Supplementary Information

Engineering Protein-Based Nanoplatform as Antibacterial Agents for Light Activated Dual-Modal Photothermal and Photodynamic Therapy of Infection in Both the NIR I and II Windows

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Experimental Section

Materials: Copper(II) nitrate trihydrate (Cu(NO₃)₂·3H₂O), Sodium sulfide nonahydrate (Na₂S·9H₂O) was obtained from Sinopharm Chemical Reagent Co. Ltd. Bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC₄(3)) and LIVE/DEAD bacLight bacterial viability kit were
received from Thermal Fisher Scientific. Sephacryl S-300 HR, HEPES buffer (1M), N-(3-Dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride (EDC), N-Hydroxysuccinimide (NHS), Chlorin E6 (Ce6), Triton-X 100, polyethylene glycol (PEG, MW 3350), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), sodium hydroxide (NaOH) were all purchased from Sigma-Aldrich. Bovine serum albumin (BSA) was obtained from Aladdin. All chemicals used in this study were of analytical reagent grade and used without further purification. Ultrapure water (18.25 MΩ·cm, 25 °C) was used in all experiments.

Synthesis Ce6-labeled BSA-CuS Nanoparticles: Copper sulfide (CuS) nanoparticles were first prepared according to our previously report with minor modifications. Briefly, copper solution (0.2 M, 1mL), dissolving Cu(NO$_3$)$_2$ in ultrapure water was added into BSA (40 mg mL$^{-1}$, 7.5 mL) with vigorous stirring. Na$_2$S (0.2 M, 2mL) was then mixed with the former solution. Temperature of the mixture was raised to 90°C and maintained for about one hour after adjusting pH value to 12. Ce6, activated by EDC and NHS which form an active ester intermediate with the carboxyl functional groups, was reacted with the obtained BSA-CuS nanoparticles for about one day under room temperature.

Purification Ce6-labeled BSA-CuS Nanoparticles: Sephacryl S-300 HR gel column was used to separate Ce6-labeled BSA-CuS nanoparticles from molecules including free Ce6, Ce6-labeled BSA, and free BSA. As described in the previously report, BSA (2% (w/v), 10 mL), triton-X 100 (0.25% (w/v), 100 mL), and PEG (5% (w/v), 100 mL) were prepared for further experiment. Ultrapure water (96 mL), HEPES buffer (1 M, 2 mL) and polyethylene glycol (5%, 2 mL) were then mixed as eluent. The gel column was then washed three times using the eluent. The sample was mixed with HEPES buffer (1M, 20μL), PEG (5%, 20 μL), Triton-X 100 (0.25%, 20 μL) and BSA (2%, 20 μL) before adding into the column for purification.
Characterization of Ce6-labling CuS Nanoparticles: Optical absorption spectra were recorded by a PerkinElmer Lambda 750 Uv–Vis–Nir spectrophotometer. Transmission electron microscopy (TEM) image was obtained by a FEI Tecnai G20 transmission microscope at 200 kV. Samples for TEM observation were prepared by dropping 10 μL of the solution with adequate concentration on a carbon-coated gold grid and allowed the sample to dry.

Photothermal Effect, Photostability, and Photothermal Conversion Efficiency: To evaluate the photothermal effect of as-prepared Ce6-labeled BSA-CuS nanoparticles, the temperature of nanoparticles with different concentrations (0, 0.125, 0.25, 0.5, 1 mg mL$^{-1}$) were recorded at an interval of 10 s by an infrared thermal imaging camera (Ti400, Fluke, USA) when it was exposed to 1064 nm laser of power density at 1.3 W/cm$^2$. The temperature of nanoparticles irradiation under different power density of 1064 nm laser (0.6, 1.3, 2 W/cm$^2$) were also measured. The photothermal effect of the as-prepared nanoparticles according to the previously report $^{[1]}$:

$$\eta = \frac{(T_{\text{max}} - T_{\text{amb}}) - Q_{\text{dis}}}{I (1 - 10^{-A_{1064}})} \times \frac{m_D c_D}{\tau_s}$$

Where $T_{\text{max}}$ is representative for the equilibrium temperature, $T_{\text{amb}}$ is the ambient temperature, $Q_{\text{dis}}$ indicates the heat dissipation from the energy absorbed by the sample, $I$ expresses incident power of 1064nm laser (1.3 w/cm$^2$), and $A_{1064}$ is the absorbance of the Ce6-labeled BSA-CuS nanoparticles at 1064 nm. $m_D$ and $c_D$ is the mass and heat capacity of water, respectively. And $\tau_s$ is the system time constant of the sample, which can be obtained by calculating the slope of the linear plot of $-\ln \theta$ versus time. The value of $\theta$ is measured according to the following equation:

$$\theta = \frac{T_{\text{amb}} - T}{T_{\text{amb}} - T_{\text{max}}}$$
**Reactive Oxygen Species (ROS)** Measurement of NPs: 2′,7′-dichlorofluorescin diacetate, which was highly sensitive to ROS, was employed here during the detection process. The generation of ROS of six group samples including ROS sensor with NPs under 660 nm laser and 1064 nm laser, ROS sensor under 660 nm laser, PBS under 660 nm laser and 1064 nm laser, NPs under 660 nm laser, ROS sensor with NPs under 1064 nm laser, and ROS sensor with NPs under 660 nm laser were measured by detecting enhanced fluorescence of ROS sensor (λexcitation = 504 nm and λemssion = 529 nm).

**Bacteria and cell culture:** *S. aureus* (Gram-positive, strain NRS 077) and *E. coli* (Gram-negative, strain ATCC 25922) solutions were cultured in tryptic soy broth (TSB) and luria-bertani broth (LB) medium, respectively, and incubated at 37 °C to an optical density at 600 nm (OD\textsubscript{600}) of 0.5. The U87 cells human primary glioblastoma cell line and human brain microvascular endothelial cells were incubated in Dulbecco’s Modified Eagle Medium (DMEM, HyClone) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) penicillin, and 1% (v/v) streptomycin. Cells were cultured in a humidified incubator at 37 °C with 5% CO\textsubscript{2}. To test the cytotoxicity of Ce6-labeled BSA-CuS NPs, the medium containing different doses of NPs (0, 125, 250, 500, 1000 μg mL\textsuperscript{-1}) was incubated with the cells. After 12h and 24h, the viability was evaluated by the MTT assay.

**Antibacterial activity measurement of Ce6-labeled BSA-CuS nanoparticles:** The Ce6-labeled BSA-CuS NPs were added to treat *S. aureus* and *E. coli* strains. The experiments were divided into different groups: group 1 is *S.aureus* or *E. coli* without laser and Ce6-labled BSA-CuS NPs; group 2 is *S.aureus* or *E. coli* under 1064 nm laser and 660 m laser; group 3 is *S.aureus* or *E. coli* with the addition of Ce6-labled BSA-CuS NPs; group 4 is *S.aureus* or *E. coli* under 660 nm laser in the presence of Ce6-labled BSA-CuS NPs; group 5 is *S.aureus* or *E. coli* under 1064 nm laser in the presence of Ce6-labled BSA-CuS NPs; group 6 is *S.aureus* or *E. coli* exposed to 1064 nm
laser and 660 nm laser in the presence of Ce6-labled BSA-CuS NPs. After treatment, 0.1 mL of the suspension was collected, diluted, and spread on an agar plate. Each dilution had three parallel groups. After being rubbed for 2 min, the substances were incubated at 37 °C for overnight, and the bacterial colonies were inspected.

*Morphological Characterization of Bacteria:* Bacteria (1 × 10^6 CFU mL⁻¹) before and after treatment with NPs under 660 nm laser and 1064 nm laser were collected by centrifugation at 8000 rpm for 1 min and fixed with 2.5% glutaraldehyde overnight at 4 °C. After washing with PBS buffer for three times, the bacterial cells were dehydrated through sequential treatments of 30%, 50%, 70%, 80%, 90% and 100% ethanol for 30 min and imaged using a Field Emission Scanning Electronic Microscope (FESEM) (ZEISS, Merlin SEM, Germany).

*Live/Dead Staining:* The LIVE/DEAD BacLight Bacterial Viability Kit provides a two-color fluorescence assay to test bacterial viability for a diverse array of bacteria. The Live/Dead staining experiments were carried out according to the manufacturer’s instructions. Bacterial cells (1 × 10^6 CFU mL⁻¹) treated with different conditions (Group 1: bacterial without any treatment; Group 2: bacterial under 660 nm laser and 1064 nm laser; Group 3: bacterial with NPs only; Group 4: Bacterial with NPs under 660 nm laser; Group 5: bacterial with NPs under laser 1064 nm; Group 6: bacterial with NPs under 660 nm laser and 1064 nm laser) were collected and washed with PBS buffer for three times by centrifugation at 2400 rpm for 10 min. After that, the bacteria were stained with green fluorescent nucleic acid stain (SYTO 9) and red fluorescent nucleic acid stain (PI) for 15 min. The bacteria samples were imaged using a Zeiss LSM 710 confocal microscope with 63x (Plan-Apochromat, NA 1.40) oil immersion objective.
Mouse infection model and treatment: In vivo animal experiments were carried out according to protocols approved by University of Macau. Six-week-old BALB/c mice were chosen to evaluate the in vivo antibacterial effect of Ce6-labeled BSA-CuS NPs. Mouse-model of bacterial infection was established by subcutaneous injection of 1 × 10^8 CFU mL⁻¹ S. aureus cells in 20 μL of PBS into the ear of BALB/c mice. Ce6-labeled BSA-CuS NPs were directly injected into the infected sites after 1 day infection. For treatment group, the infection site was then irradiated under the 1064 nm laser (1.3 w cm⁻²) and 660 nm laser (0.1 w cm⁻²) for 10 minutes simultaneously. The infected sites in the control group was applied with Ce6-labeled BSA-CuS NPs without any laser irradiation.

In vivo photoacoustic (PA) imaging: To estimate the treatment efficiency, PA signals of blood vessels around the infection site were obtained by a home-made optical-resolution PA microscopy (OR-PAM) system at the excitation wavelength of 532 nm. The output laser beam was reshaped by a filter system and then coupled into a single-mode fiber. After collimated, the laser was focused on the mouse ear with 4 x objective lens. A 30 MHz focused ultrasound transducer (80% −6dB bandwidth) was employed to detect PA signals. To do the experiment, the mouse which anesthetized by isoflurane lied on the working stage, and then its ear was put on a cylindrical agar block. The middle of the working stage was hollowed out and filled with solidified agar for coupling ultrasonic signals. To control the system, the trigger signal from the laser was applied to initiate the data acquisition to collect PA signals. The 2D platform will move to next point across x-y plane for imaging.

Statistics: The samples were measured three times to calculate the error bars. In the manuscript, the error bars were calculated as follows: the value of error bar = mean value × mean deviation.
Fig. S1. Uv-Vis-Nir absorption spectra of Ce6-labeled BSA-CuS NPs with different concentrations
Fig. S2. (a) Temperature of PBS and Ce6-labeled BSA-CuS NPs under irradiation of 660 nm laser; (b) Temperature of Ce6-labeled BSA-CuS NPs (600μg/mL) under irradiation of laser 1 and laser 2 simultaneously, and laser 2 alone; laser 1 was representative of 660 nm laser and laser 2 indicated 1064 nm laser.
Fig. S3. The effect of temperature on the fluorescence emission intensity at 525 nm of ROS probe
Fig. S4 Size of the Ce6-labeled BSA-CuS NPs dispersed in PBS (pH = 7.4) stored at room temperature for half a month.
Fig. S5. Schematic of bacterial solution irradiation with two lasers simultaneously
Fig S6. bacterial inhibition ability of the Ce6-labled BSA-CuS NPs. a) Live (green fluorescence) and dead (red fluorescence) stained *E. coli* (scale bar = 5μm), and b) photomicrographs of the colonies of *E. coli* incubated on the solid nutrient medium and c) related quantitative results under various experimental conditions: group 1 is *E. coli* without laser and Ce6-labled BSA-CuS NPs, group 2 is *E. coli* under 1064 nm laser and 660 m laser, group 3 is *E. coli* with the addition of Ce6-labled BSA-CuS NPs, group 4 is *E. coli* under 660 nm laser in the presence of Ce6-labled BSA-CuS NPs, group 5 is *E. coli* under 1064 nm laser in the presence of Ce6-labled BSA-CuS NPs, group 6 is *E. coli* exposed to 1064 nm laser and 660 nm laser in the presence of Ce6-labled BSA-CuS NPs.
Fig.S7 The viabilities of microvascular endothelial (bEnd.3) under various experimental conditions: group 1 is bEnd.3 with addition of PBS, group 2 is bEnd.3 with the addition of Ce6-labeled BSA-CuS NPs, group 3 is bEnd.3 under 1064 nm laser and 660 m laser, group 4 is bEnd.3 under 660 nm laser in the presence of Ce6-labeled BSA-CuS NPs, group 5 is bEnd.3 under 1064 nm laser in the presence of Ce6-labeled BSA-CuS NPs, group 6 is bEnd.3 exposed to 1064 nm laser and 660 nm laser in the presence of Ce6-labeled BSA-CuS NPs.
Fig. S8. (a) and (b) are SEM images of untreated cells of *E. coli*; (c) and (d) are SEM images of *E. coli* exposed to 1064 nm laser and 660 nm laser in the presence of Ce6-labeled BSA-CuS NPs. Blue circles and blue arrows indicate fragments and rough surface of *E. coli* after treatment, respectively.
Fig. S9. Flow cytometry analysis of *S. aureus* and *E. coli* before and after treatment labeled with DiBAC$_4$(3)
Fig. S10. Schematic of the experimental setup for in vivo photoacoustic imaging. SF: Single-mode Fiber; FC: Fiber Collimator; OL: Objective Lens; WS: Working Stage; UT: Ultrasound Transducer; AMP: Amplifiers; DAQ: Data Acquisition.

Reference