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Fully Zwitterionic Nanoparticle Antimicrobial Agents through Tuning of Core Size and Ligand Structure

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Abstract

Zwitterionic nanoparticles are generally considered non-toxic and non-interacting. Here, we report effective and selective antimicrobial activity of zwitterionic gold nanoparticles (AuNP) through modulation NP size and surface charge orientation. Using a set of 2, 4 and 6 nm core AuNPs, increasing particle size increased antimicrobial efficiency through bacterial membrane disruption. Further improvement was observed through control of ligand structure, generating antimicrobial particles with low hemolytic activity and demonstrating the importance of size and surface structure in dictating the bioactivity properties of nanomaterials.

Graphical Abstract

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Supporting Information Available: Nanoparticle synthesis and characterization, and additional experimental details and figures. This material is available free of charge *via* the Internet at <http://pubs.acs.org>.

Keywords

antimicrobial activity; size; charge orientation; zwitterionic gold nanoparticles

Pathogenic bacteria are a serious threat to public health, with acquired antibiotic resistance challenging the effectiveness of traditional antimicrobials.^{1,2} Nanoparticles (NPs) have attributes that make them promising antimicrobial agents.^{3–7} For example, the high surface area to volume ratio of NPs enables the efficient encapsulation/conjugation of antibiotics.^{8–10} Cationic nanoparticles provide self-therapeutic antimicrobial systems that function through bacterial membrane penetration and disruption.^{11–14} These positively charged NPs, however, can possess substantial toxicity to mammalian cells, including hemolytic activity.^{15,16} Integrating neutral zwitterionic ligands into cationic nanoparticles has created a class of NP-based antimicrobial that decrease mammalian cells toxicity and maintain antifouling properties.^{17,18} Activity in these systems, however, requires careful balancing of zwitterionic and cationic ligands to ensure the stability and antibacterial activity of the nanoparticles.^{11,19,20}

In addition to surface functionalization, there is mounting evidence indicating that size and monolayer structure of NPs also plays a significant role in determining the bactericidal activity of NPs.^{21,22} For example, we have previously found that cationic gold nanoparticles (AuNP) displayed very different interaction patterns with bacterial membrane between 2 nm and 6 nm core sizes.²³

Here, we report the design and synthesis of family of fully zwitterionic NP-based antimicrobials. The antibacterial activity of these zwitterionic NPs was readily manipulated by tuning the NP core size. Small-NPs (2 nm core) display low antimicrobial activity. Surprisingly, modestly larger 6 nm core AuNPs exhibit very potent antimicrobial activity, efficiently disrupting the bacterial cell membrane. In addition, subtle tuning of the surface charge orientations of zwitterionic AuNPs results in a substantial change of antimicrobial activity. Noteworthy, all these zwitterionic nanoparticles exhibit low toxicity to mammalian cells and maintain high hemocompatibility. The parametric engineering of NPs provides both different bactericidal therapeutics and insight into the coupled role of size and surface functionality on the antimicrobial activity.

RESULTS AND DISCUSSION

Zwitterionic surface are known to inhibit bacterial adhesion and prevent biofilm formation,^{18,24} however their bactericidal ability has not been well explored. In this study, two sets of zwitterionic ligands were designed to synthesize zwitterionic monolayer-protected gold nanoparticles. These zwitterionic ligands display different charge orientations (Figure 1A), one has positive charges in the outermost layer (SN), while the other one has the positive charge inside the ligand terminus (NS).

Three sets of zwitterionic AuNPs with different core size were synthesized by reducing chloroauric acid in the presence of precisely controlled ratio of SN or NS ligand as described in Supporting Information.^{25–28} With the molar ratio of gold salt to surface ligand increased

from 1/3 to 3/1, AuNPs with core size of 2, 4, and 6 nm were obtained (Figure 1B). Dynamic light scattering (DLS) analysis (Table S1) confirms the increased NP size under the different reaction molar ratio. Additionally, zeta-potential measurements showed nearly neutral charges of both Au-NS and Au-SN NPs. The successful surface functionalization of nanoparticles with zwitterionic ligands was also analyzed and verified using laser desorption/ionization mass spectrometry (LDI-MS, Figure S1). Molecular peaks observed at m/z value of 530.2 and 601.1 were attributed to NS and SN ligands, respectively.

We first evaluated the antimicrobial activities of these zwitterionic AuNPs against Gram-positive and Gram-negative bacterial strains. Two antibiotic resistant pathogenic strains were tested: *P. aeruginosa* (*Pseudomonas aeruginosa*, Gram-negative) and *A. azurea* (*Amycolatopsis azurea*, Gram-positive).^{29,30} The minimal inhibitory concentration (MIC) of each NP against these bacteria was determined using broth dilution method in the presence of nanoparticles of varying concentrations.³¹ As shown in Figure 1C, all zwitterionic NPs were able to completely inhibit the proliferation of *P. aeruginosa* and *A. azurea*. There was a significant effect of particle size on bacteria proliferation. As an example, the MIC concentration of Au-SN NP against Gram-negative *P. aeruginosa* decreased from 8000 nM to 50 nM when the core size of NP was increased from 2 nm to 6 nm. A similar trend was observed for the Gram-positive *A. azurea* strain (Table S2), with the 6 nm NP displaying a 40-fold lower MIC than the 2 nm analog. Surface structure likewise played a role in bactericidal efficacy: all of the Au-SN NPs were more effective than these Au-NS analogs. Our finding is consistent with the previous study where cationic-terminated particles were more toxic to both Gram-negative and Gram-positive bacterial strains than anion-terminated particles.¹¹ Collectively, our study demonstrated that both the size and charge orientation of zwitterionic NPs are crucial in determining the antimicrobial properties of these zwitterionic AuNPs.

Having demonstrated the distinct size- and charge orientation-dependent antimicrobial activity of zwitterionic AuNPs, we set out to understand the antibacterial mechanism of these NPs. To this end, we treated *P. aeruginosa* and *A. azurea* with NPs at their respective MIC concentrations for 3 hours (Table S2), followed by examining the bacterial morphology and NP-bacterial membrane interaction using transmission electron microscopy (TEM). As shown in Figure 2, both Gram-negative and -positive strains without NPs treatment exhibit typical membrane structures, with thin or thick peptidoglycans readily identified.³² The morphology and membrane integrity of the bacteria, however, were dramatically changed after NPs treatment in a size-dependent fashion. Smaller 2 nm NPs were randomly adsorbed on bacteria and on the TEM substrate without inducing noticeable membrane changes. However, 4 and 6 nm NPs penetrated deeper into the peptidoglycan layer and anchored more strongly on the bacterial surface, with 6 nm NP attaching the most. Noteworthy, with more NP accumulation on the bacterial surface a higher degree of membrane damage was observed. Moreover, some outer membrane vesicles (OMVs) began to form on the cell membrane after incubation with 4 nm Au-SN NP as denoted in Figure 2. This blebbing can be attributed to the NP aggregation formation on the bacterial surface as indicated by red arrows (Figure 2). Significantly, we observed a higher efficiency of these NPs to disrupt Gram-negative *P. aeruginosa*. The multilayered peptidoglycan coating of Gram-positive bacteria presumably passivates the interactions of NPs and bacterial membranes, leading to

less NPs penetration and decreased toxicity of NPs against gram-positive bacteria. Au-NS NP displayed a similar interaction trend with *P. aeruginosa* and *A. azurea*, but with decreased bacteria disruption efficiency compared to that of Au-SN NP. More SN nanoparticles were found sticking onto the cell surface than NS, with greater structural damage observed (Figure 2,) indicating the positive charge terminated SN ligands have higher affinity to the negatively charged bacterial membrane than the NS analogs. As expected, this effect is magnified for larger particle that feature increased surface contact areas. These observations correlate well with the MIC studies where Au-SN NP shows superior antimicrobial activities compared to that of Au-NS NP (Table S2 and Figure S2).

Having observed NPs-induced bacterial membrane distortions, we surmised that membrane damage induced by NPs provided their antimicrobial activities. To verify this hypothesis, we treated bacteria with the most potent 6 nm NPs, followed by propidium iodide (PI) staining to check the integrity of bacteria membrane. PI only leaks into cells with compromised membranes, with concomitant enhanced fluorescence.^{6,8} As shown in Figure 3, Figure S3 and Figure S4, we only observed strong red fluorescence from bacterial cells treated with NPs, indicating the integrity of bacterial membrane was indeed disrupted by NPs.

The above studies demonstrate that the larger AuNPs are efficient bactericidal agents. Effective applications of antimicrobials in or on patients, however, require a low toxicity to mammalian cells, and in particular red blood cells (RBCs).^{5,33} To this end, we first studied the toxicity of zwitterionic NPs against fibroblast 3T3 cells that have been widely used to determine the cytotoxicity and biocompatibility of antibacterial agents.³⁴ As shown in Figure S5, the treatment of 3T3 cells with AuNPs at individual MIC concentration had negligible cytotoxicity as measured by Alamar Blue assay. The hemolytic activity of these NPs against human red blood cells (RBCs) was also measured.^{15,35,36} As shown in Figure 4, Figure S6 and Figure S7, at the concentrations we tested (up to 16-fold higher than the MIC concentration, with highest dosing determined by the high optical density of the AuNPs), none of the NPs show any observable hemolytic activity, demonstrating the intrinsic biocompatibility of zwitterionic ligands with mammalian cells and human blood cells.

The observed effect of NP size is substantial compared with the relatively small increase in particle diameter. The most reasonable explanation for the greater antimicrobial activity of the larger particles is the increase in monolayer pre-organization that occurs with increasing particle core diameter. Murray's study of nanoparticle monolayers indicates that as the size of NPs increases, the surface curvature decreases, leading to a more densely packed ligand shell.³⁷ Small diameter (2 nm core) NPs feature disorganized ligand shells, whereas larger NPs (> 4.4 nm) feature highly organized "2D-like" surfaces. The greater pre-organization of the larger particles presumably presents more terminal charged groups toward cell surface, causing membrane distortion, and eventually lysis the cell (Figure 5). Furthermore, the higher surface area of larger particles would be expected to increase the interaction energy of these particles with membranes.

CONCLUSIONS

In summary, we have demonstrated an important synergy between NP core size, surface charge orientations, and antimicrobial behavior of zwitterionic gold nanoparticles. We observed a dramatically increased antimicrobial property with increasing NP size, with changes of charge orientation also substantially contributing to antimicrobial activity. Significantly, all NPs maintained their intrinsic biocompatibility toward mammalian and in particular red blood cells, making these systems promising for antimicrobial therapeutic use. In a broader context, this study provides direct demonstration that subtle changes in NP size and surface functionality can dramatically alter the interactions of these materials with biosystems, showing the importance of these attributes in predicting NP behavior.

METHODS

Determination of antimicrobial activities (MIC) of zwitterionic gold nanoparticles

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of AuNP that inhibits visible growth after overnight incubation as observed with the naked eyes.³⁸ First, bacteria were cultured in LB medium at 37 °C and 275 rpm until into stationary phase. The cultured bacteria were then harvested by centrifugation and washed with 0.85% sodium chloride solution for at least three times. Optical density measurement at 600 nm was used to determine the concentration of re-suspended bacterial solution. Then, 50 μ L of bacteria solution was mixed with another 50 μ L NP solutions in a 96-well plate, reaching a final bacterial concentration of 5×10^5 cfu/mL. NPs concentration cultured with bacteria varied in half fold according to the standard protocol.³¹ A growth control group treated without NPs and a sterile control group with only growth medium M9 were carried out at the same time. All of the cultures were performed in triplicates, and at least two independent experiments were repeated on different days.

TEM observation of cell membrane structure

For TEM grid preparation, 20 μ L of the bacteria solution (OD_{600 nm} = 0.35) was mixed with nanoparticle solution (10 μ L, 2000 nM for 2 nm, 900 nM for 4 nm, 500 nM for 6 nm) at room temperature. After incubation for 3 h, aliquots of the mixture were applied to a carbon-coated Cu grid (400-mesh). Aliquots of 2 μ L were allowed to settle on the grid for 30 s and then blotted with filter paper slightly. In order to distinguish the cell membranes, the grids were stained with 2 μ L of 1% uranyl acetate for 30 s and then blotted again. All grids were then screened with FEI TecaniT12 microscope with an accelerating voltage of 120 kV.

Propidium iodide staining assay study

First, *P. aeruginosa* (Gram-negative) and *A. azurea* (Gram-positive) (1×10^8 cfu/mL) were incubated with 6 nm nanoparticles at each MIC concentration in M9 buffer at 37 °C and 275 rpm for 3 h. Then, bacteria solutions were mixed with 2 μ M PI and incubated for 30 min in dark. After that, 10 μ L of the samples was placed on a glass slide with a coverslip and observed under a confocal laser scanning microscopy using a 543 nm excitation wavelength (Zeiss 510, Carl Zeiss, Germany).

Hemolysis assay

Hemolysis assay was performed on human red blood cells (RBCs).³⁹ In this work, citrate stabilized human whole blood (pooled, mixed gender) was purchased from Bioreclamation LLC, Westbury, NY. First, the RBCs were centrifuged for five times and resuspended in 10 mL of PBS buffer as soon as received. Then, 0.1 mL of RBC solution mixed with 0.4 mL of NP solution in PBS. After incubation at 37 °C, 150 rpm for 30 min, the mixture was centrifuged at 4000 rpm for 5 min. At last, the absorbance value of the supernatant was measured at 570 nm. RBCs incubated with PBS and water was used as negative and positive control, respectively. All samples were prepared in triplicate at one time. The percent hemolysis was calculated using the following equation: %Hemolysis = (sample absorbance - negative control absorbance)/(positive control absorbance - negative control absorbance) × 100.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) Chemical structure of zwitterionic ligands used for AuNP synthesis. Zwitterionic headgroups were conjugated to a non-interacting oligo(ethylene glycol)-functionalized interior. (B) TEM images and the corresponding size distribution histograms of zwitterionic AuNPs with 2, 4, and 6 nm core size. (C) The minimum inhibitory concentration (MIC) of zwitterionic Au-SN and Au-NS NPs against Gram-negative (*P. aeruginosa*) and Gram-positive (*A. azurea*) bacterial strains. Bacteria were cultured overnight with NPs and the experiments repeated in triplicate.

Figure 2.

Visualizing morphological changes in cell membranes using TEM. (A) Gram-negative (*P. aeruginosa*) and (B) Gram-positive (*A. azurea*) bacterial strains were treated with 2, 4 and 6 nm Au-SN and Au-NS NPs at their MIC concentration for 3 h. Red arrows indicate the morphological changes of cell membrane structures with and without NPs treatment. Scale bars: 200 nm for black and 500 nm for white.

Figure 3. Propidium iodide staining assays of Gram-negative (*P. aeruginosa*) bacterial strain after 3 h incubation with 6 nm zwitterionic NPs at each MIC concentration *via* confocal microscopy observation.

Figure 4.

Hemolysis assay study of sub-10 nm zwitterionic gold nanoparticles at several concentrations on human red blood cells for 30 min at 37 °C. The mixture was centrifuged to detect the cell-free hemoglobin in the supernatant. RBCs incubated with PBS as well as water were used as negative (–) and positive (+) control, respectively. All samples were prepared in triplicate at one time. MIC of Each NP was indicated using the red arrows above the wells.

Figure 5. Schematic illustration of interaction mechanism between bacterial membrane with 2 nm and 6 nm core sized zwitterionic nanoparticles.