

Silver-coated gold nanorods as a promising antimicrobial agent in the treatment of cancer-related infections

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Background: Many cancer patients suffer from cancer-related life-threatening infections due to immune system damage. Therefore, researchers are continuously looking for new options to treat cancer-related infections. As nanotechnology has gained tremendous interest over the past several decades, silver nanoparticles have been investigated as an effective antimicrobial agent. Here, silver-coated gold nanorods were synthesized to share similar optical properties as gold nanoparticles for cancer diagnosis and treatment, with an added advantage of antibacterial properties.

Results: Their dose-dependent antimicrobial properties were demonstrated on both Gram-negative and Gram-positive bacteria species. These nanorods were found to be highly efficient in killing bacteria and suppressing biofilm formation.

Conclusion: Collectively, such results suggest that silver-coated gold nanorods should be further investigated as a novel material, which can both decrease cancer cell functions and reduce the risk of infection for cancer patients.

Keywords: nanotechnology, bacteria, cancer cells, infrared excitation

Background

Cancer is the second most deadly disease in the USA and can easily lead to life-threatening infections in patients due to their weakened immune systems.¹⁻⁵ The immune system of cancer patients can be damaged either directly by certain types of cancer, like lymphoma, or by cancer treatments, which include any type of major surgery, radiation therapy, immunotherapy, chemotherapy, and the removal of immune system organs such as the spleen.^{4,6,7} Moreover, the increasing prevalence of antibiotic-resistant pathogens due to antibiotic overuse has posed an even greater challenge in treating cancer patients.⁸ The antibiotic-resistant bacterial strains, or the so-called “super bugs”, have been reported to evolve dramatically over the years and become resistant to even the most effective antibiotics available.⁹ Therefore, it is of great importance and necessity to research alternative approaches to combat infections, especially those happening to cancer patients.

Silver has been applied to treat infections even long before the discovery of microbes, but lost its status during the 1930s to antibiotics.¹⁰⁻¹² In recent years, silver, in the form of nanoparticles, has made a remarkable comeback as a promising antimicrobial agent after recent advances in nanotechnology have provided innovative solutions for many applications.^{13,14} Silver nanoparticles (AgNPs) have been proven to be an efficient antimicrobial agent, even to multidrug-resistant bacteria strains, due to their extremely large surface-area-to-volume ratio, which provides a better chance for contact with bacteria and generation of ROS.¹⁴⁻¹⁶ Hence, AgNPs have been widely

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researched for diverse medical applications including silver-based dressings and silver-coated medical devices, among others.^{17,18} For example, Maneerung et al demonstrated that silver nanoparticle-impregnated bacterial cellulose (a wound dressing material) exhibited strong antimicrobial properties against both Gram-positive and Gram-negative bacteria.¹⁷ However, AgNPs have been found to cause DNA damage in mammalian cells as well as chromosomal aberrations and cell cycle arrest in a dose-dependent manner.¹⁹ Therefore, in order to employ the super antimicrobial properties of AgNPs, more studies are needed to reduce the amount of AgNPs needed, while achieving a comparable antimicrobial effect toward multiple bacteria strains.

Along this line, silver-coated gold nanoparticles of various shapes have been synthesized and investigated as a potential solution.^{20–22} Among them, silver-coated gold nanorods (Ag/AuNRs) have attracted much interest due to their facile synthesis and easy tenability.^{21,23} The core-shell structure is obtained by forming gold nanorods first using the seed-mediated growth method proposed by Nikoobakht and El-Sayed, and then depositing silver onto the surface of the nanorods.^{24,25} The Au–Ag bimetallic material shares similar optical properties as those of gold nanoparticles, thus demonstrating promise in cancer detection and treatment through temperature increases after radiation, which originates from the localized surface plasmon resonance (LSPR) effect.^{26,27} Moreover, by modulating the aspect ratio of the core structure, or the gold nanorods in this case, the LSPR band of the bimetallic nanorods can be tuned into the near infrared region (NIR region), allowing for potential use in various biomedical applications.²⁴ Gold nanoparticles have been investigated for their cancer cell imaging and photothermal therapy in the NIR region.^{28–31} NIR can be used to ablate tumor cells in vivo because such light can penetrate deeper into human tissue and blood. The Ag/AuNRs can also have high absorbance of NIR light when their LSPR band lies in the NIR region.²⁴ Therefore, Ag/AuNRs are promising candidates for the combined therapy of cancer and cancer-related infections. However, the potential Ag/AuNRs for antimicrobial applications has not been well studied to date.

In the present in vitro study, we focused on the antimicrobial properties of Ag/AuNRs for cancer patients. First, the aforementioned nanorods were synthesized and characterized. The synthesis process was completed under different conditions to investigate the subsequent effect of each condition on the morphology of the Ag/AuNRs. Then, the antimicrobial properties of the Ag/AuNRs were tested on both Gram-negative and Gram-positive bacteria species.

Transmission electron microscope (TEM) images of the nanoparticle-treated and non-treated bacteria were investigated to reveal the possible working mechanisms of the Ag/AuNRs. Furthermore, fluorescence assays on *Staphylococcus aureus* and *Staphylococcus epidermidis* showed the influence of Ag/AuNRs on bacterial growth and biofilm formation compared with control groups.

Materials and methods

Synthesis of Ag/AuNRs

Gold nanorods were synthesized using the seed-mediated growth method described by Nikoobakht and El-Sayed²⁵ and the process was carried out in the same way as in the study by Zhang et al³² until before the centrifugation step. The seed solution was prepared by adding 5 mL of a 0.2 M cetyltrimethylammonium bromide (CTAB) aqueous solution to 5 mL of a 0.0005 M HAuCl₄ aqueous solution, followed by adding 0.6 mL of ice-cooled 0.01 M NaBH₄. The solution was stirred vigorously for 2 minutes and then kept at room temperature. The growth solution of AuNRs was prepared by adding 5 mL of 0.2 M CTAB to 0.3 mL of a 0.004 M AgNO₃ aqueous solution. Then, 5 mL of 0.001 M HAuCl₄ was added and the solution was gently hand-shaken. Then, 70 µL of 0.0788 M ascorbic acid was added. The solution changed from dark yellow to colorless after being hand-shaken. Soon after, 12 µL of the seed solution was added to the growth solution. After equilibrating at room temperature for 15 minutes, the solution was centrifuged at 11,000 rpm for 20 minutes at 40°C. The supernatant was taken out and the remaining pellet was dispersed in 3 mL of deionized (DI) water or CTAB.

The deposition of silver on top of the above synthesized gold nanorods was achieved by modifying the method proposed by Park and Vaia.²⁴ Briefly, after the pellet was dispersed in 3 mL of DI water or CTAB, 4 µL of 1 M NaOH was added to the gold nanorod solution in order to adjust the pH. Then, 1 mL of the 0.001 M HAuCl₄ aqueous solution, 0.75 mL of the 0.004 M AgNO₃ aqueous solution, and 25.38 µL of 0.0788 M ascorbic acid were added into the solution in this order. The solution was gently hand-shaken and kept at 37°C for 20 minutes to allow for silver deposition. The solution was then centrifuged at 11,000 rpm for 20 minutes at 40°C. The obtained pellet was dispersed in PBS and the pH was adjusted to 7.4 before use. The Ag/AuNRs were diluted into a series of concentrations for bacteria and cell experiments: 0, 50, 75, 100, 125 and 150 µg/mL for the growth curves of Gram-negative bacteria; and 0, 10, 20, 30, 40 and 50 µg/mL for the growth curves of Gram-positive bacteria.

Characterization of Ag/AuNRs

A JEOL JEM-101 TEM was used to characterize the average size and shape of the nanoparticles. Size distributions of the nanorods were obtained by measuring TEM images from at least 100 nanorods using ImageJ. Nonlinear Gaussian distribution curves were used to fit the size distributions and to get the average length, width, and aspect ratios of the nanorods. Ultraviolet-visible (UV-Vis) spectroscopy (SpectraMax M3, Molecular Devices) was used to test the difference in the absorption spectra of gold nanorods and Ag/AuNRs. Scanning electron microscopy with energy dispersive X-ray (SEM/EDX) spectroscopy was used for elemental analysis of the Ag/AuNRs to prove the successful deposition of silver.

Bacterial growth curve assays

Two Gram-negative bacteria, *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922), and two Gram-positive bacteria, *S. aureus* (ATCC 12600) and *S. epidermidis* (ATCC 35984), were used to test the antimicrobial properties of the coated nanorods. All the bacterial species were inoculated into 5 mL of 30 g/L Tryptic soy broth (TSB) from their single-isolated bacterial colonies. The bacteria were cultured overnight in a shaking incubator at 37°C and then diluted ten times in the media. The optical density (OD) of the bacteria solutions was obtained through UV-Vis spectroscopy using a microplate reader at 600 nm. Bacteria solutions were then diluted to a final density of 10⁶ cells/mL. The diluted bacteria solutions were added into a 96-well plate, with three replicates for each condition. A series of diluted nanoparticle solutions were added into the bacteria solution with 0.1 mL to each well: 0, 50, 75, 100, 125, and 150 µg/mL for Gram-negative bacteria, and 0, 10, 20, 30, 40, and 50 µg/mL for Gram-positive bacteria, making the final concentrations: 0, 25, 37.5, 50, 62.5, and 75 µg/mL for Gram-negative bacteria, and 0, 5, 10, 15, 20, and 25 µg/mL for Gram-positive bacteria. The plate was well mixed for 5 minutes and then placed inside a spectrophotometer for incubation. The OD was measured at 600 nm every 2 minutes for about 20 hours to get the bacterial growth curves.

Bacterial TEM

Gram-positive bacteria species, *S. aureus*, were treated with Ag/AuNRs for TEM imaging in order to have a closer observation of the cell membranes. Briefly, 100 µL of 10⁷ cells/mL of *S. aureus* was treated with TSB supplemented with Ag/AuNRs at a final concentration of 15 µg/mL for 24 hours at 37°C. The bacteria were centrifuged and then fixed with

2.5% glutaraldehyde in a 0.1M sodium cacodylate buffer overnight at 4°C. The bacteria pellets were rinsed three times with sodium cacodylate buffer and then post-fixed with 1% osmium tetroxide for 2 hours and dehydrated with gradient ethanol solutions (30%, 50%, 70%, 85%, 90%, and 100%). Samples were infiltrated with squetol resin. The resins were polymerized at 60°C for 24 hours and thin-sectioned using an ultra-microtome (Reichert-Jung Ultracut E, Reichert Technologies, Buffalo, NY, USA). The sectioned samples were mounted on 200-hex mesh copper-coated carbon grids for imaging.

Fluorescence assay

Two Gram-positive bacteria species, *S. aureus* and *S. epidermidis*, were used for the biofilm fluorescence assay. Biofilms were allowed to form on glass coverslips submerged with 1 mL of TSB in a 24-well plate at 37°C for 24 hours. Then, the supernatant containing non-adhered cells was removed and the coverslips were washed with sterile PBS. Existing biofilms were incubated for another 24 hours with TSB supplemented with different concentrations of Ag/AuNRs, making the final concentrations: 0, 15 and 25 µg/mL. Biofilms were fixed using a 4% paraformaldehyde solution for 30 minutes at room temperature and then were washed three times with PBS. After that, 5-(4, 6-dichlorotriazinyl) aminofluorescein was added into the wells and the samples were allowed to incubate with shaking for 2 hours at room temperature. The coverslips were washed three times in PBS and inverted onto a cover glass. Images were acquired using a Zeiss Axio Observer Inverted microscope (Carl Zeiss Meditec AG, Jena, Germany).

Statistical analysis

All experiments were performed in triplicate (N=3) with three replicates each. Data are presented as the mean value with SD. Statistical differences were determined by Student's *t*-tests. The critical *P*-value was set at 0.05; a probability value of *P*<0.05 was considered to be statistically significant.

Results

Characterization of Ag/AuNRs

The as-synthesized Ag/AuNRs were characterized using a TEM, EDX and UV-Vis spectroscopy. The results are shown in Figure 1.

Figure 1 (A and B) are the TEM images of the synthesized Ag/AuNRs under different conditions. For (A), the gold nanorods were first synthesized and then redispersed in DI water before the deposition of silver; while for (B), the gold

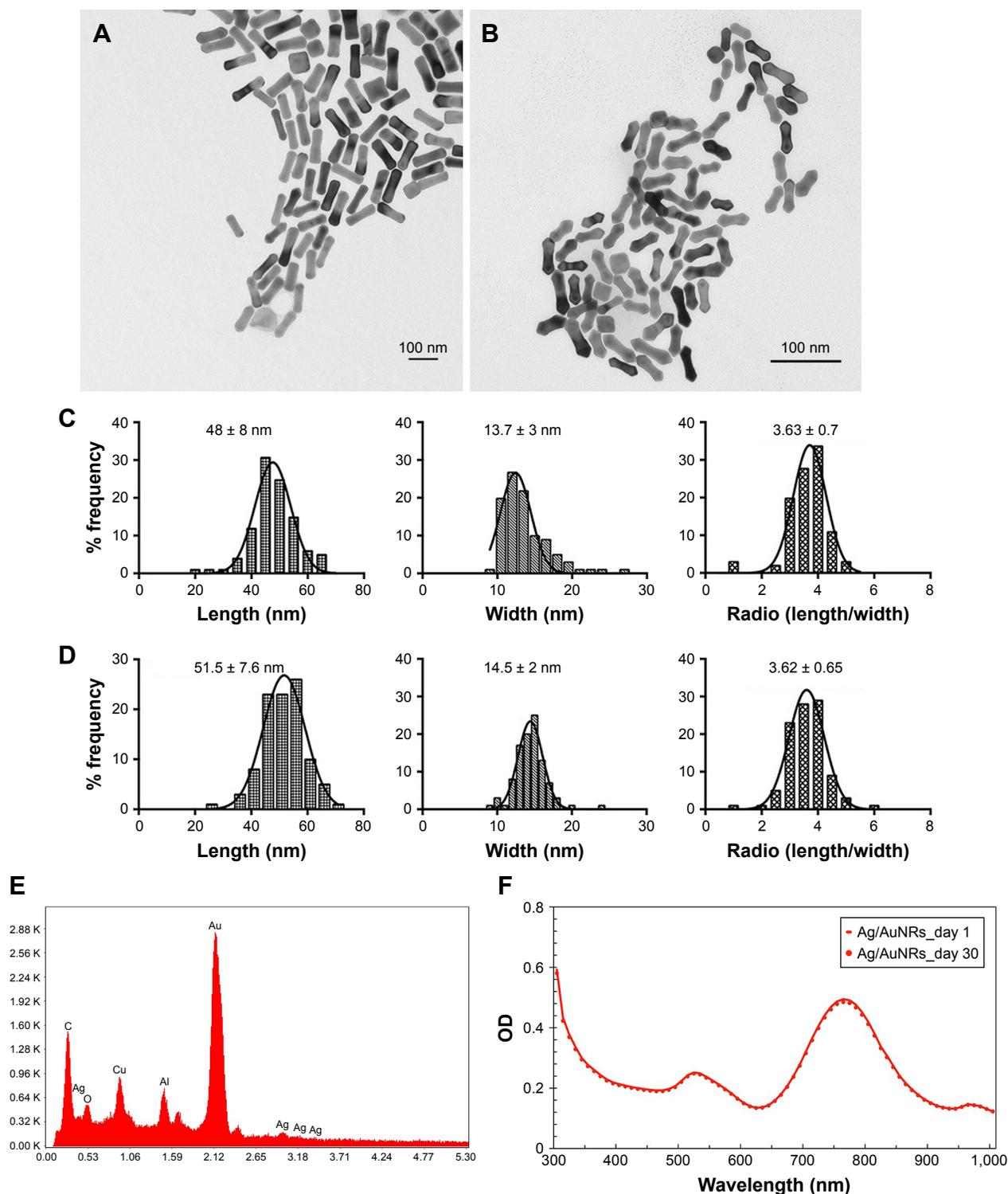


Figure 1 Characterization of the synthesized Ag/AuNRs. (A) and (B) transmission electron microscope images of Ag/AuNRs. (C) and (D) statistical analysis of the length, width and aspect ratio of the synthesized Ag/AuNRs in (A) and (B), respectively. A Gaussian distribution was fitted for each plot. (E) EDX analysis of Ag/AuNRs. (F) UV-Vis spectra of Ag/AuNRs on days 1 and 30, stored at room temperature.

Abbreviation: EDX, energy dispersive X-ray; UV-Vis, ultraviolet-visible.

nanorods were redispersed in CTAB. The morphology of Ag/AuNRs for (A) was regular-shaped rods with a rectangle side view, while that for (B) were a more dumbbell-like shape with two sharp tips on both ends.

The statistical analysis of the size distribution of the Ag/AuNRs as shown in Figure 1(A and B) was carried out through measuring the length, width, and aspect ratio (length/width) of 100 separate nanorods from the obtained

TEM images (Figure 1C and D). The “width” of the nanorods in Figure 1B was represented by the narrowest width in the middle of the dumbbell-like nanorods. The size distribution of the original gold nanorods before the deposition of silver can be found in the study by Zhang et al,³² which was conducted under exactly the same conditions. Compared with the previous results, both the length and the width increased after silver deposition. However, the aspect ratio decreased from about 4.5 to 3.6 due to a disproportional deposition along the length and width directions. Compared with the Ag/AuNRs that were redispersed in DI water (Figure 1), those that were redispersed in CTAB had a slightly larger average length and width, suggesting that CTAB enhanced the deposition of silver on top of the original surface. Since the dumbbell-like shape of Ag/AuNRs in Figure 1 had two sharp tips on both ends, this suggests that they can be used for enhancing the photothermal therapy efficacy in a future study. Elongated or sharp nanoparticles are more efficient at increasing temperatures, allowing the entire nanoparticle to be involved in heating.^{33–35} The following studies focused on the dumbbell-like Ag/AuNRs.

EDX was carried out on the Ag/AuNRs for elementary analysis (Figure 1E). Both elements, gold and silver, were found in the components of the nanorods. From the literature, silver was not observed in the gold nanorods synthesized through the same seed-mediated method.²⁴ Therefore, silver was not present until the final deposition step, demonstrating the successful deposition of silver onto the gold nanorods. Other elements shown in the analytical result include Cu from the copper TEM grid, Al from the SEM holder that was made of aluminum, and C from the carbon tape that was used to fix the TEM grid onto the SEM holder.

Moreover, there was not much difference in the absorbance spectra of the Ag/AuNRs right after synthesis (Ag/AuNRs_day 1) and those stored for 30 days at room temperature (Ag/AuNRs_day 30) (Figure 1), suggesting that the synthesized Ag/AuNRs could be stored at room temperature without much change for at least 30 days. In spectra for both storage conditions, the longitudinal and transverse surface plasmon absorption peaks existed. The transverse absorption peak was relatively fixed while the longitudinal one could be tuned into different wavelengths. In this study, the longitudinal peak located at around 740–760 nm was slightly different between batches. However, although a difference was observed, every longitudinal peak was still located in the NIR region for each batch, showing much promise for future research on the photothermal therapy of these nanoparticles.

Inhibition of bacterial growth

The antimicrobial properties of the synthesized Ag/AuNRs at different concentrations were tested on both Gram-negative bacteria species (*P. aeruginosa* and *E. coli*) and Gram-positive bacteria species (*S. aureus* and *S. epidermidis*), and the growth curves of the bacterial species were examined by measuring the OD at 600 nm (OD_{600}) for at least 20 hours after adding the nanoparticles. OD was then converted to bacteria cell number through the estimation: 1×10^9 cells/mL at $OD_{600} = 1$ and the growth curves were plotted as shown in Figure 2.

The response of bacteria toward the Ag/AuNRs was dose-dependent, and higher concentrations could both delay the growth and suppress the final bacteria concentration reached. For Gram-negative bacteria, the added nanorods started to influence the bacteria growth at a concentration of 37.5 $\mu\text{g/mL}$ and obvious suppression was found at a concentration of 62.5 $\mu\text{g/mL}$. When the concentration was 75 $\mu\text{g/mL}$, the growth was completely suppressed for at least 20 hours. The Ag/AuNRs performed better on Gram-positive bacteria species, with a total suppression for at least 20 hours at a concentration as low as 25 $\mu\text{g/mL}$. This is due to the different structures between Gram-negative and Gram-positive bacteria species. The former ones have an outer membrane that provides extra protection toward the Ag/AuNRs.

TEM was used to evaluate the morphology of the *S. aureus* after being treated with 15 $\mu\text{g/mL}$ of Ag/AuNRs for 24 hours (Figure 3). From both Figure 3(A and B), bacterial membranes showed major damage and there were “pits” formed on the cell walls, leading to leakage of the intracellular substances into the surround environment. Ag/AuNRs accumulated and coagulated near the damaged bacteria. However, there is no direct evidence of the nanoparticles penetrating the cell membrane and entering the cells.

The fluorescence assay was carried out on Gram-positive bacteria species, *S. aureus* (Figure 4(A)–(C)) and *S. epidermidis* (Figure 4(D)–(F)), as described in the Materials and methods section. There were three groups treated with different concentrations of Ag/AuNRs, 0 ((A) and (D)), 15 $\mu\text{g/mL}$ ((B) and (E)), and 25 $\mu\text{g/mL}$ ((C) and (F)). In Figure 4A and D, a large amount of bacteria and complex biofilm matrices could be observed, indicating the healthy status of the two bacterial species. In (B) and (E), although there were still a lot of bacteria, the formation of biofilms was greatly suppressed. While at the highest concentration, 25 $\mu\text{g/mL}$, most of the bacterial cells were killed compared with the control group, and no formation of biofilm was found in the fluorescence images. Therefore, the Ag/AuNRs were efficient in destroying the existing bacteria,

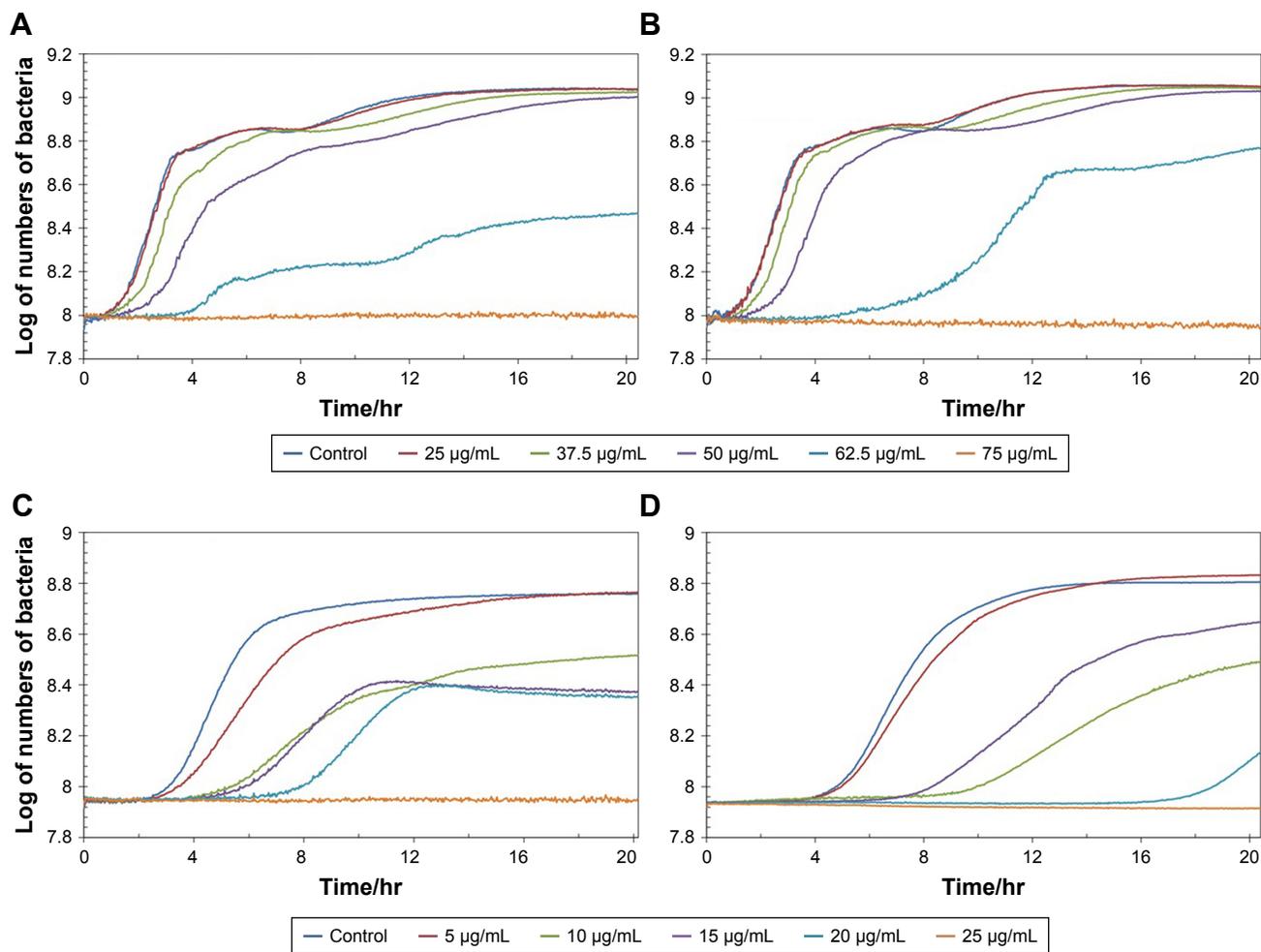


Figure 2 Bacteria growth curves for Gram-negative bacteria strains: (A) *Pseudomonas aeruginosa* and (B) *Escherichia coli* and Gram-positive bacteria strains, (C) *Staphylococcus aureus*, and (D) *Staphylococcus epidermidis* under different concentrations of Ag/AuNRs.

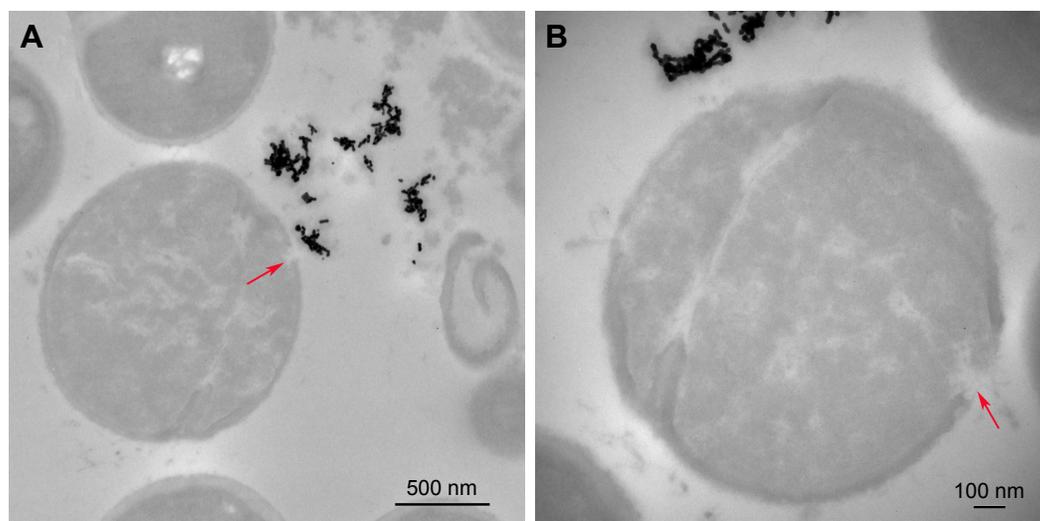


Figure 3 TEM images of *Staphylococcus aureus* after treated with 15 $\mu\text{g/mL}$ of Ag/AuNRs for 24 hours. Arrows indicate where "Pits" were formed on the cell membrane.

Note: (A) Low magnification, (B) high magnification.

Abbreviation: TEM, transmission electron microscope.

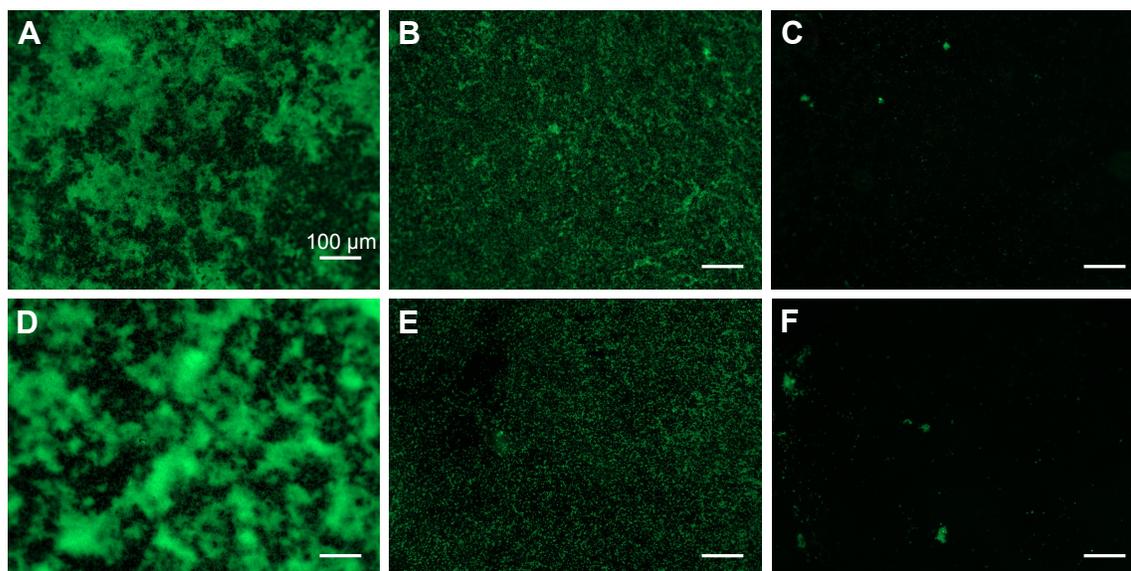


Figure 4 Fluorescence imaging of *Staphylococcus aureus* (A–C) and *Staphylococcus epidermidis* (D–F) after treating with Ag/AuNRs for 24 hours at a concentration of 0 (A, D), 15 (B, E) and 25 (C, F) µg/mL, respectively.

suppressing bacteria proliferation, and biofilm formation. This was accomplished without using antibiotics and, thus, this approach does not contribute to the growing clinical problem of antibiotic-resistant bacteria.

Discussion

The mechanism of the antimicrobial properties of AgNPs has been studied.^{15,36,37} AgNPs exhibit efficient antimicrobial properties compared with other salts due to their large surface area, providing better contact with cells, and increased generation of ROS due to their larger surface areas.¹⁴ It has been reported that AgNPs can damage the cell membrane and form “pits” in the cell walls, causing leakage of intracellular substances.¹⁵ Moreover, AgNPs can penetrate the cell membrane and interact with sulfur-containing proteins in the membrane and inside the cells and also the phosphorus-containing compounds, like DNA, in the cells.³⁶ However, in our study, there was no direct evidence of the nanoparticles entering the bacterial cells from the TEM images. This is because the antimicrobial properties of nanoparticles are size-dependent and nanoparticles smaller than 10 nm have the ability of penetrating the cell membranes.^{14,36} The Ag/AuNRs in our study had an average length of about 50 nm and average width of about 15 nm, which might be too large to penetrate the bacterial membrane. The main antibacterial mechanism observed in this study was an increased formation of “pits” on the bacterial membrane, which was caused by the interaction between silver nanoparticles and constituents of

the bacterial membrane, leading to the leakage of intercellular contents and the release of silver ions into the cell, which also interacted with bacteria to cause death.¹⁵

The bimetallic nanoparticles chosen for our study aimed to reduce the amount of AgNPs needed to achieve sufficient antimicrobial efficacy. This is because AgNPs have been found to be equally toxic to mammalian cells, which greatly limits their potential applications in the biomedical area.³⁸ It has been reported that for rod-shaped AgNPs, a total of 50–100 µg of silver is needed in order to possess antimicrobial efficacy.¹⁴ However, it was demonstrated in this study that a much lower concentration of silver is needed in order to suppress the proliferation of Gram-positive bacteria species. The efficient concentration of Ag/AuNRs was 25 µg/mL here, including both Ag and Au. Notably, since silver was only coated onto the gold nanorods, it accounts for less than 1/10 of the total nanoparticle composition, which was also confirmed by inductively coupled plasma mass spectrometry (ICP-MS) tests when measuring the concentration of the nanorods. Therefore, through the silver coating, much less silver was needed to achieve the same antimicrobial efficacy as those demonstrated in previous studies with pure AgNPs.

In this study, two different shapes of Ag/AuNRs were synthesized, the normal rod shape and the dumbbell-like shape with two sharp tips at both ends. The latter was used for further investigating bacterial and cell interactions because they had the potential to be employed for the photothermal

therapy on cancer cells in future research. It has been demonstrated that elongated or sharp nanoparticles are more efficient at increasing temperatures than massive nanostructures because the incoming electrical field can penetrate more easily inside the thin nanostructures, allowing the entire nanoparticle to be involved in heating.^{33–35} The increase in temperature can promote the photothermal therapy efficacy on tumor cells. Also, by changing the aspect ratio of the core structure of the Ag/AuNRs, their plasmon band could be easily adjusted into the NIR region. NIR can be used to ablate tumor cells in vivo because such light can penetrate deeper into human tissue and blood. Therefore, the Ag/AuNRs synthesized in our study have the potential of a combined therapy of cancer and cancer-related infections, which should be further studied. In addition, this study introduces a new field where one can potentially use the same material (without drugs or antibiotics) to kill cancer cells and bacteria for which cancer patients have increased susceptibility.

Conclusion

Ag/AuNRs were synthesized under different conditions and characterized using TEM, EDX, and UV-Vis. Then, the antimicrobial properties of the synthesized nanorods were tested on both Gram-negative and Gram-positive bacteria species and their growth was suppressed with increasing nanoparticle concentrations. The suppression of Gram-positive bacteria was more notable with complete suppression of bacteria growth for more than 20 hours at a concentration as low as 25 µg/mL due to the structural differences between Gram-negative and Gram-positive bacteria species, the former having an outer membrane that provides extra protection. Furthermore, TEM images showed “pits” on the treated *S. aureus* membrane, leading to leakage of intercellular substances into the surrounding environment. Also, fluorescence assays were carried out on *S. aureus* and *S. epidermidis*, and compared with the control groups; the groups with 25 µg/mL of Ag/AuNRs showed much less bacteria and no formation of biofilms. Moreover, since the LSPR band of the synthesized Ag/AuNRs was located in the NIR region, these nanoparticles are promising in applications such as combined cancer therapy and cancer-related infections.

Disclosure

The authors declare that there is no conflict of interests.

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