfaces modulates NP-bacteria interactions. The interface between NPs and bacteria is characterized by hydrophobic and electrostatic interactions and Van der Waals forces that can be modulated by tuning nanomaterial properties[124]. Tuning the size, surface and shape of a nanomaterial can maximize antibacterial activity, biofilm penetration, biocompatibility, biodistribution and therapeutic index[125].

Size

The size of nanomaterials regulates bactericidal activity. Small NPs (2–10 nm) cause more membrane damage than larger ones because of high surface areas of contact with bacterial cells coupled with greater curvatures[126,127]. NPs with sizes less than 350 nm can diffuse through the constrained spaces of pores within biofilms[121,122].

Surface

Nanoparticle surfaces can be functionalized with chemical groups that enable multivalent interactions with bacterial cells and the EPS matrix[17]. Nanoparticles have surface charge-dependent bacterial toxicity (i.e., the more positively charged the surface, the more toxic the NP becomes)[36]. Careful placement of the positive charge and hydrophobic moieties can enhance the antibacterial activity of polymeric NPs, while maintaining minimal cytotoxicity. Furthermore, biofilm penetration can be enhanced by surface modification[120,128,129]. As shown in the figure, anionic and zwitterionic NPs have poor matrix penetration, while cationic NPs with an appropriate hydrophobic balance can penetrate the EPS. Several strategies have taken advantage of the acidic pH of biofilms to switch from anionic or zwitterionic to cationic NPs[45, 130]. Insets are confocal images showing the biofilm penetration profile of quantum dots with different surface charges (scale bar = 20 μm). Figure reproduced, with permission, from REF 131 © (2015) The Royal Society of Chemistry. All rights reserved.

Shape

Contact-killing can be influenced by NP shape; sharp and pointed NPs can puncture bacterial cell membranes, leading to cytoplasmic leakage[32,64,125,132]. Comparisons of the activities of spherical, rod-shaped and truncated triangular silver nanoplates against planktonic cells of *E. coli* reveal that truncated triangular AgNPs possess superior bactericidal effect. This is due to the number of NP facets directly interacting with the bacterial surface. Triangular NPs have more facets than the two other shapes, causing

more membrane damage to bacteria[132]. On the other hand, rod-shaped nitric oxide-releasing silica NPs result in better biofilm eradication than their spherical counterparts, a result attributed to the higher particle aspect ratio of rod-shaped than spherical NP[133].

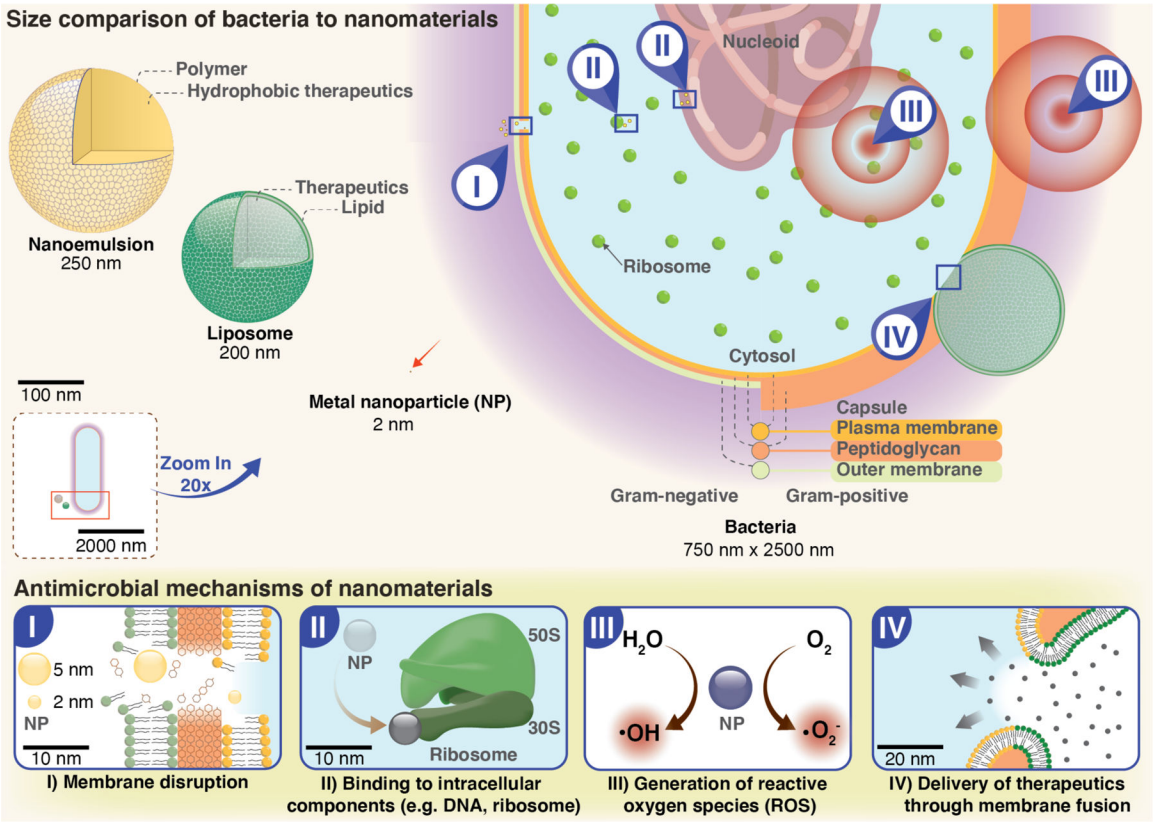


Figure 1. Nano *versus* micro – size comparison between nanomaterials and bacteria.

Bacteria typically have diameters ranging from 0.2 to 10 μm. Varying nanoparticle materials and preparation methods provide a wide range of particle sizes (2–500 nm) that facilitate maximal contact and strong interactions with bacterial membranes. Nanomaterials may display a variety of bactericidal mechanisms: **I| Membrane disruption.** Electrostatic interactions of NPs with the negatively charged groups present on bacterial surfaces results in membrane damage and cytoplasmic leakage. **II| Intracellular damage.** NPs can bind various bacterial components, such as ribosomes, proteins and/or DNA, interrupting their function. **III| ROS.** NPs with catalytic activities increase production of reactive oxygen species, such as hydroxyl radicals and superoxides, causing oxidative cellular stress. **IV| Delivery.** Nanomaterials can be used for delivery of therapeutic agents; some nanomaterials readily enter bacterial cells through membrane fusion, facilitating delivery of their cargo.

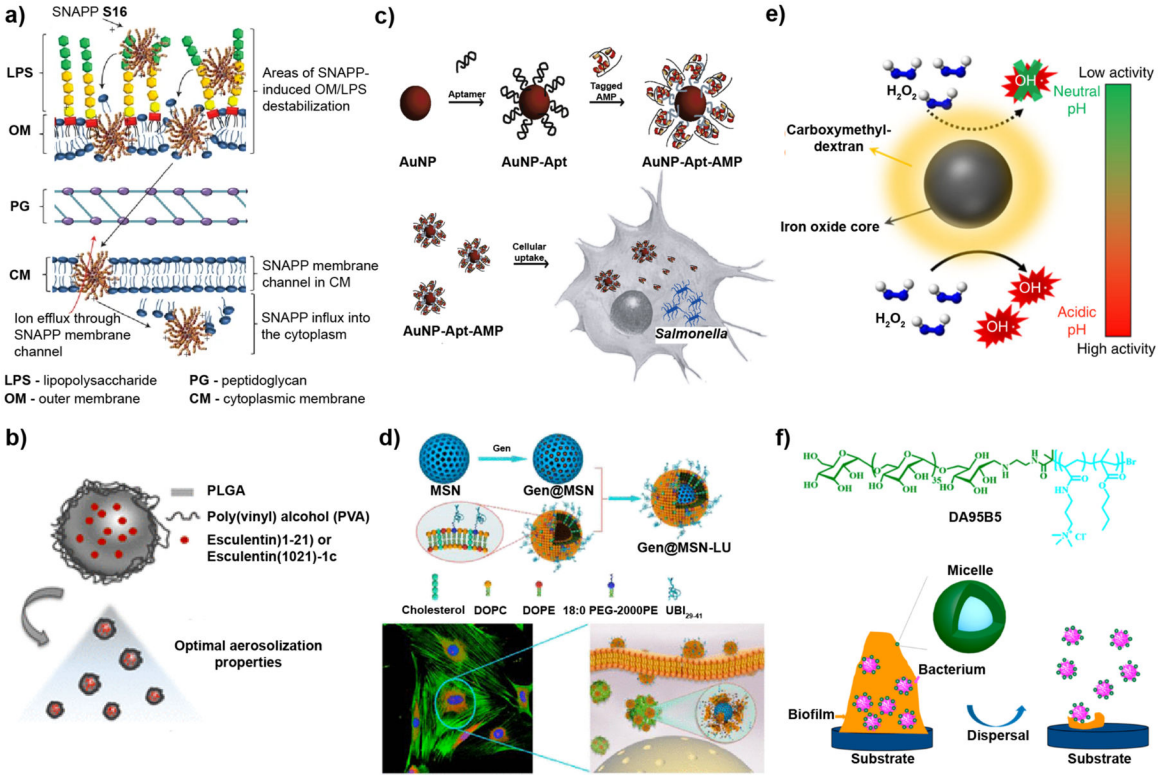


Figure 2. Examples of nanomaterial-based strategies used to combat bacterial infections.

a-b| Planktonic bacterial infections. **c-d**| Intracellular infections. **e-f**| Biofilm infections. **a**| Structurally engineered AMPs, SNAPPs, exhibited promising antimicrobial activity *in vitro* and *in vivo*. SNAPPs interacts with the outer membrane, peptidoglycan and cytoplasmic membrane layers of bacteria through electrostatic interactions, ultimately leading to cell lysis. **b**| Intratracheal administration of antimicrobial esculentin-1a formulated to be delivered to the lungs using PLGA NPs reduced *P.* aeruginosa lung infection in a mouse model. **c**| Histidine-aptamer-conjugated gold nanoparticles loaded with His-tagged AMPs were effective for treatment of *Salmonella enterica*-infected mammalian cells. **d**| Gentamicin-loaded mesoporous silica nanoparticles with a bacterial toxin-responsive lipid bilayer surface shell and bacteria-targeting peptide $UBI_{29–41}$, allowed targeted release of antibiotic for killing of intracellular *S. aureus*. **e**| A carboxymethyl-dextran-coated iron oxide nanoparticle, ferumoxytol, catalyzed ROS production of H_2O_2 in a pH-dependent manner as a treatment against oral biofilms. **f**| Dextran (green) and poly(AMPTMA-co-BMA) (light blue) form a micelle with a bactericidal core and non-fouling dextran shell used to treat wound biofilms. Electrostatic interaction of the NPs with the biofilm weakens bacterial attachment while gradually dispersing EPS matrix. Image in part a reproduced, with permission, from REF 63 © (2016) Macmillan Publishers Limited, part of Springer Nature. Image in part b reproduced, with permission, from REF 95 © (2019) American Chemical Society. Image in part c reproduced, with permission, from REF 96 © (2016) Elsevier Ltd. Image in parts d and f reproduced, with permission, from REF 97 and 98, respectively © (2018) American Chemical Society. Image in part e reproduced, with permission, from REF. 99 © (2018) Nature Communications. All rights reserved.

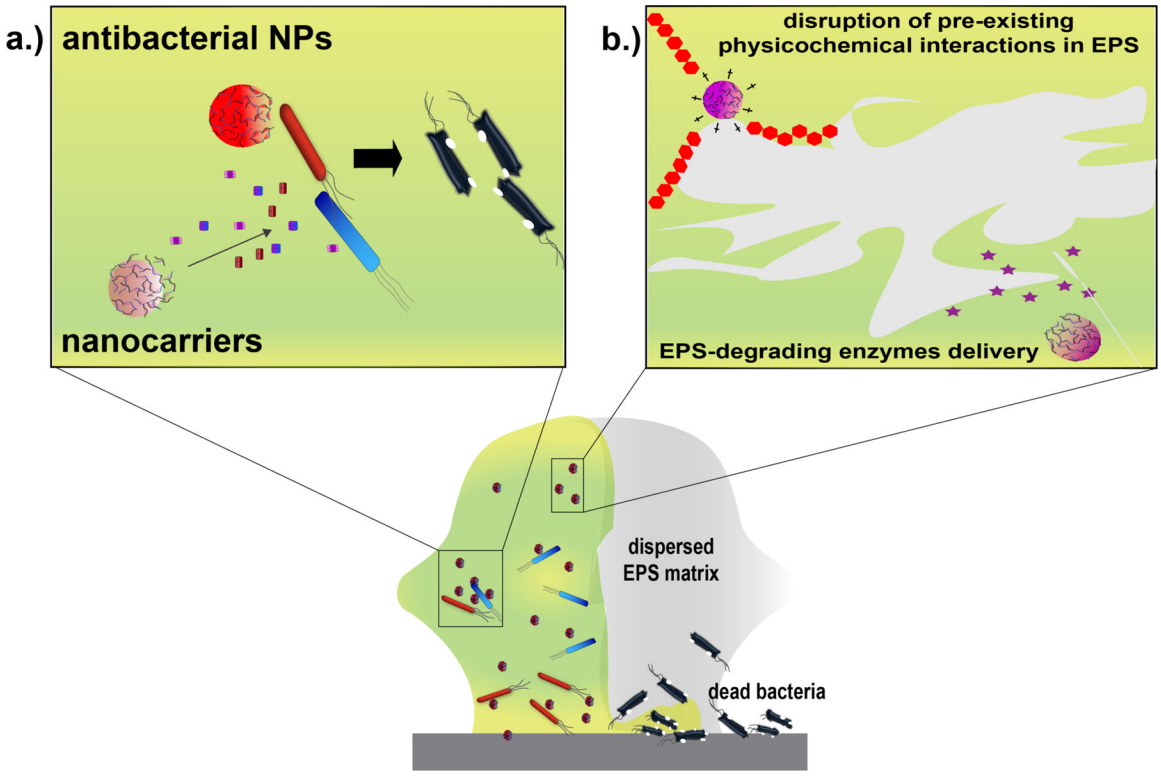


Figure 3. Eradicating biofilms using NPs.

Biofilms are comprised of cells with phenotypic heterogeneity embedded across the 3D-matrix of their self-secreted EPS. The ability of NPs to penetrate throughout the matrix allows them to **a|** interact with cells entrenched within the EPS and/or **b|** initiate disruptive interactions with the matrix that weaken physicochemical interactions responsible for keeping the 3D structure of biofilms intact. NPs can then either exert their inherent antimicrobial action or deliver therapeutic agents, such as antibiotics or essential oils, to kill the bacteria within the biofilms. NPs can alternatively deliver EPS-degrading molecules that promote dispersion of biofilms, facilitating their disruption.

Table 1.

Overcoming resistance mechanisms

Mechanism of Action	Antibiotics		Potential ways nanomaterials evade resistance
	Class	Major resistance mechanism	
Cell wall/membrane disruption	β-lactams	drug modifying enzymes[59]; binding site modifications[60]; porin changes[61]	physical damage to the cell envelope limits development of resistance[62,63,64]; flexible design space and unique physico-chemical properties can be used to maximize disruptive interactions[16]
	glycopeptides	binding site modifications[65]	
	peptide antibiotics	outer membrane modifications[66]	
Damage to intracellular components	aminoglycosides	drug modifying enzymes[61]	entry via membrane fusion overcomes resistance from limited antimicrobial entry[51]; ability to block efflux pumps[52,53]; multiple active groups available target general rather than specific bacterial pathways[47]
	macrolides	efflux pumps[67]; binding site modifications[61]	
	quinolones	binding site modifications[61]; porin changes[61]	

Table 2.

Nanomaterial-based therapeutics under clinical trials

Trade name	NP type	Active agent	Target pathogens/ infection	Clinical Trial Phase	Clinical Trial Number	Ref
Arikace	Liposomal	Amikacin	Gram-negative	III	NCT01315691	164
Pulmaquin	Liposomal	Ciprofloxacin	Gram-negative	III	NCT02104245	165
Silvasorb	Silver NP	Silver	Topical infection	III	NCT00659204	166
NanoAgCVC	Silver NPs	Silver	Central venous catheter-related infection	IV	NCT00337714	167
N/A	Polymeric NP	Doxycycline	Chronic periodontitis	II	NCT02726646	168
IABN	Polymeric NP	Ammonium polyethyleneimine	Oral infection	II	NCT01167985	169