SUPPORTING INFORMATION

Laser Activatable CuS Nanodots to Treat Multidrug-Resistant Bacteria and Release Copper Ion to Accelerate Wound Healing for Infected Chronic Nonhealing Wounds

Yue Qiao,[a,b,1] Yuan Ping,[c,1] Hongbo Zhang[d,1] Bo Zhou,[b] Fengyong Liu,[e] Yinhui Yu,[a,f] Tingting Xie,[b] Wanli Li,[b] Danni Zhong,[b] Yuezhou Zhang,[d] Ke Yao,[a,f] Hélder A. Santos,[g,j,*] and Min Zhou[a,b,h,i,*]

[a]Eye Center & Department of Nuclear Medicine, The Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou 310009, China

[b]Institute of Translational Medicine, Zhejiang University, Hangzhou, 310009, China

[c]College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, 310058, China

[d]Department of Pharmaceutical Science Laboratory, Åbo Akademi University, Turku, 20520, Finland

[e]Department of Interventional Radiology, Chinese PLA General Hospital, Beijing 100853, China

[f]Zhejiang Provincial Key Lab of Ophthalmology, Hangzhou, China

[g]Drug Research Program, Division of Pharmaceutical Chemistry and Technology, Faculty of Pharmacy, University of Helsinki, FI-00014, Helsinki, Finland

[h]Key Laboratory of Cancer Prevention and Intervention, National Ministry of Education, Zhejiang University, Hangzhou, 310009, China

[i]State Key Laboratory of Modern Optical Instrumentations, Zhejiang University, Hangzhou, 310058, China

[j]Helsinki Institute of Life Science, HiLIFE, University of Helsinki, FI-00014, Helsinki, Finland

[1]These authors contributed equally

1. Materials.

Sodium sulfide ($Na_2S•9H_2O$, ACS reagent grade), copper(II) chloride ($CuCl_2$, ACS reagent grade), bovine serum albumin (BSA, Vetec reagent grade), sodium citrate dehydrate ($Na_3Cit•2H_2O$, ACS reagent grade), propidium iodide (PI), 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) and 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypsin-EDTA, Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS), Luria-Bertani agar (LB agar), and Luria-Bertani broth (LB broth) were purchased from Gibco (Burlington, ON, Canada). Matrigel was purchased from Corning (Corning, NY, USA).

2. Experimental methods

2.1. Characterization. Transmission electron microscopy (TEM, FEI Tecnai F20, USA) was used to characterize the morphology and size of the nanoparticle or bacteria. Scanning electron microscopy (SEM, Hitachi SU-8010, Japan) with energy-dispersive X-ray spectroscopy (EDS) equipped was applied to investigate element mapping and the state of the bacterial microstructure. Zetasizer (Malvern Nano-ZS90, USA) was used to measure the hydrodynamic size and zeta potential of nanoparticles. UV−vis spectrometer (SHIMADZU UV-2600, Japan) was used to record the optical absorption of nanoparticles. X-ray diffractometer (Rigaku, Japan) was applied to characterize X-ray diffraction (XRD) patterns of nanoparticles. *In*

vivo imaging system (PerkinElmer, IVIS Lumina LT, USA) was used to observe DCF fluorescence of different treatment groups conducted by produced ROS.

2.2. Synthesis of CuS NDs/CuS NPs. CuS NDs were synthesized by a simple chemical reaction. Firstly, $CuCl_2$ (1 M, 100 μL) was added into an aqueous solution of BSA (10 g/L, 10 mL) under stirring at room temperature. After 5 min, Na_2S (1 M, 100 μL) was introduced into the mixed solution. 5 min later, the solution was heated to 90 ℃ upon vigorously stirring until the color turned dark green. Finally, free BSA and ions were removed by ultrafiltration centrifugation. For the preparation of CuS NPs, a similar method was used, while the PEG-SH (Mw=5,000) was instead of BSA during the synthesis.

2.3. Photothermal Conversion Effect. NIR laser system (Changchun Optoelectronics MDL-N-808-10W, China) was used to induce photothermal effect of nanoparticles and operate the *in vitro or in vivo* PTT experiments at wavelength of 808-nm. The experimental conditions were performed at various power densities (0.5, 1.0, 1.5, 2.0, and 2.5 W/cm^2) and CuS NDs concentrations (2.81, 5.63, 11.25, 22.5, and 45.0 μg/mL) with a duration of 300 s. The corresponding temperature change was recorded by an infrared thermal imaging camera (FLIR A350, USA). The photothermal conversion efficiency of CuS NDs was analyzed according to the whole period that the temperature natural cooling following increasing to maximum. The CuS NPs was used as a control.

2.4 Bacterial Fluorescent Assay. In this assay, a membrane-permeant

fluorescent-based nucleic acid dye (PI) which possessed the ability of staining DNA of dead bacteria with red fluorescence by permeating through the compromised or disrupted membranes was used. The treated ESBL *E. coli* suspension was acquired from CuS NDs Laser group of *In vitro* antibacterial activity analysis before the incubation step (45 μg/mL CuS NDs, 2.5 W/cm^2 for 10 min laser irradiation). 3 mL of this suspension was centrifuged and the precipitate was rinsed via PBS (pH 7.4) to collect bacterial cells (roughly 10^8 CFU/mL). After co-cultured at 37 °C for 15 min with PI (30 μM, 50 μL) fluorescent dye, the bacterial cells were observed by a fluorescence inversion microscope (Olympus IX71, Japan). CuS NDs group, CuS NPs plus laser treatment group, and the blank control group were also implemented in the above manner.

2.5 Morphological Characterization of Bacteria. ESBL *E. coli* or MRSA suspension (10^8 CFU/mL) was collected and rinsed, which was cultured with CuS NDs (45 μg/mL) and illuminated laser (808-nm, 2.5 W/cm^2) for 10 min. Then the suspension culture was harvested by centrifugation (1000 rpm) for 3 min and rinsed by PBS, followed by fixing in a centrifuge tube with diluted glutaraldehyde solution (2.5%) for 12 h, washing three times for 15 min each in PBS (pH 7.4), dehydrating with sequential treatment of ethanol-water mixtures (30%, 50%, 70%, 90%, 95%, and 100%) for 10 min in each mixing ratio at room temperature, respectively. Finally, dried bacteria were sputter-coated with gold. The size, morphology, and elements analysis of the bacteria were characterized with an SEM with EDS equipped. In addition to the above SEM image, bacterial cells

were also analyzed by TEM. As a procedure, after the samples were centrifugal precipitated and rinsed twice with PBS, the treated bacteria were immobilized with an electron microscopy fixative reagent, negative stained, and thereafter section processed over a TEM nickel grid with a carbon film for visualization. CuS NDs, CuS NPs plus laser treatment, and non-treated bacterial suspension were used as controls.

2.6 ROS Effect Assay. After DCFH-DA was added to CuS NPs/CuS NDs solution (45 μg/mL) in a 96 wells plate, laser irradiation (2.5 W/cm^2, 10 min) was performed or not. Then fluorescence images of the bacteria with different treatments were conducted by an *in vivo* imaging system (PerkinElmer, USA). The experiment procedures were as same as bacterial fluorescent death assay except that DCFH-DA (10 μM, 100 μL) was employed as fluorescent dye instead of PI.

2.7 Cu^{2+} Release Assay. The concentration of Cu^{2+} release from CuS NDs upon laser irradiation was assessed. After gently rinsed with PBS, CuS NDs (45 μg/mL) in a quartz tube was irradiated with laser (808-nm, 1.0, 2.0, or 2.5 W/cm^2) and then transferred into a centrifuge ultrafilter at different time points. After centrifugation, the CuS NDs was resuspended and continued to be irradiated with laser. The supernatant was measured by inductively coupled plasma mass spectrometry (ICP-MS) to analyze the released Cu^{2+}. CuS NPs upon laser irradiation was taken as control with the same treatment.

2.8 Cytotoxicity Assay. HFF-1 and HUVEC cells seeded in 96-well plates (5 × 10^3 cells per well) were incubated with a series of concentrations of CuS NDs (1.41, 2.81, 5.63, 11.25, 22.5, 45.0 μg/mL) upon laser irradiation (808-nm, 2.5 W/cm^2, 10 min). After incubating for 48 h, 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, 0.5 mg/mL) solution was added to each well and incubated for an additional 4 h. Afterward, the medium was removed and 100 μL of dimethyl sulfoxide was added to dissolve crystals formed by living cells. Absorbance at 490 nm was measured using a microplate reader. Cell viability was expressed as a percentage of the absorbance to that of the blank control experiment without treatment.

2.9 Pilot Toxicity Study. Toxicity experiments were carried out with BALB/c mice *in vivo*. The blood samples were obtained by cardiac puncture for clinical chemistry and hematologic analysis. Then major organs including heart, lung, liver, spleen, and kidney were removed, followed by fixing in 10% formalin, processing routinely into desiccating, embedding in paraffin and being sectioned into 5 μm sections. The sections were then processed with H&E staining for toxicology histological analysis. For body weight measurement, each mouse (n = 5) was weighed on day 0, 2, 4, 6, 8, 10, and 12 during *in vivo* study.

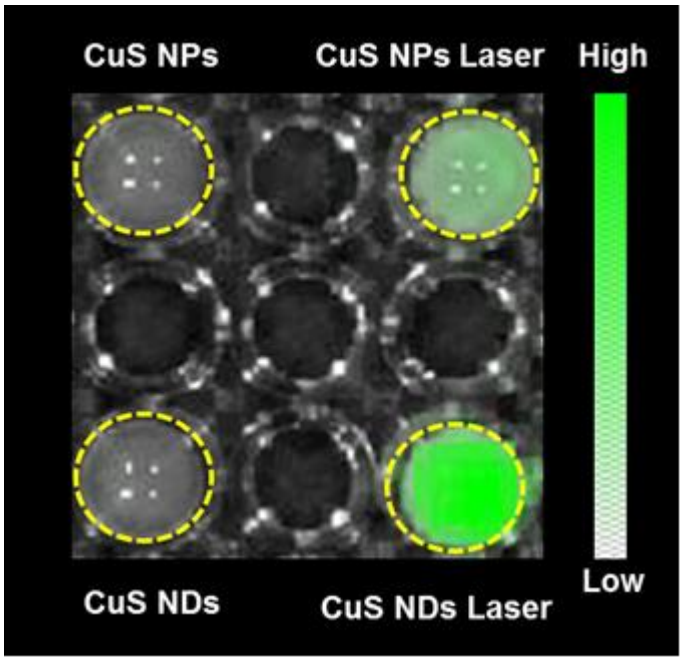

Figure S1. Fluorescence imaging of ROS generation from various treatments detected by DCFH-DA assay, samples are in yellow circles, respectively.

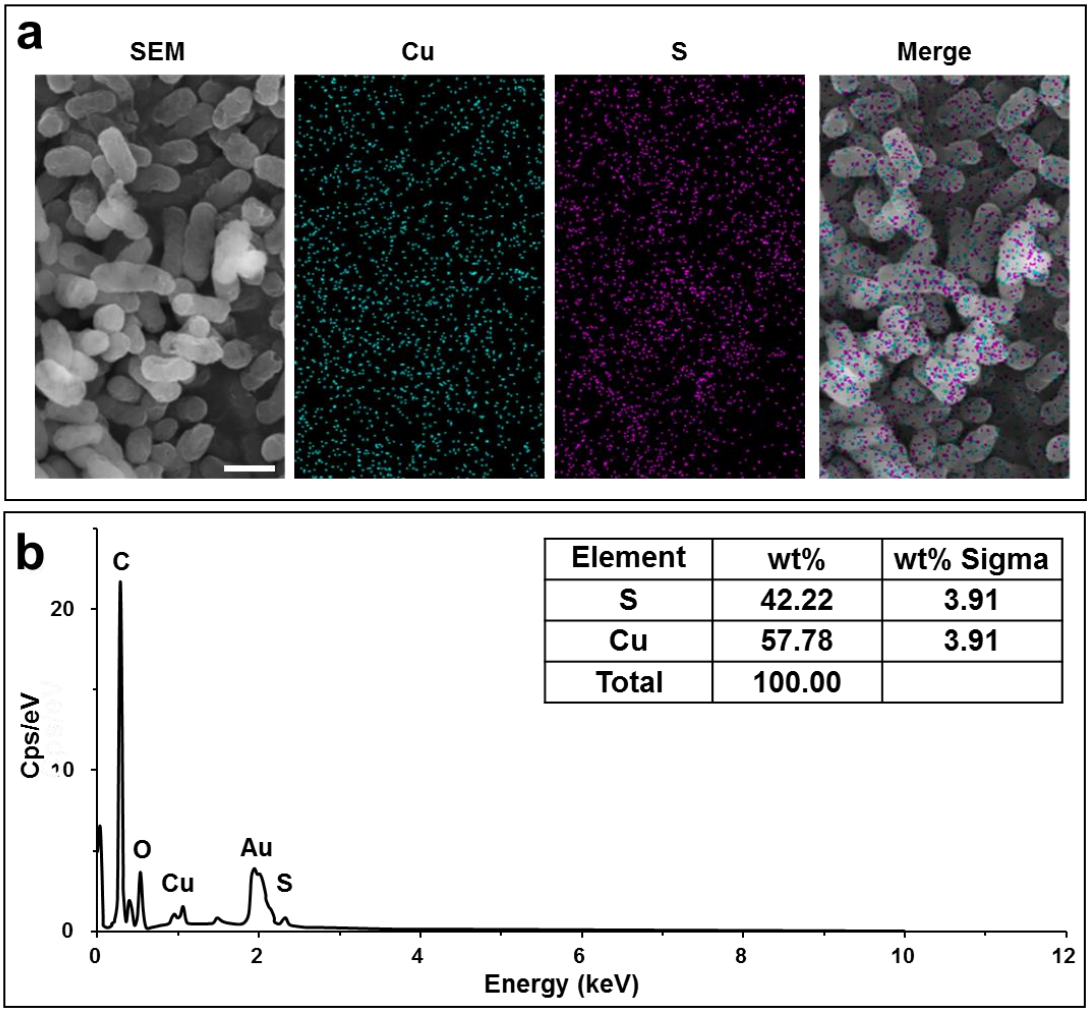

Figure S2. (a, b) EDS element maps and spectrum of ESBL *E. coli* treated with CuS NDs upon laser in SEM observation indicating the element composition is Cu and S (insert table was mass percent of elements, bar = 1 μm, Cps/ev, counts per second per electron-volt, keV, kilo-electron-volt).

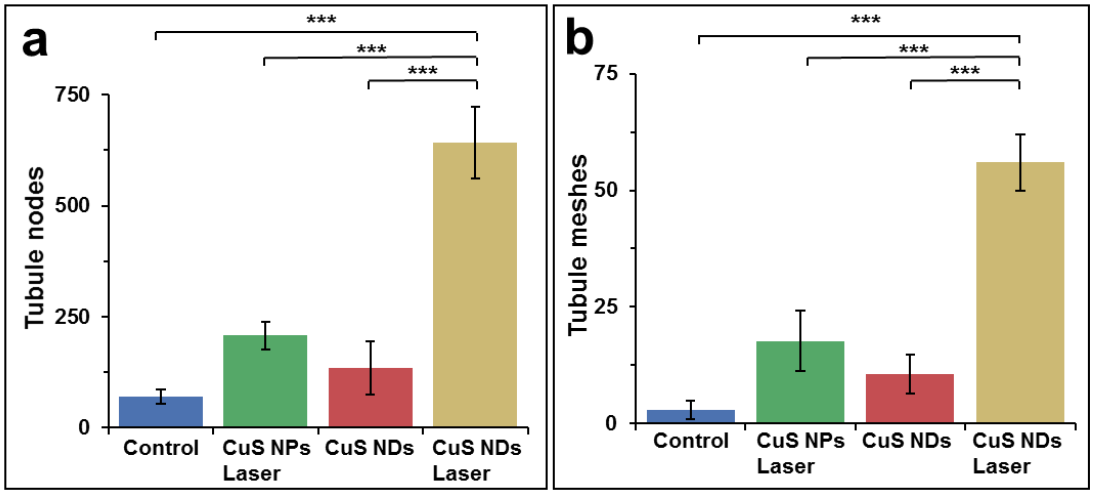

Figure S3. (a, b) Quantification of tubule nodes and meshes. Error bars represent means± SD (n≥ 3). Student‘s t-test (* P≤ 0.05, ** P≤ 0.01, *** P≤ 0.001).

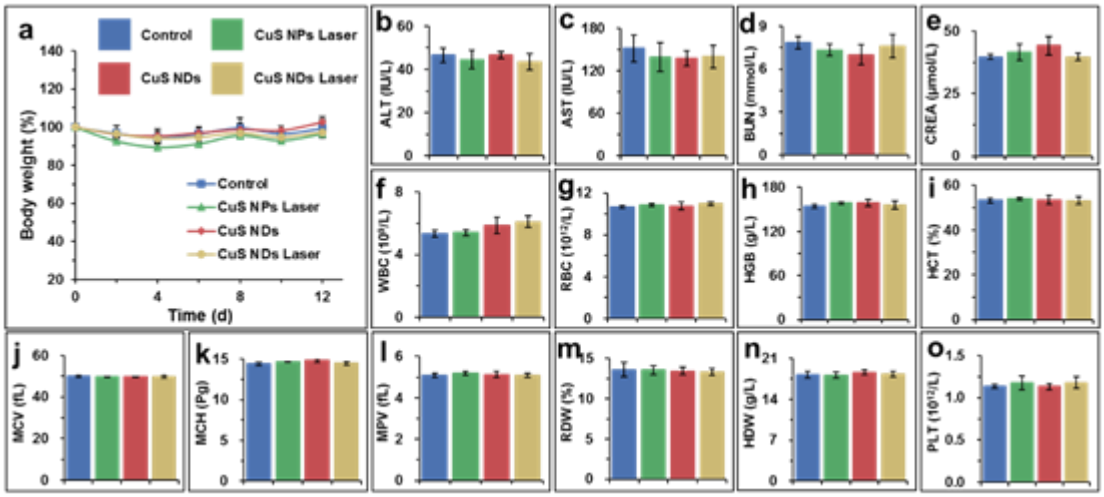

Figure S4. Influence of mice healthy. (a) Mice body weight changes after different treatments. (b-o) Blood biochemistry analysis: ALT (alanine transferase), AST (aspartate transferase), BUN (blood urea nitrogen), CREA (creatinine). (f-o) Hematology survey: WBC (white blood cells), RBC (red blood cells), HGB (hemoglobin), HCT (hematocrit), MCV (mean cell volume), MCH (mean corpuscular hemoglobin), MPV (mean platelet volume), RDW (red cell distribution width), HDW (hemoglobin distribution width), PLT (blood platelet).

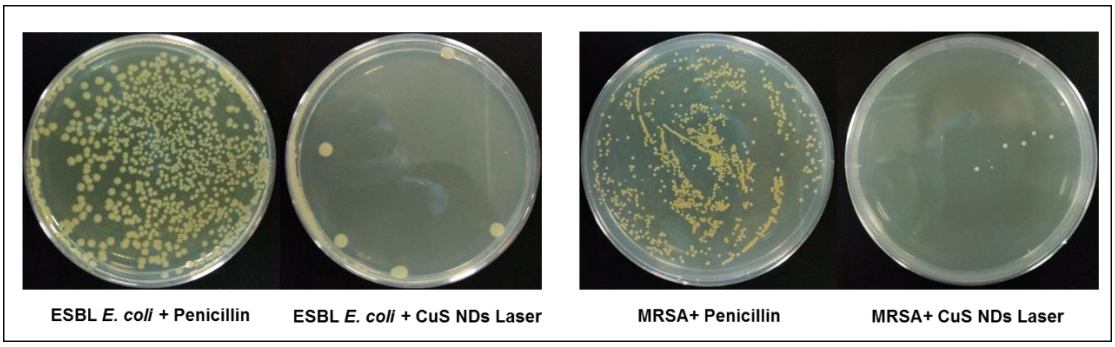

Figure S5. Images of ESBL *E. coli* and MRSA colonies formed on LB-agar plates after CuS NDs Laser (45 μg/mL, 2.5 W/cm^2, 10 min) or penicillin (45 μg/mL,) treatment, which displayed an antibacterial effect comparison of CuS NDs Laser and penicillin against ESBL *E. coli* and MRSA.

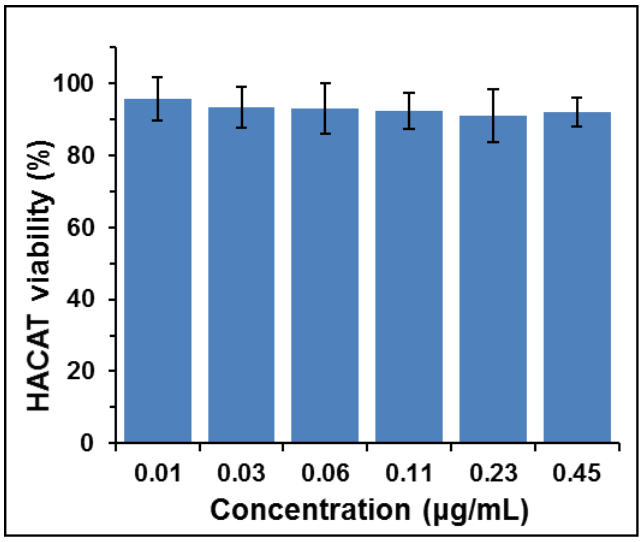

Figure S6. HACAT (immortalized human keratinocytes cell line) cells with different CuS NDs concentrations with laser irradiation (2.5 W/cm^2, 10 min).

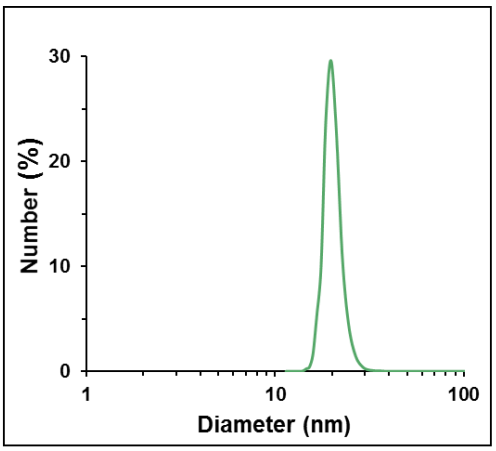

Figure S7. Hydrodynamic diameter of CuS NPs measured by dynamic light scattering.

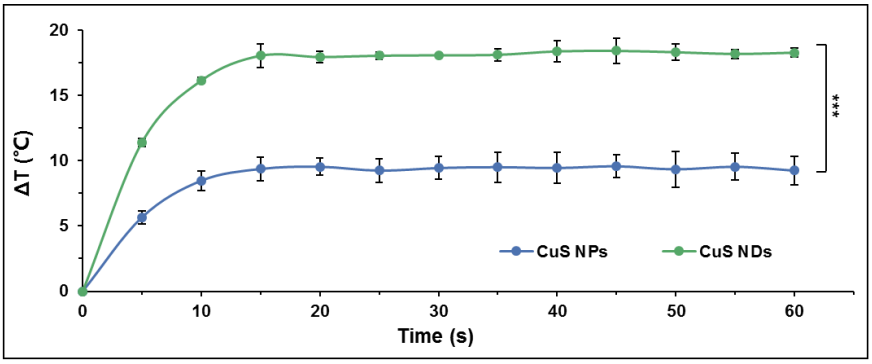

Figure S8. Temperature evolution profile of dosed mice upon NIR laser irradiation.