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Bioconjugated nanoparticles for attachment and penetration into pathogenic bacteria

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ABSTRACT

As an antimicrobial agent, silver nanoparticles functionalized with both bacitracin A and polymyxin E (AgNPs-BA&PE) were designed and synthesized with complementary antibacterial functions to act against gram-positive and gram-negative bacteria. AgNPs-BA&PE could easily get attached and penetrate into the bacterial cell membrane through surface-immobilized BA and PE with a membrane target, resulting in up to 10-fold increase in the antibacterial activity, without the emergence of bacterial resistance. Analysis of the antimicrobial mechanism confirmed that the synthesized nanoparticles caused disorganization of the bacterial cytomembrane and leakage of cytoplasmic contents. This antimicrobial agent with better biocompatibility can promote healing of infected wounds, and has promising and useful applications in biomedical devices and antibacterial control systems.

1. Introduction

Infectious diseases induced by the bacteria continue to be one of the greatest health challenges worldwide. The introduction of antibiotics has made a striking impact on the treatment of infectious diseases and has dramatically decreased mortality. However, emergence of multiple antibiotics-resistant bacteria poses a new threat to human health [1,2]. Therefore, the design and development of new antimicrobial agents that have high antibacterial activity and low propensity to induce resistance are crucial. Nanomaterials, as new antimicrobial agents, which are capable of getting attached to the bacterial membrane and disrupting its integrity, have attracted increasing attention in the field of biomedicine [3]. To date, many kinds of antibacterial nanoparticles, such as carbon nanotubes [4], fullerene [5], metal oxide nanoparticles [6], and metallic nanoparticles [7] have been examined. Among them, carbon nanotubes and fullerene C_{60} are found to exhibit strong antibacterial properties. However, tedious synthesis procedure, high cost, and use of large amounts of organic solvents have hampered their further applications. Although metal oxide nanoparticles obtained by aqueous synthesis also exhibit antimicrobial activities, problems such as induction of bacterial resistance (e.g. TiO_2 and ZnO nanoparticles) and requirement of UV illumination for antibacterial actions (e.g. TiO_2 nanoparticles) are still not resolved [6].

When compared with the above-mentioned nanomaterials, silver nanoparticles (AgNPs) are the most exceptional antimicrobial agents among metallic nanoparticles used against different bacteria, viruses, and fungi, with minimal perturbation to human cells and low propensity to induce bacterial resistance [3,8]. AgNPs can increase the permeability of bacterial cell membrane, penetrate into the cytoplasm [9], and inactivate essential respiratory enzymes and proteins responsible for RNA and DNA replication, leading to bacterial death [7]. Unfortunately, these antibacterial actions of AgNPs are often dependent on high concentration because of the random physical collision of AgNPs with the bacterial surface, leading to penetration of AgNPs into the cytoplasm [10]. Recent studies have indicated that cationic polymers-stabilized AgNPs with positively charged surface can easily bind to the negatively charged bacterial surface through non-specific electrostatic interaction [11]. However, toxicity is still an obstacle to the biomedical applications of cationic polymers [12,13].

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It is generally accepted that antimicrobial agents have single or multiple target sites within the microbial cell, and that damage to these target sites results in the antibacterial effect [14]. The major target sites for antimicrobial agents are often present at the outermost layers of the bacterial cell. In order to be effective, an antimicrobial agent should reach and interact with its microbial target site(s). In recent years, antimicrobial peptides (AMPs) have been demonstrated to be excellent antimicrobial agents for the treatment of multidrug-resistant infections [15,16]. The macrocyclic amido groups of some AMPs chelate Mg^{2+} and Ca^{2+} of the bacterial cell surface to easily fuse into the bacteria and disrupt membrane organization, eventually leading to bacterial death [17,18]. AMPs, acting as "a molecular knife" by inserting into and damaging the bacterial cell membrane, mediate the penetration of AgNPs into the bacteria [19]. Moreover, AMPs serve as stabilizers protecting AgNPs against agglomeration [20] and achieve polyvalent effects by getting concentrated on the surface of AgNPs [21]. In addition, the concurrent emergence of multiple antimicrobial agents in a single antimicrobial agent does not induce bacterial resistance [22]. To the best of our knowledge, the use of AMPs-functionalized AgNPs as antimicrobial agents against gram-positive and gram-negative bacteria has not yet been reported.

In this study, we designed and synthesized AMPs-functionalized AgNPs as an antimicrobial nanomaterial. We chose bacitracin A (BA) and polymyxin E (PE) with macrocyclic amido groups as the target molecules from commercial AMPs. BA and PE have potent bactericidal activity directed primarily against gram-positive and gram-negative bacteria, respectively. Both gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and gram-positive bacteria (*Staphylococcus aureus* and *Bacillus amyloliquefaciens*) were used as bacterial models. To achieve highly efficient antimicrobial activity, the synthesis conditions of AgNPs functionalized with both BA and PE (AgNPs-BA&PE) were optimized comprehensively. The minimum inhibitory concentration (MIC), fractional inhibitory concentration (FIC), zone of inhibition test, and LIVE/DEAD bacterial viability assay were used to characterize the antibacterial activity of AgNPs-BA&PE and the synergistic effects between AgNPs and AMPs. Furthermore, the mechanism of antibacterial action of AgNPs-BA&PE was also systematically studied. Finally, we further explored the biocompatibility of AgNPs-BA&PE using mouse fibroblast cells (NIH3T3 cells) and determined the effect of this antimicrobial agent on the treatment of bacteria-induced wound infection.

2. Materials and methods

2.1. Materials

Silver nitrate ($AgNO_3$, 99.995%, metals basis, Ag 63% min) and sodium borohydride ($NaBH_4$, 98% min) were purchased from Alfa Aesar (Ward Hill, MA, US). BA (from *Bacillus licheniformis*, ≥50,000 U/g) and PE (Colistin sulfate salt, ≥15,000 U/mg) were from Sigma–Aldrich (St. Louis, MO, US). *E. coli* ATCC 8739, *P. aeruginosa* ATCC 9027, *S. aureus* ATCC 6538 and *B. amyloliquefaciens* ATCC 23842 strains were provided by Department of Microbiology of Nankai University (Tianjin, China). The ultrapure water (Millipore, 18.25 MΩ cm) was used to prepare solutions. All solutions were stored in the refrigerator at 4 °C.

2.2. Synthesis and characterization of AgNPs-BA&PE

Briefly, 500 μL of 1.0% silver nitrate ($AgNO_3$), 50 μL of 10 mmol/L BA, and 125 μL of 2.0 mmol/L PE were added to 49 mL of ultrapure water under vigorous stirring for 10 min in an ice-water bath. Then, 350 μL of 300 mmol/L fresh sodium borohydride ($NaBH_4$) solution was rapidly added under constant stirring for 30 min. The final color of the solution was brown. The solution was dialyzed (8–14 kDa M.W. cutoff) against ultrapure water for 48 h, filtered through a 0.22 μm cellulose membrane, and stored at 4 °C until further use. The concentration of AgNPs-BA&PE was calculated to be about 2.7×10^{-7} mol/L [23].

The UV–vis absorption spectra of the samples were recorded on a UV–vis spectrophotometer using quartz cell with a path length of 1.0 cm. The FTIR spectra were measured on a Fourier Transform Infrared Spectrometer using a KBr tablet containing the sample powder. The morphology of the obtained nanoparticles was observed using a transmission electron microscope (TEM) operated at 200 kV. A droplet of AgNPs-BA&PE was placed on an ultrathin carbon film and dried at room temperature before observation. X-Ray diffraction (XRD) was performed on an X-ray diffractometer (Rigaku, D/max-2500 with a Cu tube and a diffracted beam curved graphite monochromator operating at 60 kV and 300 mA).

2.3. Antibacterial activity

The MIC of the antimicrobial agent that inhibited visible growth of the microorganism in a broth dilution susceptibility test was determined according to the guidelines of the Clinical and Laboratory Standards Institute with a modified method [24]. The microorganisms were grown overnight in Luria–Bertani (LB) broth at 37 °C. The bacteria were diluted with LB broth to approximately 2.0×10^6 CFU/mL. The bacterial suspension was mixed with an equal volume of 2-fold diluted antimicrobial agent solution and incubated for 8 h at 37 °C. The visible growth of the bacterial cells was assessed by measuring the optical density value at 600 nm (OD_{600}) using UV–vis spectroscopy. The lowest concentration was the one at which there was no turbidity greater than the faint turbidity. Each assay was carried out in triplicate.

The synergistic antimicrobial effect of AgNPs (compound A) and one of the AMPs (compound B) was studied in LB broth using a two-dimensional microdilution assay. The FIC was calculated as follows:

$$\mathrm{FIC} = \frac{\text{MIC of compound A in combination}}{\text{MIC of compound A alone}} + \frac{\text{MIC of compound B in combination}}{\text{MIC of compound B alone}}$$

The interaction was defined as synergistic if the FIC index was ≤0.5 [25].

The zone of inhibition test was employed to evaluate the synergistic effects between AgNPs and the two kinds of AMPs. The bacteria were incubated in LB broth at 37 °C overnight. The resulting bacterial suspension was diluted to approximate 1.0×10^7 CFU/mL with LB broth. Subsequently, 50 μL of the bacterial suspension were inoculated evenly on LB agar plates. Then, the sample disk containing the antimicrobial agent solution was gently placed at the center of the LB agar plates and incubated overnight at 37 °C. The antibacterial activity was measured by evaluating the diameter of the zone of inhibition around the disk.

The fluorescent dyes were prepared by mixing 10 mg of acridine orange (AO, Fluk) and 10 mg of ethidium bromide (EB, Fluk) in 10 mL of phosphate buffer solution (PBS, 0.01 mol/L, pH 7.4). The bacterial suspension (1.5 mL, 1.0×10^8 CFU/mL) was harvested by centrifuging (5000 rpm for 5 min) at 4 °C and washing with PBS for three times. The supernatant was discarded and the remaining bacteria were resuspended in 1.5 mL of PBS. Then, 100 μL of 1.0×10^{-9} mol/L AgNPs-BA&PE solution were added to the bacterial suspensions. After incubation for 30 and 60 min, respectively, the bacteria were stained with 100 μL of fluorescent dyes for 15 min. After rinsing with PBS, 10 μL of the samples were placed on a glass slide with a glass coverslip and observed under an inverted fluorescence microscope. The control assay was performed without AgNPs-BA&PE treatment.

2.4. Effect of metal ions on the antibacterial activity of AgNPs-BA&PE

Zn^{2+}, Cu^{2+}, K^+, Na^+, Al^{3+}, Fe^{3+}, Fe^{2+}, Ca^{2+}, and Mg^{2+} were added to 1.5 mL of 2.0×10^7 CFU/mL bacterial suspensions to a final concentration of 100 μmol/L, respectively. Then, 100 μL of 1.0×10^{-9} mol/L AgNPs-BA&PE solution was added to the suspensions and incubated at 37 °C for 8 h. The OD_{600} was measured using UV–vis spectroscopy. The control assay was performed without the treatment of metal ions.

2.5. Cytoplasmic membrane permeabilization assay

The cytoplasmic membrane permeabilization was assayed by determining the release of β-galactosidase from the cytoplasm of *E. coli* into the culture medium using ortho-nitrophenyl-β-D-galactopyranoside (ONPG) as the substrate [26]. Briefly, 100 μg/mL of ONPG were added to 1.5 mL of 2.0×10^7 CFU/mL bacterial suspension. Subsequently, the AgNPs-BA&PE solution was added to the above-mentioned suspension. Ortho-nitrophenol (ONP) was determined by monitoring the increase in OD_{420} using UV–vis spectroscopy. The control assay was carried out without the addition of AgNPs-BA&PE.

2.6. Preparation of ultrathin sections of bacterial samples for TEM

The bacterial suspensions (1.0×10^8 CFU/mL) of *P. aeruginosa* and *S. aureus* were washed twice with PBS and resuspended in 1.5 mL of PBS. The samples were subsequently incubated for 1 h at 37 °C with 100 μL of 1.0×10^{-9} mol/L AgNPs-BA&PE solution. Free AgNPs-BA&PE were removed by centrifuging (5000 rpm for 5 min) at 4 °C and washing with PBS for three times. The remaining bacteria were fixed with 1.0 mL of 2.5% glutaraldehyde in cacodylate buffer (0.1 mol/L, pH 7.4) for 4 h. The treated bacterial samples were washed twice with PBS and further fixed with 200 μL of 1.5% osmic acid for 2 h. The obtained residues were washed thrice with PBS,

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