Photodynamic Inactivation of Multidrug-Resistant Staphylococcus aureus Using Hybrid Photosensitizers Based on Amphiphilic Block Copolymer-Functionalized Gold Nanoparticles

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ABSTRACT: Multidrug-resistant Staphylococcus aureus (MRSA) has become one of the major causes of various infections, leading to morbidity in both healthy and immune-compromised populations worldwide. Herein, we report a novel type of hybrid photosensitizer based on amphiphilic block copolymer-functionalized gold nanoparticles. The design of the nanoparticles provides a facile means to incorporate hydrophobic photosensitizing molecules for use in aqueous media. The hybrid photosensitizers display greatly enhanced singlet oxygen generation and outstanding photodynamic inactivation (PDI) efficacy against MRSA under light illumination. These hybrid photosensitizers greatly improve the effectiveness of PDI against MRSA while not involving antibiotics.

1. INTRODUCTION

Many human and animal diseases are caused by microorganisms.1–3 Over the last several decades, treatments have been developed to overcome this problem, and the development of antibiotics to treat bacterial infections represents one of the most revolutionary advances in the scientific field.4–7 However, bacteria have gradually developed resistance to antibiotics over the time, and nowadays, many previously highly effective antibiotics have failed to combat the pathogens. One of the dangerous pathogens is the multidrug-resistant strain of Staphylococcus aureus, commonly known as MRSA. It is a gram-positive bacterium, resistant to multiple β-lactam antibiotics such as methicillin, penicillin, oxacillin, and amoxillin.8–16 MRSA can cause infections ranging from skin conditions to life-threatening pneumonia. According to the US Center for Disease Control and Prevention, over 80 000 invasive MRSA infections and 11 285 related deaths occur every year.17 Therefore, MRSA has become a major concern of public health. As such, there is an urgent need for the development of novel and convenient ways to combat these drug-resistant pathogens.

Photodynamic inactivation of bacteria (PDI) is considered as one of the promising approaches to overcome the problem of drug resistance. PDI utilizes a photosensitizer, oxygen, and light of appropriate wavelength. The combination of these components produces reactive oxygen species (ROS), such as singlet oxygen, which is highly toxic to pathogens, including the drug-resistant bacteria.18–24 Furthermore, it is not known that bacteria develop resistance toward ROS. Thus, PDI has drawn an increasing attention in recent years.25,26

Still, PDI suffers some shortcomings. For instance, most photosensitizers are highly hydrophobic and tend to aggregate in the aqueous media. Many photosensitizers show low-to-moderate quantum yield in generating ROS and cannot be excited using long-wavelength light, such as red or infrared light, which has larger tissue penetration depth than blue or green light. These factors limit their use in clinical applications.27–29 Recently, we have reported nanoparticle-based hybrid photosensitizers, which demonstrated highly efficient singlet oxygen generation because of plasmonic resonance between the silver nanoparticles and the photosensitizing molecules, and high efficacy in photoactivating broad-spectrum bacteria.30–32 These hybrid photosensitizers also displayed broadened excitation profile, allowing them to be excited by red/infrared light sources.

Herein, we report the development of a new hybrid photosensitizer, gold nanoparticles (AuNPs) functionalized with a block copolymer (BCP) loaded with Chlorin e6 (Ce6), for efficient antibacterial applications. Our experimental results show that the hybrid photosensitizers display greatly enhanced singlet oxygen generation and high efficiency in photoactivating the MRSA.

2. RESULTS AND DISCUSSION

The schematic illustration of the synthesis of AuNP@BCP@Ce6 is shown in Figure 1. An amphiphilic BCP, poly(NIPAAm-
b-styrene), is used in this study. The AuNPs (∼40 nm) were first synthesized using the citrate method according to the literature. Poly(NIPAAm-b-styrene) was then conjugated to these AuNPs through its thiol end to the Au surface, forming AuNP@BCP, which could be well-dispersed in water because of the hydrophilic poly(NIPAAm-b-styrene) block of the polymer. The photosensitizing molecule, Ce6, is hydrophobic and hardly soluble in water. However, when mixing with AuNP@BCP in aqueous solution, Ce6 can be entrapped in the hydrophobic styrene block of AuNP@BCP. The resulting AuNP@BCP@Ce6 hybrids are stable for weeks in aqueous solution without aggregation. This design facilitates Ce6 to be dispersible in aqueous solution while simultaneously brings Ce6 close to the AuNPs, which is required for the plasmonic effect described later.

AuNP, AuNP@BCP, and AuNP@BCP@Ce6 were characterized using transmission electron microscopy (TEM), particle size analysis, and ultraviolet–visible (UV–vis) absorption spectroscopy. TEM images show a uniform distribution of AuNPs with an average size of ∼40 nm, whereas AuNP@BCP@Ce6 hybrids show an average size of ∼100 nm (Figure 2). The change in the overall nanoparticle size was likely due to the increased contrast of the BCP coating after the loading of Ce6 molecules. The zeta potentials were found to be −30 mV for AuNPs and −34 mV for AuNP@BCP@Ce6 hybrids, suggesting their good stability in aqueous solution.

UV–vis absorption spectra of pure Ce6, AuNP@BCP, AuNP, BCP, and AuNP@BCP@Ce6 are shown in Figure 3a. AuNP@BCP has a strong peak at ∼530 nm, typical for AuNPs of the similar size. Ce6 has three peaks at around 400, 530, and 640 nm. The spectral overlap between AuNP@BCP and Ce6 indicates the possibility of resonance coupling between the two in the AuNP@BCP@Ce6 hybrids. It is one of the reasons that Ce6 was chosen for this study.

Fluorescence spectra of pure Ce6 and AuNP@BCP@Ce6 are shown in Figure 3b. The Ce6 concentrations in both pure Ce6 and AuNP@BCP@Ce6 were maintained the same while taking the fluorescence spectra. As shown, the fluorescence intensity of pure Ce6 was much higher than that of AuNP@BCP@Ce6. This could be due to self-quenching of the entrapped Ce6 in AuNP@BCP@Ce6.

The singlet oxygen generation was measured by monitoring its phosphorescence emission at ∼1280 nm. As shown in Figure 4a, the singlet oxygen generation of AuNP@BCP@Ce6 was...
higher than that of pure Ce6 and AuNP@BCP under 400 nm excitation. Note that in these measurements the concentration of Ce6 in both pure Ce6 and AuNP@BCP@Ce6 was 0.24 μM and that AuNP@BCP and AuNP@BCP@Ce6 have the same amount of AuNP. Compared to pure Ce6, there is a ~twofold enhancement for AuNP@BCP@Ce6 in singlet oxygen generation. We attribute the increase in singlet oxygen generation by AuNP@BCP@Ce6 compared to pure Ce6 to the plasmonic effect of the AuNPs. In addition, the singlet oxygen excitation spectra in Figure 4b show that the excitation profile for AuNP@BCP@Ce6 is broadened, extending into the red and near-infrared regions. This has a great implication for applications requiring tissue penetration.

The cytotoxicity of the AuNP@BCP@Ce6 hybrids without light illumination was evaluated by a standard methyl thiazolyl tetrazolium (MTT) assay using MCF-7 cell line (ATCC HTB-22). Results shown in Figure 5 indicate that the AuNP@BCP@Ce6 hybrids at the concentrations used in the PDI assay display little cytotoxicity toward the MCF-7 cells under ambient conditions.

PDI of bacteria was carried out using AuNP@BCP@Ce6, pure Ce6, and AuNP@BCP against MRSA (ATCC BAA-44) with or without light illumination. From the results shown in Figure 6, none of the AuNP@BCP@Ce6, pure Ce6, or AuNP@BCP killed S. aureus at the tested concentrations without light illumination. Under white light illumination for 3 min, pure Ce6 and AuNP@BCP show negligible photoinactivation efficacy against S. aureus, whereas AuNP@BCP@Ce6 demonstrates much higher photoinactivation efficiency under the same conditions. Note that in all these PDI experiments, similar to the spectroscopic measurements, AuNP@BCP@Ce6 and pure Ce6 have the same amount of Ce6 concentration and AuNP@BCP@Ce6 and AuNP@BCP contain the same amount of AuNP. These results demonstrate that the (1) photothermal effect of AuNP@BCP is negligible under the experimental conditions and (2) AuNP@BCP@Ce6 displays a synergistic PDI effect as compared to pure Ce6 and AuNP@BCP. The enhancement of PDI efficiency is defined as log10(enhancement) = log10(AuNP@BCP@Ce6_killing) − log10(AuNP@BCP_killing) − log10(Ce6_killing). The results for the enhancement of AuNP@BCP@Ce6 in PDI are shown in Figure 6c. It is worthwhile to point out that AuNP@BCP@Ce6 demonstrates up to ~6 orders of magnitude in the enhancement of PDI efficiency against S. aureus when compared with AuNP@BCP and pure Ce6.

Note that the hybrid photosensitizer developed in this study, AuNP@BCP@Ce6, is different from another hybrid photosensitizer shown in our previous report, AgNP@BCP@HP, where the Ag nanoparticles had to be synthesized in the presence of the BCP.31 By contrast, the AuNPs in AuNP@BCP@Ce6 can be synthesized independently, not necessarily limited by the citrate method adopted in this study. This opens up the possibility of screening AuNPs of optimal sizes to have high plasmonic resonance with the photosensitizing molecule, Ce6, in this instance. In essence, this type of hybrid nanostructures serves as a platform to assemble plasmon-enhanced hybrid photosensitizers, where the two key components, metal particles and photosensitizing molecules, can be individually tailored to achieve a large degree of resonance coupling and improve the singlet oxygen generation and overall PDI performance of the hybrid.

In summary, we have reported the development of a type of hybrid photosensitizers, BCP-functionalized, Ce6-entrapped AuNPs, which display greatly enhanced singlet oxygen generation, and significant PDI efficiency against the MRSA under white light illumination. This design allows to incorporate hydrophobic photosensitizing molecules for use in aqueous media. These novel hybrid photosensitizers demonstrate great potential as an alternative to antibiotics for antibacterial applications.

3. METHODS

3.1. Chemicals and Reagents. Gold chloride (99%), sodium citrate dihydrate, tetrahydrofuran (THF), Ce6, ethanol, and phosphate buffer saline (PBS, 10× solution) were purchased from Fisher Scientific. Trypticase soy broth (TSB) and Mueller Hinton II broth were purchased from Becton Dickinson. S. aureus (ATCC BAA-44) and MCF-7 cell line (ATCC HTB-22) were purchased from the American Type Culture Collection (ATCC, USA). N-Isopropylacrylamide (NIPAAm, 99%), 2,2’-azobis(2-methylpropionitrile) (99%),

![Figure 4. Singlet oxygen emission (a) and excitation (b) spectra of Ce6, AuNP@BCP, and AuNP@BCP@Ce6 hybrid.](Image 108x240 to 253x361)

![Figure 5. Cell viability of MCF-7 cells treated with various concentrations of AuNP@BCP@Ce6 hybrids without light illumination.](Image 162x626 to 462x749)
and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were purchased from Sigma-Aldrich. Sodium borohydride was purchased from Fluka. All chemicals were used as received.

3.2. Synthesis of BCP-Functionalized AuNPs. 3.2.1. Synthesis of AuNPs. The AuNPs (~40 nm) were prepared using a citrate stabilized method based on the previously published procedure.33 In brief, 125 μL of gold(III) chloride was dissolved in 9.8 mL of water. The solution was then heated to boil under stirring. Upon boiling, 100 μL of 1 w/w % sodium citrate was added rapidly. The colloid was boiled for 5 min to ensure complete reduction. The resulting AuNPs were pelleted by centrifugation at 7000 rpm for 20 min. The AuNPs were washed with deionized (DI) water to remove excess ions and dispersed in DI water for later use.

3.2.2. Synthesis of AuNP@Poly(NIPAAm-b-styrene) Nanoparticles. The poly(NIPAAm-b-styrene) polymer used in this study is the same as reported in the previous study, where the synthesis of poly(NIPAAm-b-styrene) was described in detail.31 For the synthesis of the poly(NIPAAm-b-styrene)-stabilized AuNP, 10.0 mg of poly(NIPAAm-b-styrene) was dissolved in 1.00 mL of THF. While stirring, 1.00 mL of AuNP (~40 nm) solution was added to the THF solution. The mixture was stirred for 1 h at room temperature. The products were centrifuged at 14 000 rpm for 20 min and washed three times with DI water. The as-synthesized AuNP@poly(NIPAAm-b-styrene) nanoparticles, noted as AuNP@BCP, were dispersed in DI water for later use.

3.2.3. Synthesis of AuNP@BCP@Ce6 Hybrids. The freshly prepared AuNP@BCP nanoparticles were used for the synthesis of AuNP@BCP@Ce6 hybrids. In brief, 10.0 mg of Ce6 was dissolved in 2.00 mL of ethanol and centrifuged at 5000 rpm to collect the supernatant. From the freshly prepared Ce6, 2.00 mL of supernatant was added into the freshly prepared AuNP@BCP under vigorous stirring. The mixture was stirred overnight at room temperature, and the resulting nanoparticles were pelleted by centrifugation at 14 000 rpm for 20 min. The resulting nanoparticles, noted as AuNP@BCP@Ce6 hybrids, were then washed three times with the ethanol/water mixture (50/50) to remove any excess of Ce6. The as-synthesized nanoparticles were dispersed in DI water and stored at 4°C for later use.

3.3. Characterization. The freshly synthesized nanoparticles (AuNP and AuNP@BCP@Ce6) were characterized using a Phillip BioTwin 12 TEM. A drop of the sample aqueous solution was placed and dried onto a carbon-coated copper grid (300 mesh, EMS). A particle size analyzer (Microtrac) was used to measure the zeta potential and the hydrodynamic diameter of the particles.

A UV−vis spectrometer (USB4000-ISS, Ocean Optics) was used to determine the loading amount of Ce6 using an absorbance at 400 nm. A series of solutions with different concentrations of Ce6 in dimethyl sulfoxide (DMSO) were used to establish a calibration curve. Then, the absorbance at 400 nm of a known amount of the Au@BCP@Ce6 hybrid was measured, and the loading amount of Ce6 in the Au@BCP@Ce6 hybrid was calculated using the calibration curve.

The fluorescence and phosphorescence measurements were carried out using a QM-40 spectrometer (PTI) equipped with a photomultiplier tube and a high-performance InGaAs photodiode. Singlet oxygen generation was detected by monitoring its phosphorescence emission at ~1280 nm. The light source was a xenon arc lamp, and the output was passed through an optical chopper operating at a fixed frequency. A long-pass filter (850 nm cutoff) was used to remove higher-order artifact signals. All fluorescence and phosphorescence measurements were done in a quartz cuvette, and the samples were dispersed in the ethanol/water solvent (50/50). The excitation wavelength used to collect fluorescence and singlet oxygen emission spectra of Ce6 was 402 nm.

Figure 6. Colony count of Ce6, AuNP@BCP, and AuNP@BCP@Ce6 hybrid against MRSA (ATCC BAA-44) under (a) no light and (b) white light illumination. (c) Bacterial killing efficacy of Ce6, AuNP@BCP, and AuNP@BCP@Ce6 hybrid. Enhancement in bacterial killing efficacy is defined in the text. Results are expressed as mean ± SD (n = 3, p < 0.05).
3.4. PDI Assay of Bacteria. Overnight cultures of MRSA (ATCC BAA-44) were prepared on a TSB medium. Typically, S. aureus was inoculated in the PBS buffer solution (pH = 7.4) and mixed with different concentrations of AuNP@BCP@Ce6, pure Ce6, and AuNP@BCP in PBS. All bacterial suspensions (200 μL, ~10^7 cfu/mL), including nontreated controls, were transferred to the wells of a 96-well plate and illuminated under a white light source with an interchangeable fiber bundle (model LC-122, Lumacare) for 3 min. The white light intensity was measured using a laser power meter (model 840011, Sper Scientific) and found to be 408 mW/cm². The irradiation fluence was 73 J/cm². After light illumination, the plate count method was used to determine the viable bacterial numbers (cfu/mL) in each suspension. Dark controls were run in parallel. All experiments were performed in triplicate. The data were presented as the means with standard deviations. Statistical significance was analyzed using the two-sample t-test, and the probability value of <0.05 was considered significant.

3.5. Cell Viability Assays. The in vitro cytotoxicity assay for the AuNP@BCP@Ce6 hybrids was carried out using a standard MTT assay on the MCF-7 cells without light illumination. Briefly, the MCF-7 cells were maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units per mL penicillin, and 0.1 mg/mL streptomycin at 37 °C. The cells (100 μL) were seeded in the wells of a 96-well plate at the density of 10,000 cells/well. After overnight incubation in the culture incubator (37 °C, 5% CO2), the cells were treated for 24 h with various concentrations of the AuNP@BCP@Ce6 hybrids. Next, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (5 mg/mL) was added to the culture medium to reach a final concentration of 0.5 mg/mL. After the cells were incubated at 37 °C for 4 h, the supernatants were removed, and the formazan dye was dissolved in 100 μL of DMSO. The absorbance was measured on a microplate reader at 490 nm with a reference wavelength at 650 nm. Each experiment was done in six wells in parallel.

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Notes
The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS
P.Z. acknowledges partial support from the University of Cincinnati Technology Accelerator award. N.A. acknowledges the donors of the American Chemical Society Petroleum Research Fund (S1850-DN17) for the support of this research.

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