The Use of Nanoparticles to Prevent and Eliminate Bacterial Biofilms

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A bacterial biofilm is generally defined as a structured community of bacterial cells, enclosed in a self-producing matrix and adhered to a living or abiotic surface. By producing biofilms bacteria gain advantages of resistance to antimicrobial agents including antibiotics, disinfectants, and sanitizers, as well as to harsh environment such as heat, osmotic stresses, and UV lights over their planktonic counterparts. Bacteria within biofilms can increase resistance to antimicrobial agents up to 1000 times comparing with free living bacterial cells. Biofilms play a key role in the development of infectious diseases in human, accounting for 80% of bacterial infections in human. Because bacterial biofilms are an important concern for human health and food safety, multiple approaches have been used for preventing biofilm formation in clinical and industrial settings. Most commonly used antibiotics and detergents are less effective in eliminating bacterial biofilms. To prevent and eradicate biofilms, the use of nanomaterials as anti-biofilm agents is one of the most promising strategies. A nanoparticle is a small particle with at least one dimension between 1 to 100 nanometers, renowned for its antimicrobial activity. In this mini review article, the steps of biofilm formation, potential anti-biofilm mechanisms of nanoparticles, and the methods employed for detecting the anti-biofilm effects and safety of NPs will be addressed, focusing on metal and metal oxide nanoparticles.

Key words: nanoparticles, biofilm, extracellular polymeric substances, anti-biofilm, antibacterial activity

Introduction

A bacterial biofilm is generally defined as a structured community of bacterial cells, enclosed in a self-producing matrix composed of extracellular polymeric substances (EPS) and adhered to a living or abiotic surface [1]. It is known that bacteria spend their 80-90% of times within biofilms, and biofilms play a critical role in bacterial survival in harsh environments.

The most common biofilm-forming bacteria associated with human disease are *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella* species, *Cronobacter* species, *Klebsiella pneumoniae,* and *Enterococcus faecalis*, etc. [2, 3, 4].

Biofilms affect human in many ways as they can form in natural, industrial, and hospital settings. Biofilm forming on medical devices such as catheters or implants often results in difficult-to-treat chronic infections [5, 6]. A number of bacteria can produce biofilms on silicon, latex, polycarbonate, stainless steel, glass, and polyvinyl chloride, which are commonly used in food process, storage, and reconstitution equipment [7]. Biofilm formation is linked to the infections on human surfaces such as teeth, skin, and the urinary tracts. Biofilms are responsible for a number of diseases, such as otitis media (an acute ear infection), bacterial endocarditis, cystic fibrosis, Legionnaire's disease (an acute respiratory infection), kidney stones, urinary tract infections, osteomyelitis, periodontal diseases, and hospital-acquired infections. It is reported that biofilms are associated with more than 80% of human bacterial infections [1, 3, 8].

Bacteria within biofilms can increase resistance to antimicrobial agents up to 1000 times compared with free living bacterial cells [9]. Current conventional antibiotics are less effective in the treatment of biofilm related infections [10].

The demand of developing new antimicrobial drugs and detergents to prevent and eliminate bacterial biofilm is a pressing task. Many approaches have been used for preventing and eliminating biofilm related infections in medical and industrial settings. One of the most promising approaches to overcome biofilm-mediated and drug-resistant infections is the use of nanoparticles [11-16].

A nanoparticle (NP) is a particle of any shape with at least one dimensions of their tridimensions between 1-100 nm. Nanoparticles utilized in biology are divided to three categories: inorganic, organic, and hybrid nanoparticles [17]. Due to their unique properties such as size, shape, surface charge, and surface area-to-volume ratio, nanoparticles have great potentials in hospital and industrial settings [18, 19]. Inorganic NPs such as metal and metal oxides are more stable than organic NPs, which may result in effective antibacterial effects [20, 21]. Commonly used nanoparticles obtained from metals and metal oxides include gold (Au), silver (Ag), copper (Cu), zinc (Zn), nickel (Ni), titanium (Ti), magnesium (Mg), aluminum (Al), silicon (Si), iron (Fe), calcium (Ca), etc.

The mini review will address the anti-biofilm activity of nanoparticles, highlights the basic steps of biofilm formation, the potential anti-biofilm mechanisms of nanoparticles, and the methods of examining anti-biofilm effects and safety of NPs.

Biofilm formation and bacterial resistances

The processing of biofilm formation can be divided to five basic steps, which make itself gradually becoming resistance to environmental stresses, especially antibiotics and detergents [22].

1. Forming a conditioning surface.

On a living or abiotic surface, organic substances in nature or proteinaceous substances in humans such as blood, urine, tears, saliva, and intervascular fluid may alter the surface properties and allow bacteria to adhere to the surface [23].

The conditioning step may begin within seconds when these substances exposure to the surface. In this stage, washing and detergent treatments could easily stop the further biofilm formation.

2. Bacterial attachment to the conditioned film.

When bacteria approach the conditioning surface about 10-20 nm, planktonic bacterial cells are attracted and attach to the surface by Waals forces, as well as by using their flagella, fimbriae, pili, and adhesins [24, 25]. The primary attachment is weak and reversible, and at this initial stage, the microorganisms are easily removed. The further attachment is irreversible, and can tolerate stronger physical or chemical shear forces [26, 27]. In the step, bacterial flagella, fimbriae, pili, and other bacterial adhesins play an important role.

3. Bacterial growth and colonization.

Bacteria produce EPS that are composed of polysaccharides, proteins, extracellular DNA, lipids, etc. Extracellular polymeric substances **c**an anchor the bacteria to the surface and allow colonies to grow. After the initial colonization, the biofilm grows via cell division and entrapment of microbes and cell debris.

4. Biofilm formation.

In the step, biofilms are formed and may only change the shape and size. A fully developed biofilm contains an EPS matrix, which mediates cell-cell communication (i.e. quorum sensing), holds the microbes within the biofilm, and protects the organisms from environmental stress, especially from antibiotics and detergents. A fully developed biofilm often contains complex diffusion channels in which nutrients, oxygen, and other elements can be transported, and metabolic wastes and cell debris can be removed.

5. Detachment (Dispersal).

Once a mature biofilm is formed, individual cells or aggregated cells may disperse from the biofilm into the surrounding environment. The dispersal step may result in the infection aggravation and spread, and bacterial colonization on new sites [28]. Several factors may associate with biofilm detachment such as mechanical forces, lacking of nutrients, and micro-environmental changes.

The sizes, shapes, and features of biofilms are associated with the species of bacteria, surface composition, and environmental factors. Despite the increased understanding of biofilm formation, preventing and eliminating bacterial biofilms remain a challenging task. Bacteria within a biofilm behave dramatically differently from free living cells. Bacterial biofilms possess some specific abilities, such as attachment to solid surfaces, transmission of resistance genes within biofilms, protection of bacteria from phagocytic killing, and blockage of antibiotic diffusion [29]. These abilities make bacteria within biofilms up to 1000 times more resistant to antibiotics than their planktonic counterparts [9].

The potential anti-biofilm mechanisms of nanoparticles

Although the antibacterial and anti-biofilm mechanisms of nanoparticles are not understood very well, there are several properties determining the activity of NPs such as the size, shape, surface area, surface charge, and biocompetence of NPs. These properties provide NPs numerous advantages to overcome the limitation of conventional antibiotics and detergents.

1. Size-dependent antibacterial properties.

Compared to bulk particles of the same compositions, extra-small sizes provide NPs great potentials, which include increased surface areas and penetration abilities. It has been known when the particle size of some material decreases to the nanometer range, its surface area will increase and its antibacterial activity will also increase [30]. For example, magnesium oxide NPs have been observed against *Escherichia coli* and *S. aureus*, only when their sizes are in 23, 18, 15, 11, or 8 nm, and the 8 nm particles shows the strongest inhibition to bacterial growth [31]. The size-dependent

antimicrobial property observed in iron oxide only occurs when it is formulated into nanometer, rather than micron or any larger sizes [32].

2. Surface area-to-volume ratio.

The surface area of nanoparticles will enlarge as particle size increases. Large surface area of NPs favors intimate interaction with bacterial membranes and results in a wide spectrum of antimicrobial activity [33].

3. Nanoparticles can penetrate biofilm.

Bacterial biofilm forms a protective barrier against the diffusion of antibiotics and detergents by its complex architecture, slow movement, and a EPS matrix [34]. Nanoparticles can penetrate biofilms due to extra-small sizes, shapes, surface charges, and biocompetence. For example, Mg NPs can adhere to and diffuse into biofilms [35].

4. Shapes.

Nanoparticles may present many kinds of irregular shapes. These shapes of NPs have a remarkable effect on biofilm destruction. It is reported that NPs with a rod like shape are more effective in anti-biofilm than NPs with a spherical shape [36].

5. Charges.

Even with the same composition, different tridimensional structures may result in different NP surface charges. The charges of NPs favor binding to bacterial cell walls, causing membrane disruption via direct interaction or free radical production [37].

6. Antibacterial activity.

The antimicrobial actions of NPs may include the alteration of microbial cell wall, the destruction of cell membrane, and the blockage of enzyme and nucleic pathways. For example, ZnO NPs can interact with membrane lipids and disrupt the membrane structures, which result in membrane damages and cell death [37]. ZnO NPs also can penetrate into bacterial cells, inhibit the bacterial growth, and stimulate the production of toxic oxygen radicals, which also lead DNA and protein damages, and bacterial death [38].

7. Drug delivery.

NPs can act as a carrier of antibiotics. The physicochemical features of NPs like ultra-small size, large surface to mass ratio, and highly reactive surface provide numerous advantages to deliver drugs [39]. Currently, inorganic NPs used for drug delivery include magnetic NPs, silica NPs, quantum dots, etc.

The methods used in investigation of NPs against bacterial biofilms

Cell culture-based and microscope-based methods have been extensively used for investigation of the behavior of nanoparticles within biofilms, the mechanisms of NPs on anti-biofilms and the effects and safety in using NPs. The detection methods include the antibiotic and antimicrobial susceptibility tests, anti-biofilm assay, cell cytotoxicity evaluation assay, electronic scanning and transmission microscopy.

1. Antibiotic susceptibility test.

The minimal inhibitory concentration (MIC) values of antibiotics against the target bacteria are determined by using disc diffusion method of Clinical and Laboratory Standards Institute [40].

2. Antimicrobial susceptibility test.

The antimicrobial activity of NPs is evaluated by standard agar disk diffusion method [41, 42]. Target bacteria (Gram-positive and Gram-negative bacteria) are cultured on LB agar medium for 18-24 h, then inoculated into nutrient agar plates. NPs are diluted in a serial of concentrations, and loaded into holes of the agar plate or added to Whatman filter paper No. 1 put on the agars of petri dishes. The dishes are incubated for 24-48 h at 37°C, and the MIC values are determined.

3. Anti-biofilm activity assay.

Anti-biofilm activity of selected NPs is evaluated on biofilms produced by target bacterial strains [43]. An overnight bacterial culture in LB broth is diluted and adjusted to OD_{600} of 0.02, and 100 µl of bacteria suspension are loaded in a

96-well polystyrene plate (U bottom) for 24 h at room temperature or 37°C depending on the target bacteria species. Then 20 µl of NPs solution at different concentrations are added into the wells of the plate, and the plate is incubated for another 24 h at room temperature or 37°C. After the incubation, the medium is removed, and the wells are washed in order to remove any planktonic cells. Biofilm is stained using 0.1% (w/v) crystal violet for 30 min. After wash with distilled water, the dye is solubilized in 100 ml of 95% ethanol and the absorbance is assessed spectrophotometrically (OD_{595} nm).

4. Transmission electron microscope (TEM).

TEM analysis is employed to examine the size and shape of NPs, the effects of NPs on target bacteria, and the attachment of NPs to the target bacteria or biofilms. After the preparation of NPs or un-treatment/treatment of target bacteria and/or biofilms with NPs, the samples are washed with sterilized distilled water, and a drop of the sample solution is loaded onto carbon-coated copper grids or other grids, and excess liquid is removed by using absorbent papers. The grids are visualized under a TEM. The particle sizes and shapes are determined through the image processing software [44]. The samples also can be observed after ultrathin section of the samples [45].

5. Scanning electron microscope (SEM).

SEM is performed to examine the morphological changes of bacteria or bacterial biofilm untreated and treated with NPs. The bacterial cells or biofilms are washed with PBS, fixed with 2.5% glutaraldehyde. After washed again and dehydrated, the samples are viewed under a SEM [45].

6. Cell cytotoxicity evaluation assay.

Cell cytotoxicity is determined by measuring the release of LDH into selected host cell culture supernatants of both untreated and treated with NPs. LDH activity is evaluated using the Cytotoxicity Detection Kit according to the manufacturer's instructions. Selected host cells are seeded into a 96-well plate in triplicate at a density of 10^5 cells/100 µl of cell culture medium. After 24-48 h, cells are exposed to different concentrations of NPs and incubated for 24 h or appropriate time. Absorbance is recorded at 570 nm [46].

Commonly used NPs with anti-biofilm activity

In exploring various options to deal with microbial resistance to antibiotics, inorganic NPs like metal and metal oxide NPs are promising candidates due to their higher stability and lower toxicity compared to organic NPs. Metal and metal oxide NPs have been extensively studied and applied in hospital and industrial settings.

A number of studies suggest that some metal and metal oxide NPs can prevent and eliminate bacterial biofilms [11-16]. These NPs are considered as important materials to develop novel medical implants, devices, and food-related equipment, as well as antimicrobial agents.

Silver NPs are effective for the destruction of various pathogens [47-48]. The antibacterial and anti-biofilm effects may result from their ultra-small size, increased surface area, and high biocompetence. Silver NPs can damage cell membranes, cause cell structure change, and penetrate into bacterial cells and biofilm matrixes. Silver NPs are able to interact intimately with various microbes and have an effect on both the developing and matured biofilms. Due to the high antibacterial and anti-biofilm capability and low toxicity towards mammalian cells, AgNPs have been extensively applied in hospital setting as broad spectrum antimicrobials [49].

Titanium dioxide NPs (TiO_2 NPs) are one of the most highly manufactured, and are widely used in many products such as toothpastes, cosmetics, food products, and pharmaceutical additives. The potential toxicity of TiO_2 NPs has been reported [50].

Zinc oxide NPs are spherical metal particles with a high specific surface area. The applications of ZnO NPs include antimicrobial and anti-biofilms. ZnO NPs are also widely applied in personal care products and industrial products such as sunscreens, cosmetics, floor coating, solar cells, etc. [51-52].

Table 1 shows several commonly used metal and metal oxide NPs that may prevent and eliminate bacterial biofilms.

Table 1. Commonly used inorganic nanoparticles against bacteria and biofilms

Inorganic nanoparticle		Antimicrobial activity	Anti-biofilm activity	References
Metal NPs	Silver (Ag)	+	+	[13, 48]
	Gold (Au)	+	+	[11]
	Copper (Cu)	+	+	[52]
Metal Oxide NPs	Titanium oxide (TiO_2)	+	+	[53]
	Zinc oxide (ZnO)	+	+	[54, 55]
	Iron oxide (Fe_2O_3)	+	+	[32]
	Aluminum oxide (Al_2O_3)	+	+	[53]
	Silicon dioxide (SiO_2)	+	+	[56]

Conclusion

Bacteria within biofilms can dramatically increase resistance to antimicrobial agents and detergents compared with their planktonic cell counterparts [9]. Because bacterial biofilms are an important concern for human health and food safety, multiple strategies have been explored for preventing and eliminating biofilms. Current conventional antibiotics are less effective in the treatment of biofilm related infections [10].

In recent years, NPs have been developed for diverse applications in medicine and industries as an alternative approach against multiple resistant bacterial pathogens due to their high antimicrobial effects with low toxicity. Inorganic NPs such as metal and metal oxide NPs are promising candidates for prevention and elimination of biofilms. Inorganic nanomaterials present high stability and low toxicity comparing with organic NPs. Metal and metal oxide NPs have been investigated and applied in the development of novel medical implants, devices, and food-related equipment, as well as antimicrobial agents. The detection methods of NPs are critical for determination of the effects and safety of NPs. Understanding the mechanisms of NPS on biofilm growth inhibition, biofilm structure disruption and eradication of biofilm formation is an important task. More researches should focus on studying of the anti-biofilm mechanisms of NPs and developing rapid and effective methods for detection their effects and safety.

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