Mercaptoypyrimidine-Conjugated Gold Nanoclusters as Nanoantibiotics for Combating Multidrug-Resistant Superbugs

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Supporting Information

ABSTRACT: Widespread bacterial resistance induced by the abuse of antibiotics eagerly needs the exploitation of novel antimicrobial agents and strategies. Gold nanoclusters (Au NCs) have recently emerged as an innovative nanomedicine, but study on their antibacterial properties especially toward multidrug resistant (MDR) bacteria is scarce. Herein, we demonstrate that a novel class of Au NCs, mercaptoypyrimidine conjugated Au NCs, can act as potent nanoantibiotics targeting these intractable superbugs in vitro and in vivo, without induction of bacterial antibiotic resistance and noticeable cytotoxicity to mammalian cells. The Au NCs kill these superbugs through a combined mechanism including cell membrane destruction, DNA damage, and reactive oxygen species (ROS) generation, and exhibit excellent treatment effects in both macrophages and animal infection models induced by methicillin-resistant Staphylococcus aureus as representative. Moreover, the induction of intracellular ROS production in bacterial cells mainly attributed to the Au NCs’ intrinsic oxidase- and peroxidase-like catalytic activities has been demonstrated for the first time.

INTRODUCTION

Antibiotics have made great contributions to rescuing human life in the past several decades. However, the abuse of antibiotics has led to the recent emergence of a great deal of multidrug resistant (MDR) bacterial strains, including the most notorious “ESKAPE” superbugs (i.e., MDR Acinetobacter baumannii, MDR Pseudomonas aeruginosa, MDR Klebsiella pneumoniae, MDR Enterobacter species, methicillin-resistant Staphylococcus aureus (MRSA), and vancomycin-resistant Enterococcus faecium (VRE)), which have become among the most serious global public health crises.1,2 Reversal of the bacterial resistance to antibiotics has been attempted by exploiting new antimicrobial drugs and chemically modifying extant antibiotics.3,4 Unfortunately, the pace of antibiotic development cannot catch up with the development of bacterial resistance, and the increasing rates of MDR also invalidate the utility of even the most potent antibiotics.5 Therefore, it is urgently needed to develop new antimicrobial approaches, especially non-antibiotic drugs to fight superbug infections.

Based on high specific surface area and exceptional antimicrobial performance (either intrinsic or chemically incorporated), engineered nanostructures have been widely studied as among the most promising antimicrobial substances, while the microbe may not be able to develop resistance to them.6−9 For example, metals and metal oxide nanomaterials which possess excellent antibacterial activity have been used as potential agents to combat MDR bacterial infections.10−13 However, although elemental nanoparticles (NPs), such as Ag NPs, have an intrinsic antimicrobial capacity, they also raise a severe safety menace for practical application due to their cytotoxicity to the human body.14,15 Therefore, it is necessary to find alternative antibacterial candidates with proper human safety to ensure the clinical transformation.

In this regard, the “noblest” of metals—gold—has a greater advantage over silver due to its bioinertia and high stability. Nanostructures based on this element have also been extensively proven to possess good biocompatibility in animal systems.16,17 This biocompatibility still remains even if the size is further reduced to the nanoclusters (NCs) range.18 Interestingly, many Au NPs have also been used as antibacterial materials after they were grafted with known antibacterial compounds, such as small molecule antibiotics,19,20 antimicrobial peptides,21,22 and cationic ligands,23 on their surface, presumably using Au nanostructures as driven antibiotic carriers. Unlike large Au NPs, smaller Au NCs have recently emerged as innovative nanomedicine with interesting physicochemical properties, such as size-dependent fluorescence and catalytic activity.24−26 Recent research has demonstrated that ultrasmall Au NCs (∼2 nm) exhibit antibacterial activity through inducing intracellular reactive oxygen species (ROS) production, whereas larger Au nano-
particles (∼6 nm) are incapable. However, studies on the impact of Au NCs on superbugs are lacking, whether in vitro or in vivo.

Herein, we synthesized Au NC conjugated mercaptopyrimidine as nanoantibiotics for combating ESKAPE superbugs. Four mercaptopyrimidine analogues, namely, 4-amino-2-mercaptopyrimidine (AMP), 4,6-diamino-2-mercaptopyrimidine (DAMP), 4-amino-6-hydroxyl-2-mercaptopyrimidine (AHMP), and 4,6-dihydroxyl-2-mercaptopyrimidine (DHMP), were used as ligands for the synthesis of Au NCs. These ligands have been also used in the synthesis of Au NPs in the previous study. Unlike mercaptopyrimidine-capped Au NPs, the as-synthesized Au NCs especially AuDAMP have superior antibacterial performance against both Gram-positive and Gram-negative bacteria. The antibacterial properties of AuDAMP are mainly attributed to their oxidase- and peroxidase-like activities. In contrast, large-sized Au NPs exhibit relatively weak antibacterial activity due to their weak mimic enzyme activity, particularly against Gram-positive bacteria. In addition, the Au NCs exhibit detectable cytotoxicity and hemolysis for mammal cells but have excellent therapeutic effects against superbug infections, as demonstrated through macrophage infection models as well as mouse infection models.

### RESULTS AND DISCUSSION

We first synthesized mercaptopyrimidine-conjugated Au NCs via reduction of gold precursor (HAgCl) by relying on the ligand’s inherent reducibility in the presence of the mercaptopyrimidine ligands in 50% ethanol aqueous solution. The transmission electron microscopy (TEM) images show that the sizes of ultra-small AuAMP, AuDAMP, AuAHMP, and AuDHMP NCs are less than 2 nm. The zeta potential measurements indicate that amino-mercaptopyrimidine-conjugated Au NCs (AuDAMP, AuAMP, and AuAHMP) are positively charged, and hydroxyl-mercaptopyrimidine-conjugated Au NCs (AuDHMP) are negatively charged (Figure 1).

Then we investigated the antibacterial activities of mercaptopyrimidine-conjugated Au NCs by determination of minimal inhibitory concentration (MIC) using a broth-dilution method. First, we select two antibiotic-sensitive strains *Escherichia coli* ATCC35218 (Gram-negative bacteria) and *S. aureus* ATCC29213 (Gram-positive bacteria) as bacterial model to test the antimicrobial abilities of these Au NCs. AuAMP, AuDAMP, AuAHMP, and AuDHMP are active against two representative strains, with a MIC ranging from 2 μg/mL to 32 μg/mL, suggesting that mercaptopyrimidine-conjugated Au NCs may have a broad spectrum of antibacterial properties. The antimicrobial ability

![Figure 1. Characterization of mercaptopyrimidine-conjugated Au NCs.](image-url)
of AuDAMP is much better than that of AuAMP, AuAHMP, and AuDHMP. Furthermore, we also assess the MIC of these mercaptopyrimidine-conjugated Au NCs against clinically isolated ESKAPE strains, i.e., MDR E. coli, MDR A. baumannii, MDR P. aeruginosa, MRSA, MDR K. pneumoniae, and VRE. These superbugs are isolated from the sputum samples of clinical patients with respiratory tract infections. The resistance of ESKAPE is shown in Table S1. MDR Gram-negative strain, MDR A. baumannii was resistant to 15 common antibiotics and just susceptible to aminoglycosides and tetracyclines. MDR E. coli, MDR K. pneumoniae, and MDR P. aeruginosa were resistant to 14, 12, and 10 antibiotics, respectively. MDR Gram-positive strains, MRSA and VRE, were resistant to more than 10 antibiotics. Moreover, three classes of conventional antibiotics were used as control, which are levofloxacin (a type of quinolones, broad spectrum), vancomycin (a type of glycopeptides, the first-line drug for the treatment of MRSA infections), and colistin (a type of polymyxins, to treat Gram-negative bacteria). Their antibacterial effects are not satisfying. ESKAPE were all resistant to levofloxacin. As a typical superbug’s therapeutic agent, colistin was also found to be inactive against MDR A. baumannii (Table 1, Table S1). In contrast, we found that AuDAMP can inhibit the multiplication of MDR E. coli, MDR A. baumannii, MDR P. aeruginosa, MDR K. pneumoniae, MRSA, and VRE, with a MIC of 4 μg/mL, 2 μg/mL, 4 μg/mL, 2 μg/mL, 2 μg/mL, and 8 μg/mL, respectively (Table 1). By contrast, AuAMP, AuAHMP, and AuDHMP show relatively weak antibacterial properties against clinical ESKAPE groups. In particular, AuDHMP are shown to be virtually inactive against VRE. The lower MIC values of AuDAMP indicate that their antibacterial capacity is superior to that of other mercaptopyrimidine-conjugated Au NCs. In addition, AuDAMP had a red-emitted fluorescence (620 nm) with a longer fluorescence lifetime (Figure S1), and almost no agglomeration or fluorescence quenching occurs during long-term storage in aqueous solution (Figure S2). Considering that AuDAMP has excellent stability and shows superior antibacterial characteristics to conventional antibiotics, we used the NCs in the following studies.

To compare the size effect of particles on antimicrobial activity, we also tested the antibacterial properties of DAMP-Au NPs with larger size (∼6 nm) via synthesis by NaBH4 reduction (Figure S3). Compared to AuDAMP NCs, AuDAMP NCs have relatively weak antibacterial activity, particularly against Gram-positive bacteria (Table 1). Considering the difference in surface ligand density caused by the different sizes of Au NPs and Au NCs, we will also compare the antimicrobial properties of the two kinds of Au nanostructures based on the surface DAMP concentrations. The elemental analysis results show that the Au-to-DAMP molar ratios in Au NPs and Au NCs are 1:1 and 1:1.4, respectively. The MIC data based on the same DAMP concentrations also show that the Au NCs have better antibacterial activities (Table S2). This result is different from previous reports on 6-mercaptophexanoic acid stabilized Au NPs, indicating that in addition to the size effect, ligands might also significantly affect the antibacterial properties of nanostructures (Table 1). In fact, DAMP is an analogue of 2-mercaptopypyrimidine in E. coli tRNA, and although it has almost no direct pharmacological action in itself (Table 1), it is usually used as a pharmaceutical intermediate for the synthesis of antimicrobial agents. A previous report has also shown that DAMP-Au NPs can kill some Gram-negative bacteria but are inactive against Gram-positive bacteria.

Furthermore, we performed the killing kinetic assays to determine the changes in the antibacterial action (i.e., AuDAMP) activity over time. As illustrated in Figure S4, AuDAMP shows concentration- and time-dependent antibacterial behavior against test bacteria, with different dynamics for different strains. In particular, AuDAMP caused a marked decrease in the amount of S. aureus ATCC29213 and MRSA colonies within 6 h and all bacterial cells were eradicated within 2 h at 2x MIC concentration (Figure S4Gg). These data show that the AuDAMP NCs possess fast killing kinetics, besides high antibacterial activity against superbugs.

Bacterial biofilm is the main source of persistent infections and can enhance drug resistance. Thus, we also evaluated the effects of AuDAMP on biofilm formation and mature biofilms using MRSA as a model. Remarkably, AuDAMP has a highly effective antibiofilm activity, and very low concentrations of relevant Au NCs can significantly inhibit the formation of MRSA biofilms (Figure S5). This result is similar to our previous report on inhibition of the biofilm formation of MRSA by organometallics, indicating excellent antibiofilm formation features by AuDAMP. In addition, AuDAMP can also eliminate mature biofilms of MRSA at lower concentrations (Figure S6), whereas most known antibacterial agents, including noble metal nanoparticles, cannot exhibit a similar effect.

Long-term bacterial resistance is one of the biggest challenges in the current therapy of infections. The use of any new antibiotics, without exception, has led to the emergence of drug-resistant strains, and even bacteria that tolerate Ag NPs have emerged. To evaluate the potential resistance of bacteria to Au NCs, standard strain S. aureus ATCC29213 was repeatedly exposed to AuDAMP at a sub-lethal concentration (one-third of MIC). As expected, S. aureus ATCC29213 rapidly developed significant resistance to conventional antibiotic vancomycin at sub-lethal dose within a few days. However, no resistance development toward the AuDAMP was demonstrated even after continuous passage for 30 days (Figure 2). Indeed, it is well-known that vancomycin-resistant S. aureus is a severe threat to public health.
contrast, there is no evidence that bacteria can be resistant to antibacterial Au nanomaterials. These results all indicate that AuDAMP are a novel and effective nanoantibiotics against superbugs and do not cause drug tolerance.

The antimicrobial mechanism of the Au NCs was visited using MDR E. coli and MRSA as models. The high positive charge on the surface of AuDAMP can promote electrostatic adsorption onto the bacterial surface, enabling easy and efficient internalization (Figure S7). To study whether AuDAMP destroy bacterial cell membranes, we performed the bacterial life and death identification experiments using our previous method.29 Figure 3a shows the confocal laser scanning microscopy (CLSM) images of MDR E. coli and MRSA treated with AuDAMP. The control group bacteria show only green fluorescence, demonstrating that the bacterial cells are living. However, after treatment with AuDAMP (final concentration, 20 μg/mL), almost all bacterial cells emit strong red fluorescence, which shows negligible emission by the green fluorescence channel, indicating that the bacterial cell membranes have been completely disrupted and superbugs are killed.

To further confirm the antibacterial mechanism of AuDAMP, the scanning electron microscope (SEM) study was carried out. The untreated MDR E. coli and MRSA cells show intact and smooth morphology (Figure 3b). After treatment with 20 μg/mL of AuDAMP at 37 °C for 2 h, the MDR E. coli and MRSA cells change into a wizened and damaged state. Moreover, for most of the pathogenic cells especially MDR E. coli, cellular content leakage can be observed (Figure 3b). These results confirm that AuDAMP can strongly interact with the pathogenic cell membranes, which are not only Gram-positive but also Gram-negative and readily kill such superbugs by cell damage.

Following the destruction of comprehensive cell membrane, the Au NCs can easily enter the bacteria and interact directly or indirectly with the bacterial genomic DNA, thereby
inducing the DNA destruction. To confirm this, we extracted and electrophoretically analyzed the genomic DNA of bacteria after treatment with Au DAMP, as presented in Figure 3c. The untreated and DAMPAu(I)-treated MRSA shows a compact DNA band, indicating there is no DNA destruction. However, the Au NCs and Au NPs treated group showed remarkable smearing on its DNA band, suggesting the presence of shorter DNA fragments and genomic DNA destruction caused by the treatment.

Many nanoparticle-based antibacterial agents, including Au NCs, attribute their efficiency to their ability to modulate the ROS production that ultimately eliminates the bacteria.27,37,39 As such, we called on whether the antibacterial efficiency of AuDAMP was driven by ROS generation. As illustrated in Figure 3d, AuDAMP treatment induces a dramatic increase in the intracellular ROS production in bacteria when compared to the water-treated group. Consistent with previous reports10,27, mercaptopyrimidine-stabilized Au NPs do not induce any visible increase of ROS in bacterial cells (Figure 3d). When antioxidant N-acetyl-L-cysteine (NAC) was incorporated, the ROS production induced by Au NCs was eliminated, pulling down the ROS to the original level (Figure 3d). These results clearly demonstrate that intracellular ROS production was the key factor in the potent antibacterial capability of AuDAMP.

Based on the above observations, we further studied the reason AuDAMP can induce intracellular ROS generation and Au NPs cannot. This result allows us to accurately identify the fundamental mechanism that leads to the ROS production and the subsequent antibacterial ability. It is noteworthy that previous studies have found that the ROS-induced antibacterial performances of noble metal-based nanoantibiotics are generally attributed to their intrinsic oxidase-like and peroxidase-like catalytic capacity.12,40−42 Considering that the size of the Au NPs significantly affects its catalytic performance,45,46 we therefore hypothesize that Au NCs can induce ROS generation possibly because of their oxidase-like and peroxidase-like nature, whereas the Au NPs cannot. To explore the oxidase-like nature of Au nanoantibiotics, we employed the 3,3′,5,5′-tetramethylbenzidine (TMB) as probe, which has a maximum absorbance at 653 nm when it is oxidized (producing a blue product). The results show a time-dependent increase in TMB oxidation catalyzed by Au NCs (Figure 4a). However, there is no obvious oxidized TMB characteristic absorbance after the addition of Au NPs, even after 30 min (Figure S8). To further confirm the oxidase-like property of the Au nanoantibiotics, we detected the generation of hydrogen peroxide (H2O2) in the catalytic oxidation process of ascorbic acid (AA, an effective antioxidant in cells) by the Au nanoantibiotics. As shown in Figure 4b, a remarkable increase of H2O2 is detected in the presence of Au NCs compared with control group (AA only), while there is almost no increase in the presence of large-size Au NPs. These data unarguably affirm that the Au NCs can catalyze the oxidation of antioxidants and accompany H2O2 generation.

We then evaluated the peroxidase-like performance of the Au nanoantibiotics by following the catalysis of the TMB by H2O2. The results from the UV−vis absorption spectra suggest that the relevant Au NCs can catalyze the oxidation of TMB by H2O2 (Figure 4c), as the expected one-electron oxidation intermediate product TMB+ was detected (inset in Figure 4a).

Figure 4. Oxidase- and peroxidase-like properties of the Au nanoantibiotics. (a) Time-dependent absorbance spectra of the TMB catalyzed by Au NCs with the characteristic absorption maximum of oxidized TMB at 653 nm. (b) Concentration of H2O2 generated in the catalytic system: AA alone and AA with the different Au nanoantibiotics. The data indicate the means and standard deviation (SD) from three parallel experiments. P-values were calculated by the Student’s t-test: **p < 0.01. (c) Au NCs+H2O2+TMB, where the maximum absorbance values for the TMB+ intermediate (responsible for the characteristic blue color) are at 370 and 653 nm. The inset images (tubes) represent the visual color changes of TMB in different reaction systems from left to right: H2O2+TMB; Au NCs+TMB; Au NCs+H2O2+TMB. (d) Fluorescence spectra for detection of hydroxyl radicals (•OH) from the different reaction systems. Here, the intermediate fluorescence product, 2-hydroxy-TA, presents a maximum emission wavelength at 435 nm.
which in turn is a symbol of the Au NC’s peroxidase-like activity. The catalytic oxidation rates of these Au NCs increase with increasing concentrations and reaction times (Figure S9). Similarly, the Au nanoantibiotics also exhibit a highly size-dependent peroxidase-like performance because the large-sized Au NPs have almost no catalytic activity (Figure S10).

Despite the specific mechanism of the mimic peroxidase catalysis is not yet clear, one possible reason for the peroxidase-like nature of the Au NCs might originate from the Au NC’s ability to decompose H$_2$O$_2$ species to produce hydroxyl radicals ($\cdot$OH). To further understand the reason the Au NCs have a better catalytic property than the Au NPs, we therefore compared their productivities in producing $\cdot$OH from H$_2$O$_2$ using terephthalic acid (TA) as a molecular probe, which can easily combine $\cdot$OH to form fluorescent substance 2-hydroxy-TA (inset in Figure 4d). As illustrated in Figure 4d, that strong fluorescent was observed in the systems containing TA, Au nanoantibiotics, and H$_2$O$_2$, whereas only a very feeble fluorescent was observed in the absence of either Au nanoantibiotics or H$_2$O$_2$. This finding indicated that the $\cdot$OH species are mainly generated from the decomposition of H$_2$O$_2$ in the presence of Au nanoantibiotics as catalysts. Notably, the fluorescent intensity of the system of H$_2$O$_2$, Au NCs, and TA is much stronger than that of the system of H$_2$O$_2$, Au NPs, and TA. This data demonstrates that the Au NCs are more efficient in the decomposition of H$_2$O$_2$ to generate $\cdot$OH than large-sized Au NPs, which may be related to their better catalytic performance toward the oxidation of TMB by H$_2$O$_2$ relative to Au NCs.

In general, the data from both membrane integrity assay and SEM experiments uncover that AuDAMP can effectively damage the bacterial cell membranes and change their permeability. The change in cell membrane permeability then allows AuDAMP to easily internalize into bacterial cells, inducing the destruction of bacterial genomic DNA. More importantly, due to the intrinsic oxidases- and peroxidase-like catalytic properties, AuDAMP could strongly induce intracellular ROS production after their entry. The generation of intracellular ROS further accelerates the death of bacteria. Due to the fact that bacteria have difficulty developing resistance to antimicrobial agents that cause membrane damage, the ability to damage bacterial cell membranes also effectively enables AuDAMP against drug resistance.

To evaluate the preliminary biocompatibility of these Au NCs toward mammalian cells, we performed MTT assay by using human normal liver L02 cells and normal lung AT II cells as mammalian cells models. As illustrated in Figure S11a, the AuDAMP show negligible toxicity to L02 and AT II cells at doses up to 256 μg/mL, which is much higher than the MIC for bacteria (Table 1). Meanwhile, the hemolytic behavior was also investigated by treatment of human erythrocytes with AuDAMP. Similar to the results of cytotoxicity experiments, no noticeable hemolysis was observed for AuDAMP at a relatively higher dose (Figure S11b). In addition, the $in vivo$ cytotoxicity was also evaluated. The histopathological results showed no observable damage in the main organs of mice between the AuDAMP-treated group and the untreated group (Figure S11c). Overall, AuDAMP exhibit excellent biocompatibility to both mammalian cells and the living body, and may be suitable for further $in vivo$ applications.

To further reveal the potential of biocompatible AuDAMP for the $in vivo$ applications, the $in vitro$ antibacterial capacity was also evaluated with coculture model of bacteria (i.e., MRSA) and macrophages (i.e., RAW 264.7), a conventional
mononuclear phagocyte system infection model. MRSA were marked with SYTO 9 dye before coculture with RAW 264.7 macrophages. Comparing the untreated group with the DAMPAu(I)-treated group, the number of intracellular MRSA significantly decreased in the presence of AuDAMP (Figure 5a). The amount of MRSA colonies further tested the high antibacterial property of AuDAMP toward MRSA inside the macrophage cells (Figure 5b). In the meantime, the cell nuclei of macrophages (blue stained by Hoechst 33342 dye) remain intact after treatment with DAMP and AuDAMP, indicating that the RAW 264.7 cells are still alive. These results clearly demonstrate that AuDAMP can kill superbugs inside the macrophages with high efficiency, but with no harm to the macrophages due to excellent biocompatibility.

To assess the application of AuDAMP to fight superbugs in vivo, we first used AuDAMP in a mouse skin infection model. MRSA (10^9 CFUs/wound) was selected to infect the wounds, which was followed by single-dose treatment of AuDAMP (0.1 mL, 10 μg/mL). Vancomycin (0.1 mL, 10 μg/mL) and normal saline (NaCl, 0.9%) were employed for assaying as positive control and negative control, respectively. Treatment effect was evaluated by measuring the wound sizes every 2 days and by calculating the colonies counts for the tissue homogenate of the infected sites on day 10 (Figure 6). After 10 d treatment, the wounds of mice are basically healed in the AuDAMP treatment group and the vancomycin treatment group, while the relative wound size of the negative control group was still as high as about 35% (a and b of Figure 6). Considering the whole healing process, AuDAMP as well as vancomycin treatment can expedite wound healing and reduce the probability of infection and death. Indeed, after normal saline treatment, quantification of the bacterial burden in the wound showed 6.3 × 10^6 CFU/g of MRSA. Mice treated with AuDAMP showed a bacterial burden of 3.4 × 10^4 CFU/g, which was a noteworthy reduction compared to the normal saline-treated group (with 99% bactericidal efficacy) (Figure 6c). In addition, a mouse pneumonia model was also used to further evaluate the treatment effect of AuDAMP (Figure S12). In the normal saline-treated group, all mice died after 5 days of treatment, and the amount of MRSA in the lung was as high as 10^7 CFU/g. Compared with normal saline-treated group, all mice survived after 6 days of AuDAMP therapy, and the amount of MRSA in the lung was decreased significantly by an order of magnitude. These results all show that the as-prepared Au NCs have excellent therapeutic effect, comparable to traditional antibiotics, and have great potential for clinical application of MDR superbug’s infections.
CONCLUSION

In summary, a new class of Au NCs stabilized with selected mercaptopyrimidine analogue ligands has been shown to be superior nanobiotics against both MDR Gram-negative and Gram-positive superbugs in vitro and in vivo, without inducing drug resistance and showing noticeable cytotoxicity and hemolysis to mammalian cells. The broad spectrum antibacterial property attributed to the Au NCs’ oxidase-like and peroxidase-like catalytic activities has been demonstrated for the first time. When the enzyme-like Au NCs were internalized, this intrinsic catalytic performance could induce an increase of intracellular ROS generation, conferring bactericidal efficacy as the end result. The present study not only extends the versatility of nanomaterial-based antibiotics, but also offers a new perspective for biomedical applications of ultrasmall Au NCs.

EXPERIMENTAL SECTION

Synthesis and Characterization of AuAMP, AuDAMP, AuAHMP, and AuDHMP. The preparation of Au NCs was described in our previous study. For typical synthesis of mercaptopyrimidine-stabilized Au NCs, 2 mL of mercaptopyrimidine (10 mM, dissolved in 50% ethanol solution) and 1 mL of HAuCl4 (10 mM) were added to 7 mL of ultrapure water. The mixed solution was heated to 70 °C and continually reacted under gentle stirring (300 rpm) for 12 h. The synthesized Au NCs were purified using ultrafiltration and centrifugation to remove bulk gold and free mercaptopyrimidine. The purified Au NCs solution were stored at 4 °C in the dark for further characterization. We synthesize AuAMP, AuAHMP, and AuDHMP using the same method. The morphologies of Au NCs were observed through transmission electron microscope (TEM, JEM-2100, Japan). The zeta potential was measured by Nano ZS Zetasizer 90 (Malvern, UK).

Antibacterial Activity Measurement. For evaluation of antimicrobial activity of Au NCs, E. coli ATCC35218, MDR E. coli, MDR A. baumannii, MDR P. aeruginosa, MDR K. pneumoniae, S. aureus ATCC29213, MRSA, and VRE were selected for use in this study. The MIC was determined using a broth dilution method. Bacteria (~1.0 × 10^7 CFU/mL) were cultured into LB medium of 2 mL supplemented with the Au NCs, antibiotics, or other agents. The final concentrations range from 0.5 to 128 μg/mL. The as-prepared bacterial solutions were inoculated at 37 °C for 24 h. The viable colony counts were determined before and after incubation.

Macrophage Infection Model. MRSA infected RAW 264.7 macrophages were built as an infection model to evaluate the antibacterial capacity of AuDAMP inside the macrophages. For visualization, MRSA (~1.0 × 10^8 CFU/mL) were marked with SYTO 9 dye though coincubated with SYTO 9 for 30 min. Similar, the RAW 264.7 macrophage cells (~1.5 × 10^6 cells/mL) were stained with Hoechst nuclear dye for cell visualization. Then, Hoechst-labeled macrophages were subsequently with SYTO 9-labeled MRSA at a MOI (multiplicity of infection) of 10:1 and cultured for 2 h at 37 °C in medium without antibiotics. After culture, the suspensions were centrifuged at 5000 rpm for 5 min, and the cells were washed with sterile normal saline three times. Afterward, RAW 264.7 cells were incubated with medium containing 10 μg/mL AuDAMP for another 2 h after washing for three times. The treatment of 10 μg/mL vancomycin was employed as a control. The mixtures were observed using a CLSM. To further quantify the efficacy, 100 μL diluted mixtures with and without the treatment of AuDAMP were then coated onto LB plates. The amount of bacterial colonies was computed after incubation.

Mice Skin Infection Model. Four-week-old BALB/c female mice were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). All animal experiments were performed according to the guidelines for Animal Experimentation with the approval of the National Institute of Biological Science and Animal Care Research Advisory Committee of Southeast University. To investigate the in vivo antibacterial capacity of Au NCs, the MRSA-infected mice model was prepared. 200 μL of MRSA (1.0 × 10^9 CFU/mL) in normal saline was subcutaneously injected into the mice. One hour after infection, the mice in the experimental group are intraperitoneally injected with AU NCs (0.1 mL, 10 μg/mL), the positive control group is injected with vancomycin (0.1 mL, 10 μg/mL), and the blank control group is injected with normal saline (NaCl, 0.9%). After 10 d of treatment, the bacterial amounts in the wounds were counted.

Pneumonia Model. The pneumonia model was established by our previous method. Mice were intranasally infected with 100 μL suspension of MRSA (1.0 × 10^7 CFU/mL) in normal saline added dropwise to the nares. After administering Au NCs (0.1 mL, 10 μg/mL) once a day, the mice were euthanized and the lung was harvested. The MRSA amounts in the lung samples were counted.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.8b00452.

Additional experimental section, including bactericidal kinetic assay, biofilm formation inhibition test, CLSM observation of biofilm, mature biofilm elimination assay, membrane integrity test, bacterial morphological characterization, drug resistance development, ROS assay, mimic enzymes properties of Au Nanoantibiotics, MTT assay, hemolysis assay, and histopathology; fluorescence features, stability, TEM, time-kill curves, biofilm inhibition, UV–vis absorbance spectra, biocompatibility evaluation, and plate counting results (PDF)

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Notes

The authors declare no competing financial interest.

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REFERENCES


