Antimicrobial peptides and antibacterial nanostructures are two emerging classes of antimicrobial agents that differ from conventional small-molecule antibiotics. Combining these two types of antimicrobial agents into one entity may be an effective strategy to improve their antimicrobial efficiency. In this study, we demonstrated an effective antibacterial hybrid formed by covalently conjugating antibacterial gold nanoclusters (Au NCs, a novel antimicrobial nanostructure) and daptomycin (Dap, a cyclic lipopeptide antimicrobial peptide). The as-synthesized hybrid structure (Dap\textsubscript{C0}Au NCs) not only inherits the intrinsic properties from both agents but also renders an enhanced synergistic effect. Compared with the physically mixed Au NCs and daptomycin (Dap+Au NCs), the Dap\textsubscript{C0}Au NCs hybrid structure has a stronger bactericidal effect toward methicillin-resistant Staphylococcus aureus, a representative of multidrug-resistant bacteria. Dap\textsubscript{C0}Au NCs could effectively disrupt bacterial membranes by creating more and/or larger holes in the membranes due to the localized daptomycin within the conjugated structure. These larger (and possibly more) holes motivate the entry of Dap\textsubscript{C0}Au NCs into bacterial cells and lead to more serious damage of the bacteria at subcellular levels. Moreover, bacterial genomic DNA fragmentation was further quantified to show that Dap--Au NCs may induce severe DNA breaks. The strong DNA destruction benefited from localized high concentrations of reactive oxygen species (ROS) induced by the localization of Au NCs in the antimicrobial conjugation. The conjugated Au NCs could serve as a critical free radical generator to continuously produce ROS within the bacteria. The continuous ROS bombings also limit the capacity of the bacteria to develop drug resistance. In addition, a significant fluorescence enhancement of the hybrid structure was observed due to a novel aggregation-induced emission (AIE) pattern caused by the Au NCs and daptomycin conjugation. This conjugation strategy provides a new perspective for the synthesis of new antimicrobial agents as well as AIE-type fluorescence materials.
1. Introduction

Since the discovery of penicillin, a series of small-molecule antibiotics have been developed and applied to the treatment of bacterial infections, making a significant contribution to human health [1–4]. Unfortunately, unreasonable antibiotic treatments and the indiscriminate use of antibiotics have resulted in the emergence of serious multidrug-resistant (MDR) infections. For example, worldwide drug-resistant bacteria, such as ESKAPE epidemics, have rendered previously effective antibacterial treatments ineffective, which consequently impose serious public health challenges [5–7]. In addition, the pace of conventional antibacterial development lags far behind the development of bacterial resistance [1].

Antibacterial nanostructures, including metals, metal oxides, and carbon-based nanomaterials have been applied as potential antibacterial alternatives to combat superbug infections and overcome the drug resistance of conventional small-molecule antibiotics [8–12]. The excellent antibacterial capability of engineered nanomaterials, with ultrasmall sizes and high surface-to-volume ratios, is mainly attributed to their large contact surface with bacterial cells that results in the disruption of the cell permeability and physiological functions of bacterial membranes [13,14]. Moreover, the internalized nanoparticles (NPs) further interact with intracellular components such as proteins and DNA, which disrupts normal cellular functions [6,15]. In addition, the induction of reactive oxygen species (ROS) generation by nanomaterials may also cause bacterial damage [14]. The multiple antibacterial mechanisms also greatly reduce the probability that bacteria will develop a resistance to nanomaterials [1,15,16]. However, although antibacterial nanomaterials possess a high potential for the treatment of MDR bacteria, they also present a serious threat due to their toxicity to human cells [9,17]. To reduce cytotoxicity, nanomaterials functionalized with antibiotics, antimicrobial peptides (AMPs), and lysozymes have been fabricated [13,18,19]. In addition, significant efforts have been committed to the development of less toxic noble metal-based nanomaterials including gold nanoclusters (NCs) [6,20–22], platinum NPs [23], and palladium nanocrystals [9,24] for antibacterial applications.

Combination therapy of bactericides, which can synergistically improve the performance and reduce the adverse effects of individual agent, is one of the most commonly used strategies for the treatment of serious MDR bacterial infections [25–28]. This effective strategy can attack bacteria from two distinct fronts, and bacteria need to overcome more difficult evolutionary hurdle in order to develop resistance. Gold nanomaterials have been known to possess excellent biocompatibility and have attracted great attention in the biomedical field [29–31]. It is worth noting that when the size of gold nanomaterials decreases to sub-nanometer dimensions (i.e., NCs), these ultrasmall Au NCs begin to have distinctive physicochemical and biological properties. For example, Xie’s group showed that 6-mercaptopentanoic acid-stabilized Au NCs (~2 nm) can kill test pathogenic bacteria through inducing ROS oxidative damage, while larger Au NPs (~6 nm) cannot show a homogeneous effect [14]. Our previous results also demonstrated that thiolated Au NCs with good biocompatibility show strong antibacterial properties against superbugs [6]. In other words, ultrasmall Au NCs can possess superior biocompatibility and good antibacterial activity at the same time. Such properties make Au NCs an excellent candidate for the intended combination therapy with other antimicrobial agents, such as AMPs. As a successful practice, Chen and coworkers demonstrated that nanocomplexes obtained by the self-assembly of surfactin and Au nanodots possess a lower (~80-fold) minimal inhibitory concentration and a better biocompatibility than those of surfactin [15].

On the other hand, aggregation-induced emission (AIE) enhancement, developed by Tang’s group in 2001, has opened new doors for producing highly fluorescent materials [32–35]. Thiolated metal NCs have been demonstrated to exhibit the AIE phenomenon, and several investigations have obtained strongly fluorescent composite materials, employing their AIE properties [36–42]. Previous studies have shown that the AIE enhancement of metal NCs is mainly induced by solvents and ions [36–40]. In this work, we have reported an AIE enhancement caused by the conjugation of Au NCs and AMPs (Scheme 1). We have shown that 4,6-diamino-2-mercaptopyrroline (DAMP)-mediated Au NCs (AuDAMP) have a weak fluorescence and that the NCs can strongly eliminate MDR bacteria via a combined bactericidal mechanism (i.e., ROS production, membrane damage, and DNA destruction) [6]. DAMP is an analogue of 2-mercaptopryroline in Escherichia coli RNA. Although DAMP presents almost no direct pharmacological action, it can disturb bacterial tRNA synthesis and is commonly used as a pharmaceutical intermediate for the synthesis of antimicrobial agents, including Au NPs [15,43–45]. AMPs are a class of well-known natural antimicrobial agents with a potent antibacterial ability since their unusual amino acid sequences can be easily inserted into bacterial cell membranes to cause cell membrane rupture [7,46–48]. Daptomycin (Dap), a cyclic lipoprotein antimicrobial peptide, is composed of a cyclic 13-member peptide linked to a lipophilic decylic acid side chain and has a strong antibacterial activity against most Gram-positive bacteria including MDR and drug-susceptible strains (e.g., vancomycin-resistant Enterococcus faecalis, Streptococcus pyogenes, and methicillin-resistant S. aureus (MRSA)) [49]. It exerts an antibacterial efficacy via a proposed mechanism, where the lipophilic tail of Dap can easily insert into the bacterial cell membrane with the assistance of calcium to induce rapid cell membrane damage and potassium ion efflux [49,50]. The hybrid Dap–AuDAMP NCs were prepared through the covalent conjugation of Dap on DAMP-anchored Au NCs by the strong covalent bond (amide) between the carboxyl of Dap and the amino of DAMP molecules (Scheme 1). The conjugated particles could provide a maximum of ~4-fold enhanced fluorescence due to the AIE effect. The conjugation of AuDAMP and Dap into one entity allows the NCs to possess a high specific area for intracellular ROS production and helps Dap, with its lipophilic tail insertion for optimal membrane damage, to be highly localized on the surface of NCs. Therefore, relative to physically mixed Dap and Au NCs (Dap+AuDAMP), Dap–AuDAMP possessed a superior antibacterial activity toward MRSA, a representative of MDR Gram-positive bacteria. This conjugation strategy may also significantly reduce the bacterial exposure to Dap, which will curtail the likelihood of the development of bacterial resistance to Dap in the long run.

2. Experimental section

2.1. Reagents and instruments

DAMP (~>98%) and N-(3-(dimethylamino)propyl)-N'-ethylcarbodiimide hydrochloride (EDC, ~97%) were purchased from Sigma-Aldrich (USA). The LIVE/DEAD BacLight Bacterial Viability Kit was obtained from Invitrogen (USA). The TIANamp Bacteria DNA Kit was purchased from Tiangen Biotech Co. Ltd. (China). Lipid Peroxidation (LFD) MDA Assay Kit, ROS Assay Kit, Hoechst 33342, and Colorimetric TUNEL Apoptosis Assay Kit were obtained from Beyotime Biotech (China). Chloroauric acid (HAuCl4), daptomycin (~>98%), agaro, agar, N-acetyl-L-cysteine (NAC, ~>98%), glutaraldehyde (25%), Triton X-100, dimethylsulfoxide, N-hydroxysuccinimide (NHS, 98%), and other common reagents were purchased from Sinopharm Ltd. (China). Ultra-pure water (18.2...
MΩ cm, Millipore) was employed throughout the solutions preparation.

UV–vis and fluorescence spectra were obtained using a Biomate 3S spectrophotometer (Thermo Fisher, USA) and a RF-5301PC fluorimeter (Shimadzu, Japan), respectively. The fluorescent lifetime measurement and X-ray photoelectron spectroscopy (XPS) were performed on a FLS920 fluorescent spectrometer (Edinburgh, UK) and a ULVAC-PHI 5000 VersaProbe XPS (Japan), respectively. The fluorescent lifetime measurement and X-ray photoelectron spectroscopy (XPS) were performed on a FLS920 fluorescent spectrometer (Edinburgh, UK) and a ULVAC-PHI 5000 VersaProbe XPS (Japan), respectively. The ultimate analysis was performed using a Spectroblue inductively coupled plasma optical emission spectrometer (ICP-OES) (Speicher, Germany). The hydrodynamic size and zeta potential were obtained by a Malvern Nano ZS Zetasizer 90 (UK). Fourier transform infrared spectra (FTIR) were recorded by a Nicolet iS5 spectroscopy (Thermo Fisher, USA), and the thermogravimetric analysis (TGA) utilized a TG209F3 analyzer (Netzsch, Germany) under a N2 atmosphere. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) images were obtained with a Zeiss Ultra Plus SEM (Zeiss, Germany) and a JEM-2100 TEM (JEOL, Japan), respectively. Confocal microscopic images were acquired with a Nikon Ti-E confocal microscope (Japan).

2.2. Synthesis and characterization of gold nanoclusters

The synthesis of AuDAMP was based on our previous description [6,51]. For a typical synthesis procedure, 100 μL of HAuCl4 (0.1 M) and 200 μL of DAMP (0.1 M) were added to 9700 μL of H2O. The solution underwent a hydrothermal reaction (70°C/176°C) under gentle stirring (300 rpm) for half day, and the red-emitting AuDAMP was formed. The synthesized AuDAMP was purified using an ultrafiltration tube (molecular weight cut-off, 3 KDa) to remove free DAMP. The purified AuDAMP solution was stored in a refrigerator for further use and characterization.

For the preparation of Dap–AuDAMP, the as-synthesized AuDAMP (10 mL) were first mixed with N-(3-(dimethylamino) propyl)-N-ethylcarbodiimide hydrochloride (EDC, 0.1 mL, 0.8 M) and N-hydroxysuccinimide (NHS, 0.1 mL, 0.4 M) and followed by the addition of Dap (0.1 mL, 5 mM) to the solution, which was stirred continually for 30 min (300 rpm). The Dap–AuDAMP product was collected after centrifugation (10,000 rpm) and air drying.

2.3. Bacteria culture

MRSA were cultured in Luria-Bertani (LB) agar at 35 °C. A bacterial suspension was obtained by inoculating a MRSA single colony into LB medium that was then cultured for 8 h at 35 °C and 180 rpm. Freshly harvested cells were used during each experiment.

2.4. Agar plates assay

Phosphate buffer saline (PBS, negative control), Dap, AuDAMP, Dap+AuDAMP, and Dap–AuDAMP were mixed with fresh MRSA suspensions (~1.0 × 10⁸ CFU/mL). After incubation for 1 h at 35 °C, 0.1 mL of the diluted bacterial suspension was plated onto the LB agar. The colony counts were performed after 24 h incubation.

2.5. Zone of inhibition measurements

The inhibitory effect of Au NCs was tested using the double-layer agar method [52]. An aliquot of 0.2 mL of Au NCs solution was pipetted into each hole. The culture dishes were cultured at 35 °C, and the diameters of the inhibition zone were measured after 24 h. The same volumes of PBS and Dap were used as controls.

2.6. Bactericidal dynamics

Bacterial suspensions (~1.0 × 10⁸ CFU/mL) were subjected to different final concentrations of Au NCs at 35 °C for 1 h on a shaking incubator (180 rpm). The colony counts were performed at different times during the incubation process.
2.7. Membrane integrity assay

The LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen) was employed for the determination of bacterial membrane integrity. The MRSA were dealt Au NCs in LB at 35 °C for 1 h. The working samples were stained with SYTO 9 and propidium iodide (PI) and treated for 30 min before observation using confocal laser scanning microscopy (CLSM, Nikon).

2.8. Measurement of internalized gold

MRSA were treated with Au NCs for 1 h and then washed several times until all uninternalized NCs were removed. The cleaned bacterial cells were air dried and digested with *aqua regia*. The internalized gold content was measured by an ICP-OES (Speicher, Germany).

2.9. Reactive oxygen species assay

MRSA were treated with Dap and Au NCs for 1 h at 35 °C and then were collected by centrifugation. Then, the intracellular ROS were measured using an ROS Assay Kit (Beyotime, China). The bacterial samples were treated with 2,7′-dichlorofluorescein diacetate (DCF-DA) for 1 h in the dark. The fluorescence intensity of bacterial suspensions was registered with a Shimadzu RF-5301 PC fluorospectrophotometer. A similar procedure was also used for the measurement of ROS in a cell-free system.

2.10. Lipid peroxidation test

The MRSA incubated with Dap and Au NCs for 1 h were centrifuged down. The lipid peroxidation (LPD) test was conducted by an LPD MDA Assay Kit (Beyotime, China) according to the manufacturer’s protocol.

2.11. Bacterial genomic DNA damage

The bacteria were incubated with Dap and Au NCs for 1 h and then were collected by centrifugation, respectively. The DNA was extracted by a TIANamp Bacteria DNA Kit (Tiangen, China). After the genomic DNA was extracted, 10 μL DNA of each sample was separated on a 1% agarose gel by electrophoresis.

2.12. Terminal deoxynucleotidyl transferase dUTP nick end labeling assay

The MRSA incubated with Dap and Au NCs for 1 h were fixed on slides with 2.5% glutaraldehyde in water for 1.5 h and permeabilized with Triton X-100 (1%) in PBS for 20 min. The positive control was prepared by using a 10 U/mL DNaseI solution to incubate for 30 min at room temperature. The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was conducted by a colorimetric TUNEL Apoptosis Assay Kit (Beyotime, China) per the manufacturer’s instructions.

3. Results and discussion

Ultrasound AuDAMP can be facilely prepared by the hydrothermal reduction of an Au precursor and by the reducibility of DAMP itself. The as-synthesized AuDAMP was dark yellow in solution with weak a red fluorescence under UV light (inset in Fig. 1a). We noted that the as-synthesized AuDAMP exhibit no characteristic UV absorption peaks, which is consistent with our previously reported mercaptopyrimidine-directed Au NCs that possess an excellent antibacterial capacity [6,51]. Since DAMP and Dap are rich in amino and carbonyl groups (Fig. S1 in the Supplementary data), respectively, the ultrasmall AuDAMP and Dap can form a stable hybrid structure by a simple amidation reaction in the presence of EDC and NHS (Scheme 1). The color of the solution became pale yellow after the conjugation reaction (inset in Fig. 1a), suggesting that particle aggregation may occur. Further, we found that the formed hybrid structure (Dap–AuDAMP) exhibits a strongly fluorescence enhancement accompanied by a slight blueshift (~15 nm) in the fluorescence emission peak (Fig. 1a). Using different Dap concentrations results in a maximum ~4-fold boost in the fluorescent intensity for the conjugated Dap–AuDAMP prepared with 100 μM Dap (Fig. S2). The fluorescent intensity decreases at higher concentrations of Dap, maybe due to the precipitation of aggregates. In addition, the fluorescence enhancement of Dap–AuDAMP excited by different excitation wavelengths is almost unchanged (Fig. S3), demonstrating that light scattering by the conjugated system is not responsible for the fluorescence enhancement [53]. Combining these results, we hypothesized that the AuDAMP and Dap conjugates undergo AIE enhancement.

The TEM image visually shows the occurrence of aggregation, and the conjugated Dap–AuDAMP, with an agminated network shape, is observed (Fig. 1b). Moreover, the sizes of AuDAMP particles are same in both AuDAMP and Dap-conjugated samples. This result is in line with the AIE of metal NCs, indicating that the conjugation-induced AIE enhancement does occur [42,53–55]. Furthermore, the dynamic light scattering (DLS) analysis also demonstrates the size increase following the conjugation of AuDAMP and Dap. As illustrated in Fig. 1c, the mean hydrodynamic size of the conjugated Dap–AuDAMP has a size of approximately 190 nm, while the unconjugated AuDAMP was approximately 6 nm.

The XPS analysis was applied to obtain the valence states of the gold species of AuDAMP before and after Dap conjugation. It is worth noting that the oxidation states of metal NCs are dictated by their structure and size [56]. However, the Au 4f7/2 binding energies are identical at 83.8 eV, regardless of whether the fluorescent AuDAMP was conjugated to Dap or not (Fig. 1d), implying that the conjugation of Dap to the NC surface cannot affect the oxidation states of the gold species in AuDAMP [51,57]. These data is also in good agreement with our TEM results in Fig. 1b.

The fluorescence properties of the conjugated Dap–AuDAMP and AuDAMP were further examined by fluorescence lifetime measurements. As illustrated in Fig. 1e, the fluorescence lifetime decay profile implies the existence of long fluorescence lifetime (microsecond level) components in the conjugated Dap–AuDAMP, namely, 4.659 μs (99.97%) and 26 ns (0.03%) (Detailed fitting data see Supplementary data). The microsecond-scale lifetime in Dap–AuDAMP was similar to the fluorescence lifetime of the recently reported AIE-type fluorescence Au NCs [36]. These results further demonstrate that the fluorescence emission of Dap–AuDAMP is derived from the AIE effect induced by AuDAMP and Dap.

To demonstrate the successful conjugation of AuDAMP and Dap, the FTIR analysis was performed. As shown in Fig. 1f, we detected a prominent peak at 1280 cm⁻¹ for Dap–AuDAMP, which is characteristic of the amid bond (arylamino) within the hybrid structure. This result is incontrovertible in confirming the successful conjugation of AuDAMP and Dap (Dap–AuDAMP). In addition, to measure the content of daptomycin within the Dap–AuDAMP conjugated structure, we performed the TGA analysis. As shown in Fig. 5, the TGA analysis showed that daptomycin accounted for ~10% (w/w) of Dap–AuDAMP, giving a mass ratio close to 1:9 for daptomycin and AuDAMP within the Dap–AuDAMP hybrid structure.

To examine the antibacterial efficacy of our conjugated structure, a series of antibacterial experiments were performed using MRSA as a model. Dap–AuDAMP was used at a concentration of 10 μg/mL. Since the TGA analysis revealed that the mass ratio of
Dap to AuDAMP was 1:9 (Figure S4), we introduced 1 \( \mu g/mL \) Dap and 9 \( \mu g/mL \) AuDAMP individually or physically mixed with MRSA as control groups. Compared to the other treatments, Dap–AuDAMP showed the best bactericidal effect, and the system was able to effectively eliminate 100% of the bacterial cells after an incubation of 1 h (Fig. 2a). The treatments with Dap alone and
AuDAMP alone showed similar antibacterial activities, and the relative viable bacteria that remained after treatment for 1 h were 37.5% and 41.7%, respectively (Fig. 2a). Interestingly, the Dap–AuDAMP treatment indicated a significantly enhanced antibacterial property compared to that of the unconjugated counterpart (physical mixture of Dap+AuDAMP) with the same concentrations of Dap and AuDAMP (Fig. 2a). Similar results were also demonstrated by inhibition zone measurements. The group treated with Dap–AuDAMP showed a significantly enhanced and clear transparent inhibition zone compared to that of the Dap+AuDAMP group and other groups (Fig. S5), demonstrating its superior antibacterial activity. In addition, Dap–AuDAMP presents rapid bactericidal kinetics and can eliminate all bacteria in half an hour (Fig. 2b). In contrast, the viable bacteria that remained in the other groups were higher than 50% after the same treatment time (Fig. 2b). All these results indicate the conjugated structure including localized Dap and AuDAMP could induce a synergy that led to the enhanced antibacterial activity.

In a previous study, we found that AuDAMP possesses an intrinsic mimic enzyme activity and can destroy bacterial membranes by inducing intracellular ROS production [6]. Here, we also examined the membrane integrity through a SYTO9/PI bacterial viability kit. PI is a DNA-binding fluorescent dye that enters cells and binds to DNA only when the cell membrane is damaged (red fluorescence), whereas SYTO9 can label all bacteria regardless of being intact or damaged (green fluorescence). As such, these two stains could help us evaluate the degree of damage to the bacterial membrane after treatment. As illustrated in Fig. 3a, the MRSA cells in the PBS-treated group showed almost only green fluorescence, whereas SYTO9 hybrid could enter the cells more easily and then interact with genomic DNA to induce DNA destruction. To examine this regard, the agarose gel electrophoresis analysis of bacterial genomic DNA after treatment with Dap–AuDAMP was performed. As presented in Fig. 3d, PBS- and Dap-treated groups of MRSA showed an intact DNA band, suggesting that no DNA fragmentation occurred. This result is consistent with expectations because the main mechanism of Dap bactericidal action is to destroy the bacterial cell membrane, rather than cause damage to the genomic DNA. In contrast, the AuDAMP-treated, Dap+AuDAMP-treated and Dap–AuDAMP-treated groups all showed various degrees of smearing in their electrophoresis patterns, suggesting the presence of fragmented DNA fragments and genomic DNA destruction. Although these treatments showed a certain degree of DNA fragmentation, the longest smear exhibited in the electrophoretic bands also suggested the most severe DNA damage occurred in the Dap–AuDAMP-treated group. Moreover, bacterial DNA damage was further quantified by TUNEL assays, and the results also showed that Dap–AuDAMP induced severe DNA scission (Fig. 3e and f). The bacterial cells with fractured DNA were stained green after the TUNEL analysis, while the cells without DNA damage were stained blue by Hoechst 33342 (Fig. 3e). We determined the bacterial DNA damage index by calculating the fluorescent ratio of bacterial cells stained with TUNEL to bacterial cells stained with Hoechst 33342 (Fig. 3f). To compare the relative DNA damage efficiencies, DNase I was employed as a positive control, and the DNA damage index was approximately 0.42. In good agreement with the electrophoresis patterns (Fig. 3d), the DNA damage index was close to 0 in the PBS- and Dap-treated groups. The DNA damage index was approximately 0.14 for the Dap+AuDAMP-treated group, indicating that AuDAMP did cause minimal damage to the MRSA genomic DNA. In contrast, the treated bacterial DNA damage index reached 0.23 for the Dap+AuDAMP-treated group and may be related to the destruction of the bacterial cell membrane by Dap to allow more AuDAMP to enter the cell and induce DNA damage. Correspondingly, stronger DNA damage is presented for the Dap–AuDAMP-treated group, and the DNA damage index is as high as 0.31 (Fig. 3f).
Fig. 3. (a) CLSM images of MRSA treated with PBS, Dap, AuDAMP, Dap+AuDAMP, and Dap–AuDAMP at 35 °C for 1 h. The dead cells were visualized by PI staining (red), while SYTO 9 (green) was used to identify all cells. The scale bar is 20 μm. (b) The percentage of PI-stained bacteria that show damaged bacterial membrane. NAC is N-acetyl-l-cysteine, an antioxidant that can scavenge ROS production. Data are means ± S.D., n = 3, t test. Compared to the PBS-treated group, * is significant against the PBS-treated group, p < 0.05. (c) SEM micrographs of bacteria treated with PBS, Dap, AuDAMP, Dap+AuDAMP, and Dap–AuDAMP. The arrows point to leakages and holes. The scale bar is 2 μm. (d) The agarose gel electrophoresis results of the extracted genomic DNA from MRSA treated with PBS, Dap, AuDAMP, Dap+AuDAMP, and Dap–AuDAMP (from left to right). (e) Fluorescence images of MRSA after 1 h treatment. The cells were visualized by staining the DNA with Hoechst 33342 (blue), while the DNA damage status of the bacterial cells was ascertained with a TUNEL assay (green). The scale bar is 20 μm. (f) DNA damage index, i.e., the ratio of DNA-damaged bacteria to normal bacteria, shows the degree of DNA damage. Data are means ± S.D., n = 3, t test. Compared to the PBS-treated group, * is significant against the PBS-treated group, p < 0.05. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
According to the antibacterial mechanism of inducing oxidative damage through ROS generation (by AuDAMP) and creating holes in the bacterial cell membrane (by Dap) [6,49], we hypothesize that the enhanced antibacterial activity shown by Dap–AuDAMP is a benefit of the localized high concentration of drug and ROS due to the hybrid conjugate. To verify that intracellular ROS production is involved in the enhanced bactericidal performance of Dap–AuDAMP, we first determined the intracellular ROS production in bacteria from each treatment group (Fig. 4a). Compared with the PBS-treated group, the ROS production level shows no changes after treatment with Dap. This is reasonable because the mechanism by which Dap destroys bacterial cell membranes is achieved by the insertion and interaction of its lipophilic tail into the lipid layer of the cell membrane. In contrast, a significantly enhanced ROS generation can be observed for the AuDAMP, Dap +AuDAMP, and Dap–AuDAMP treatment groups (Fig. 4a). When N-acetyl-l-cysteine (NAC, an antioxidant that can scavenge ROS production) is incorporated, the AuDAMP-induced ROS enhancement is erased, reducing the ROS level close to the initial value (Fig. 4a). The CLSM observations also confirm that the bacterial membrane damage is associated with the ROS production (Fig. 3b and Fig. S6). The incorporation of NAC in the treatment groups (i.e., AuDAMP, Dap+AuDAMP, and Dap–AuDAMP) could significantly ameliorate the bacterial membrane destruction induced by ROS. These results irrefutably support our prior view that the AuDAMP-induced ROS were involved in destroying the bacterial membrane (Fig. 3a–c). These intracellular ROS were derived from the catalytic oxidation process mediated by AuDAMP, as a catalyst. Fig. S7 also confirms that the catalytic process only occurs in the presence of bacteria. The intrinsic oxidase- and peroxidase-like activities enable AuDAMP to catalyze the production of intracellular ROS, and subsequently, to induce cell damage [6].

It should be noted that the ROS produced after the conjugation of AuDAMP and Dap did not increase significantly when compared to that of the unconjugated counterpart (Fig. 4a), while the overall bacterial membrane destruction in the Dap–AuDAMP group was markedly higher than that in the Dap+AuDAMP group (Fig. 3a–c). This paradox indicates that ROS might not be the only contributor to the antibacterial properties of the hybrid structure. Therefore, we further investigated the role of Dap in Dap–AuDAMP. We first investigated the activity of Dap in our hybrid structure by measuring the diameter of the inhibition zone after the introduction of calcium. Since Dap is a calcium-dependent antimicrobial peptide, an increase in the concentration of calcium can enhance its bactericidal ability [50]. As shown in Fig. 4b, Dap–AuDAMP exhibited a stronger antibacterial activity against MRSA on the LB agar containing calcium than on the agar without calcium. This result demonstrates that the activity of Dap in the hybrid structure is maintained. Next, the LPD analysis was used to decouple the role of Dap by separating the ROS influence from the overall influence of Dap–AuDAMP on the destruction of the bacterial cell membrane. LPD can oxidize and degrade the lipids, and the degradation products are mainly reactive aldehyde species, such as malondialdehyde, which can be further reacted with thiobarbituric acid to produce fluorescent complexes. It should be noted that ROS are generated by AuDAMP, rather than by Dap, in the conjugated structure. Consequently, LPD could be employed to recognize the role of AuDAMP in ROS production. This would also allow us to...
clarify the contributions of Dap and AuDAMP and to further understand the interaction mechanism of the bactericide hybrid. As illustrated in Fig. 4c, Dap+AuDAMP showed a high LPD level compared to that of Dap–AuDAMP, indicating more ROS were produced by Dap+AuDAMP, which is in good agreement with our ROS assay results (Fig. 4a). This observation is in line with our expectations. Because the AuDAMP within the Dap+AuDAMP group maintains its original size, it induces a higher ROS production due to its contact surface being larger than that of AuDAMP wrapped in the Dap–AuDAMP hybrid. Combined with the results of membrane damage, we can determine that the localized Dap within the Dap–AuDAMP hybrid could promote the destruction of bacterial membranes and form more and larger holes. This conclusion can also be confirmed by SEM revealing that the Dap–AuDAMP group induces a greater leakage of cellular contents (Fig. 3c). Moreover, the ICP-OES analysis also showed that the Au content in bacterial cells treated with Dap–AuDAMP was higher than that of the control groups of Dap+AuDAMP and AuDAMP (Fig. 4d), suggesting that more Dap–AuDAMP was internalized into bacterial cells. This result implies that Dap–AuDAMP promoted the production of larger or possibly more holes in the bacterial cell membranes, which in turn facilitate the internalization of Dap–AuDAMP into the bacteria. The internalized Dap–AuDAMP could further induce damage to bacterial subcellular components by catalyzing the generation and release of a locally high concentration of ROS, subsequently leading to bacterial cell death. In contrast, the unconjugated AuDAMP within the Dap+AuDAMP group cannot possess the advantage of localizing their ROS production site. In summary, the above evaluation of the results highlights the benefits of the conjugation of Dap and AuDAMP into one entity, both in enhancing their optical properties and in generating synergistic antibacterial properties.

4. Conclusion

On the basis of our previously reported antibacterial Au NCs [6], this work has presented a highly potent antibacterial hybrid obtained by covalent conjugation of Au NCs and the antimicrobial peptide daptomycin. The as-synthesized conjugated structure not only inherits the intrinsic properties from both agents but also renders an enhanced synergistic effect. The conjugated structure could effectively destroy the bacterial membrane by creating holes in the membranes via the action of the localized daptomycin. The membrane damage motivates the entry of the antibacterial hybrid into the bacteria and leads to more serious bacterial damage at the subcellular level. In addition, bacterial genomic DNA fragmentation showed that the antibacterial hybrid may induce severe DNA breakage. The strong DNA destruction benefited from localized high concentrations of ROS induced by the localization of Au NCs in the antibacterial hybrid. The conjugated Au NCs could continuously generate ROS within the bacteria, and continuous ROS bombings could also limit the capacity of bacteria to develop drug resistance. In addition, a significant fluorescence enhancement of the hybrid structure was observed due to a novel AIE pattern caused by Au NCs and daptomycin conjugation, which differs from the previously reported solvent-induced and ion-induced AIE patterns [36–40]. Our current strategy provides a new perspective for the synthesis of new antibacterial agents as well as AIE-type fluorescence materials. Further study is underway to clarify the specific mechanisms behind the conjugation-induce AIE effect. Simultaneously, we will also develop more effective antibacterial compositions to cope with severe multidrug-resistant infections.

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Appendix A. Supplementary material

Additional spectral features, molecular structure, TGA, inhibition zone, CLSM, and ROS results associated with this article can be found in the Supplementary data. Supplementary data to this article can be found online at https://doi.org/10.1016/j.jcis.2019.03.052.

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