Supplementary Information

Using visible light-triggered pH switch to activate nanozymes for antibacterial treatment

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Materials and Methods

Materials

$CuCl_2 \cdot 2H_2O$ and bovine serum albumin (BSA) were purchased from BBI Life Science Corporation (Shanghai, China). Sodium sulfide (Na_2S) and 3, 3', 5, 5'-tetramethylbenzidine (TMB) were bought from Sigma-Aldrich (USA). 30% H_2O_2 and sodium acetate ($NaAc \cdot 3H_2O$) were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). 2, 3, 3-trimethylindolenine, propane sultone and 2-hydroxybenzaldehyde were purchased from Adamas-Beta (Shanghai, China). The bacterial strains were obtained from the Department of Microbiology, Yangzhou University (Yangzhou, China). All reagents were from commercial sources and used without any further purification.

Synthesis of protonated merocyanine (MEH)

The synthesis of MEH was conducted as a previous report.[1] First, 2, 3, 3-trimethylhypoindole (1.65 g, 0.01 mmol) and propane sultone (1.26 g, 0.01 mmol) was mixed and stirred at 90 °C for 4 hours under N_2. Then, the purple solid product [2, 3, 3-trimethyl-1-(3-sulfonylpropyl)-3H-indole] was collected by filtration, washed with cold diethyl ether and dried under vacuum. Next, 2, 3, 3-trimethyl-1-(3-sulfonic acid propyl)-3H-indole (100 mg, 0.36 mmol) and 2-hydroxybenzaldehyde (48 mg, 0.39 mmol) were added to absolute ethanol (2 mL). The mixture was refluxed overnight, and the obtained yellow product MEH was collected by filtration, washed with cold ethanol and dried under vacuum. ^{1}H NMR (400 MHz, DMSO-d_6) δ: 11.04 (s, 1H), 8.60 (d, J = 16.4 Hz, 1H), 8.28 (dd, J_1 = 8.0 Hz, J_2 = 1.2 Hz, 1H), 8.03 (dd, J_1= 8.0 Hz, J_2 = 1.2 Hz, 1H), 7.89 (d, J = 13.2 Hz, 1H), 7.87-7.85 (m, 1H), 7.66-7.59 (m, 2H), 7.50-7.46 (m, 1H), 7.05-7.03 (m, 1H), 7.01-6.97 (m, 1H), 4.83-4.79 (m, 2H), 2.66 (t, J = 6.4 Hz, 2H), 2.22-2.15 (m, 2H), 1.77 (s, 6H).

Synthesis of CuS Nanoparticles

For synthesis of albumin-stabilized CuS nanoparticles (CuS NPs), $CuCl_2$ (1 M, 100 mL) was first added to BSA (10 g/L, 10 mL) solution. After stirring for 5 min, Na_2S (1 M, 100 mL) was added. The above mixture was stirred vigorously, and then heated to 90 ℃ until the color of solution turned into dark green (about 15 min).[2] After centrifugation and washing, the product CuS NPs were freeze-dried for further use.

Characterization

Transmission electron microscopy (TEM, Tecnai 12, Philips, Netherlands) was used to observe the morphology and size of CuS NPs. Scanning electron microscopy (SEM, Gemini SEM 300, Carl Zeiss, Germany) was used to observe the morphology of bacteria after different treatments. The crystal form of CuS NPs was studied by X-ray

powder diffractometry (XRD, D8 ADVANCE, Germany). X-ray photoelectron spectroscopy (XPS, ESCALAB250Xi, ThermoFisher Scientific, USA) was used to analyze the surface chemistry of elemental composition and chemical state of the CuS NPs. Thermogravimetic analysis (TGA, Pyris 1, PerkinElmer, USA) was uesed to determine the mass percentage of CuS in BSA-CuS nanocomposites. The experiments were performed at a constant rate of 10 °C/min (20-800 °C), using nitrogen as a carrier gas.

Determination of Cu^{2+} release

The CuS NPs were separately dispersed in 3 mL of pH 4.5 and pH 7.0 NaAc buffer (0.1 M), and then added to a pretreated 27 mm dialysis bag. The dialysis bag was placed in 50 mL NaAc buffer under stirring conditions (37 °C, 100 rpm). Partial release medium (3 mL) was removed for inductively coupled plasma mass spectrometry (ICP-MS) testing at given times (Elan DRC-e, PerkinElmer, USA), followed by the addition of free release medium (3 mL) for continuous observation of release behavior.

Determination of the peroxidase-like activity (POD) of CuS NPs

Peroxidase activity of the CuS NPs was studied by using 3, 3′, 5, 5′-tetramethylbenzidine (TMB) as an organic substrate in the presence of H_2O_2. Absorbance of the color reaction (λ_{max} = 652 nm) was recorded at a certain reaction time to evaluate the POD-like activity. In a typical experiment, reagents were added to 200 μL of buffer solution (0.1 M NaAc, pH 4.5) in the order of CuS NPs (final concentration: 20 μg/mL), TMB (final concentration 1.25 mM), and H_2O_2 (final concentration 0.5 M).

Steady-state kinetics assays were conducted at 25 °C in 200 μL of reaction buffer solution (0.1 M NaAc, pH 4.5) with CuS NPs (20 μg/mL) as a catalyst in the presence of H_2O_2 and TMB. (1) H_2O_2 as the substrate: TMB (final concentration: 1.25 mM) and different amounts of H_2O_2 were added to the reaction system containing CuS NPs, with change in absorbance recorded at 652 nm with time at 25°C. (2) TMB as the substrate: H_2O_2 (final concentration 0.5 M) and different amounts of TMB were added to the reaction system containing the CuS NPs, with the change in absorbance recorded at 652 nm with time at 25 °C. The pH dependence of the POD-like activity of the CuS NPs was detected under different pH buffer solutions, with increased from pH 2 to 11, and temperature dependence was detected under different temperatures, which increased gradually from 27 °C to 87 °C.

Antibacterial activity of CuS NPs

Antibacterial abilities of the CuS NPs against *Escherichia coli* (*E. coli*, 1403) and *Staphylococcus aureus* (*S. aureus*, 26003-20) were determined via the number of colony-forming units (CFU) using the plate counting method. The experiment was divided into seven groups: (1) bacteria; (2) bacteria + MEH + Light; (3) bacteria + H_2O_2; (4)

bacteria + MEH + CuS; (5) bacteria + MEH + CuS + Light; (6) bacteria + MEH + CuS + H_2O_2; (7) bacteria + MEH + CuS + H_2O_2 + Light. The concentration of H_2O_2 was 1.0 mM for *E. coli* and 10 mM for *S. aureus*, respectively, which was much lower than the effective concentration of H_2O_2 (0.5-3%, wt%) used in clinics.[3] Concentration of the MEH was 500 μM, and the CuS NPs was 500 μg/mL. For the groups without light treatment, the mixtures were incubated together for 15 min. For the groups with visible-light irradiation, the mixtures were exposed to the light for 15 min at 375 mW/cm (λ= 350-780 nm, CEL-HXF300, Beijing China Education Au-light Co., Ltd). Finally, all the groups were placed on Luria-Bertani (LB) solid medium and cultured at 37 ℃ for 18 h, and the numbers of colonies were counted.

Internal ROS generation analysis

Oxidant-sensitive dye 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, Invitrogen, Carlsbad, CA, USA) was used to measure the intracellular ROS levels. After treatments, bacteria were stained with 10 μM DCFH-DA for 30 min in the dark at room temperature and twice washed with phosphate buffer solution (PBS), followed by treatments (1) Control; (2) CuS + MEH + Light; (3) H_2O_2; (4) CuS + H_2O_2 + MEH + Light. Initial pH was 7.0 unless otherwise stated. For the group with light exposure, the power density was 375 mW/cm for 15 min. The intracellular ROS levels were measured by a Tecan Spark 20M Multimode Microplate Reader (Switzerland) with excitation and emission wavelengths at 488 nm and 525 nm, respectively.

Determination of internal malondialdehyde (MDA)

As a natural product of lipid oxidation in organisms, MDA can be used to detect the level of lipid oxidation. Bacteria were first treated with different groups: (1) Control; (2) CuS + MEH + Light; (3) H_2O_2; (4) CuS + H_2O_2 + MEH + Light, and then were lysed by lysozyme and proteinase K for MDA measurement at 4 ℃. After lysation, the supernatant was centrifuged at 12,000 g for 10 min to determine lipid peroxidation using a Micro-MDA Assay Reagent Kit (KeyGEN Biotech, China). Initial pH was 7.0 unless otherwise stated. For the group with light exposure, the power density was 375 mW/cm for 15 min.

Morphological observation of bacteria

After the antibacterial abilities were assessed, the bacterial suspensions in the control group and CuS + H_2O_2 + MEH + Light group were centrifuged and re-dispersed in glutaraldehyde (2.5%, Sigma-Aldrich, USA) for 4 h at 4 °C under dark conditions. The bacterial cells were then dehydrated with 30, 50, 70, 80, 90, and 100% of ethanol, respectively, for 10 min. Finally, the dried bacteria were sputter-coated with gold for imaging using scanning electron microscopy.

In vivo mice wound model and healing process

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Yangzhou University and approved by the Animal Ethics Committee of Medical College of Yangzhou University. Male Balb/c mice (6-7 weeks old, 18-20 g) were obtained from Yangzhou University Laboratory Animal Center. Mice were divided into 7 groups (n=6): (1) bacteria; (2) bacteria + MEH + Light; (3) bacteria + H_2O_2; (4) bacteria + MEH + CuS; (5) bacteria + MEH + CuS + Light; (6) bacteria + MEH + CuS + H_2O_2; (7) bacteria + MEH + CuS + H_2O_2 + Light. A wound was surgically cut on the back of each mouse and covered with 1 × 10^7 CFU/mL of *S. aureus* after anesthesia to establish an infected wound model. After infection for 12 h, 20 μL suspension containing 500 μg/mL CuS NPs and 500 μM MEH, and 20 μL H_2O_2 (10 mM) were dropped onto the wound areas in the corresponding groups. For the group with light exposure, the power density was 375 mW/cm for 15 min. A photo of each wound was taken at a set time and the size of the wound was measured simultaneously. The CuS, MEH, H_2O_2 and visible illumination were applied at day 1 and day 3, and the whole course of treatment was defined as 11 days. For the iPhone group, the distance between light source and tested samples was 1 cm, and the illumination time was 15 min.

Statistical analysis

Data were shown as mean ± s. d. Statistical analysis was performed using Student's test for two-group differences. $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$.

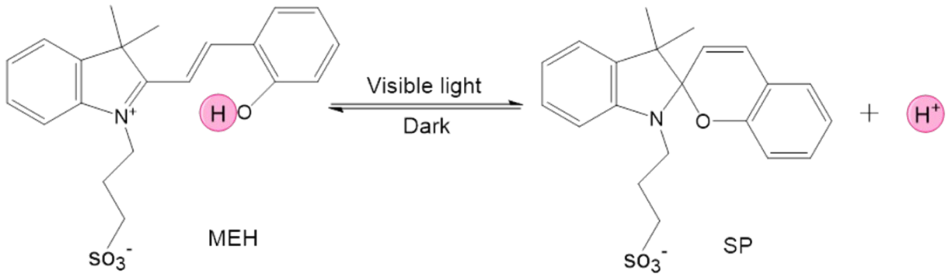

Fig. S1 MEH photoreaction under visible-light illumination. MEH: protonated merocyanine; SP: deprotonated spiropyran.

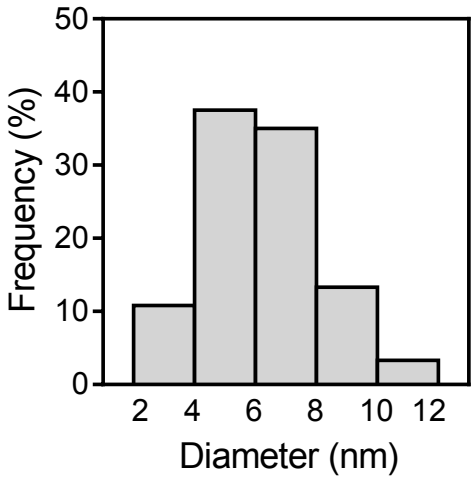

Fig. S2. Size distribution of CuS NPs.

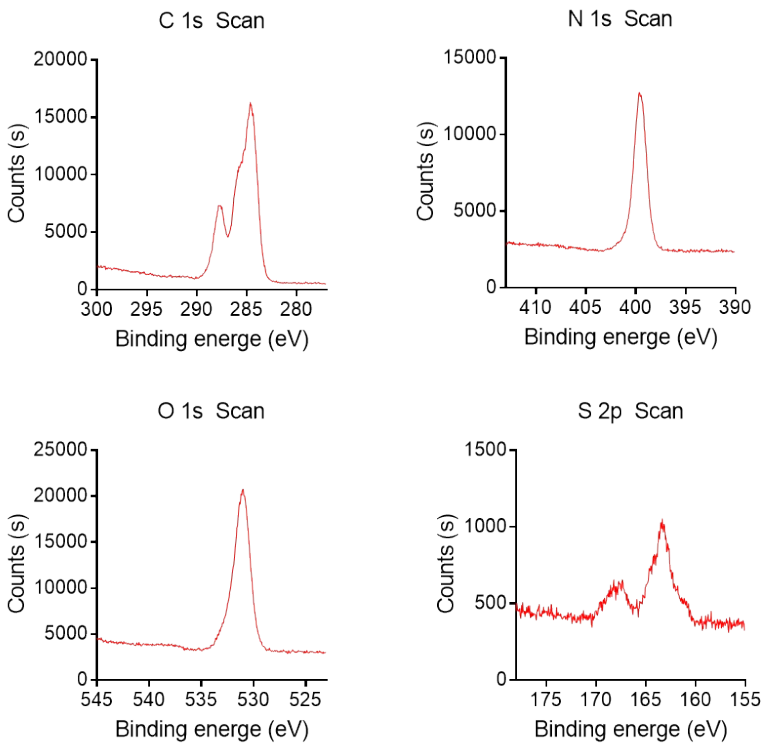

Fig. S3 XPS spectra of C 1s, N 1s, O 1s, and S 2p orbits for CuS NPs.

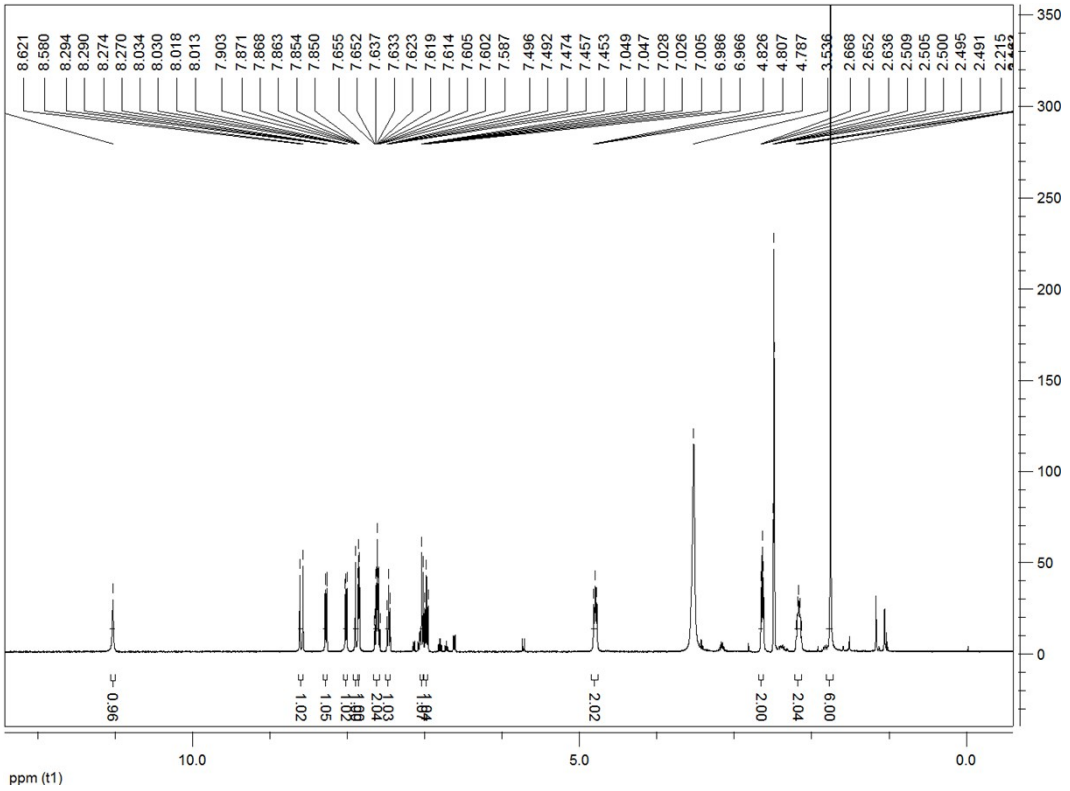

Fig. S4 ^{1}H NMR of MEH.

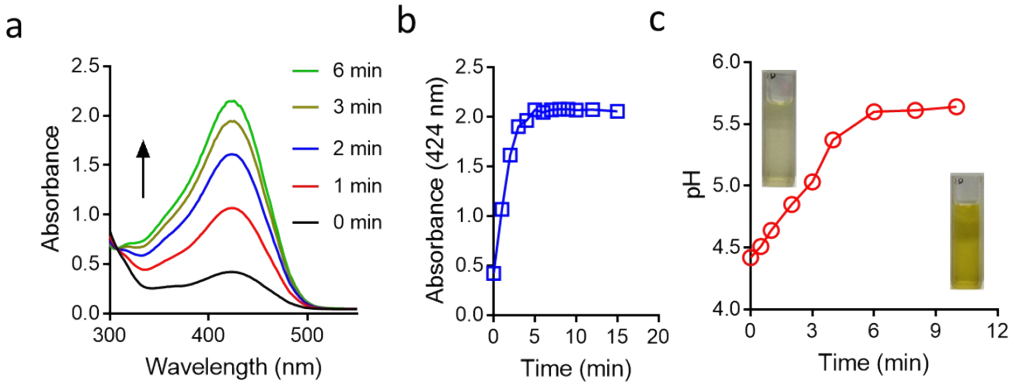

Fig. S5 (a) Time-dependent UV-Vis spectra of deprotonated spiropyran (SP) aqueous solution in the dark. (b) Absorbance of SP at 424 nm vs time. (d) Time-dependent pH of SP aqueous solution in the dark. Insets are relevant photos. Initial concentration of MEH was 200 μM. [MEH] = 200 μM.

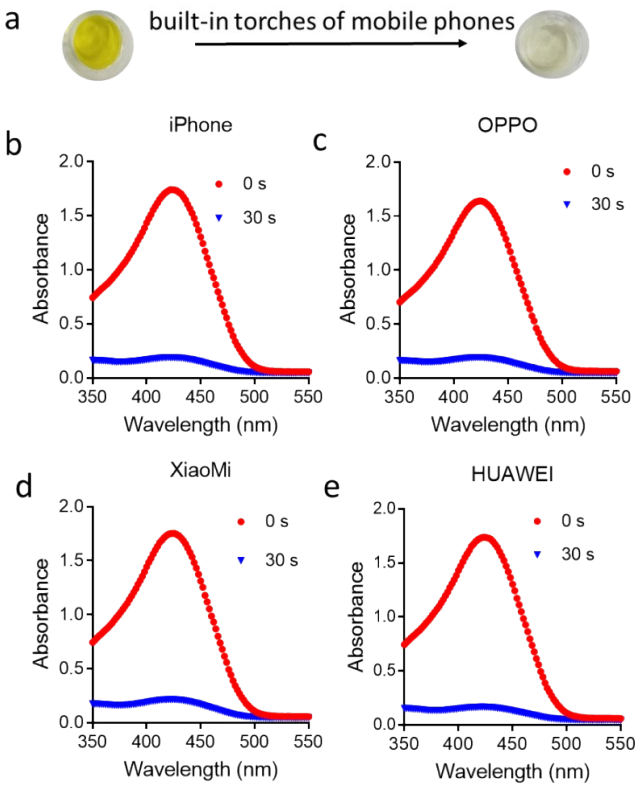

Fig. S6 (a) Color fading of MEH solution (200 μM) under illumination of the built-in torches of mobile phones. (b-e) UV-Vis spectra of MEH (200 μM) solution under illumination of different built-in torches of mobile phones. The distance between light source and tested samples was 1 cm.

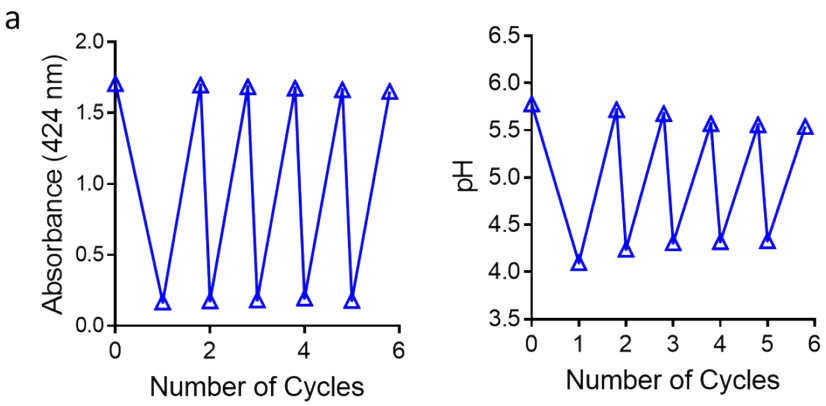

Fig. S7 Reversible transformations of (a) absorbance at 424 nm and (b) pH of MEH solution with on/off light cycles. Concentration of MEH: 200 μM. Illumination conditions: P = 37.5 mW/cm, 2 min.

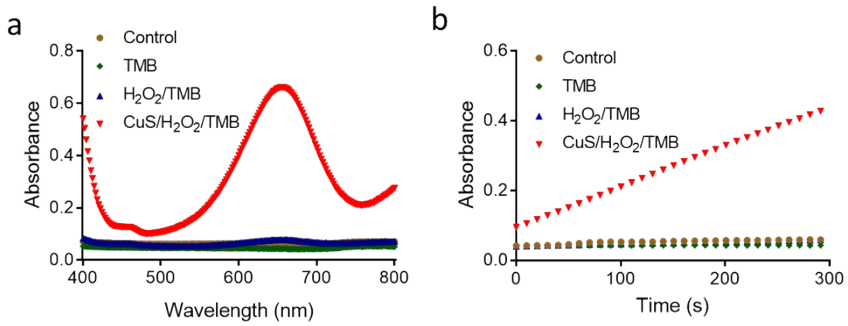

Fig. S8 (a) Absorbance spectra and visual color of TMB in different systems: (1) control (NaAc buffer), (2) TMB (1.25 mM); (3) H_2O_2 (0.5 M)/TMB (1.25 mM); (4) CuS (20.0 μg/mL)/H_2O_2 (0.50 M)/TMB (1.25 mM) in pH 4.5 NaAc buffer (0.1 M) after 5 min incubation. (b) Time-dependent absorbance changes of TMB (1.25 mM) at 652 nm in the presence of CuS NPs (20 μg/mL) and H_2O_2 (0.5 M).

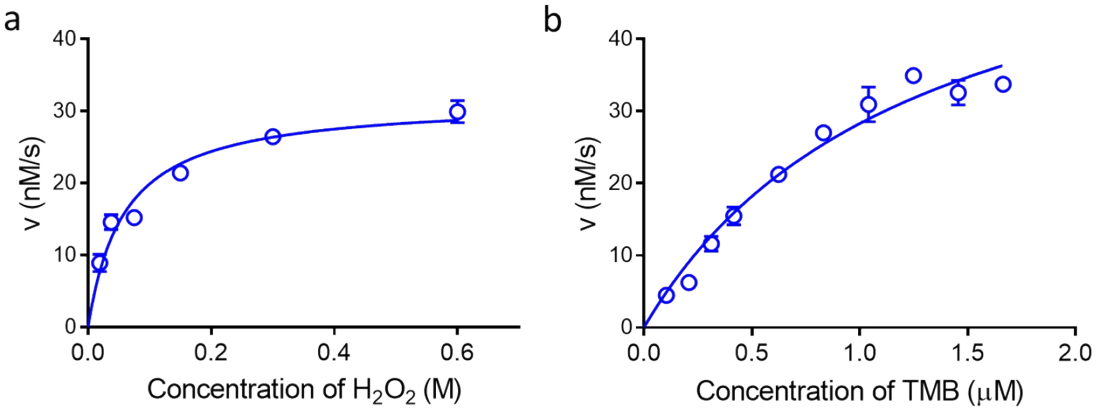

Fig. S9 Steady-state kinetic assay of CuS NPs. Experiments were carried out in 0.1M NaAc buffer (pH = 4.5) using 20 μg/mL CuS NPs.

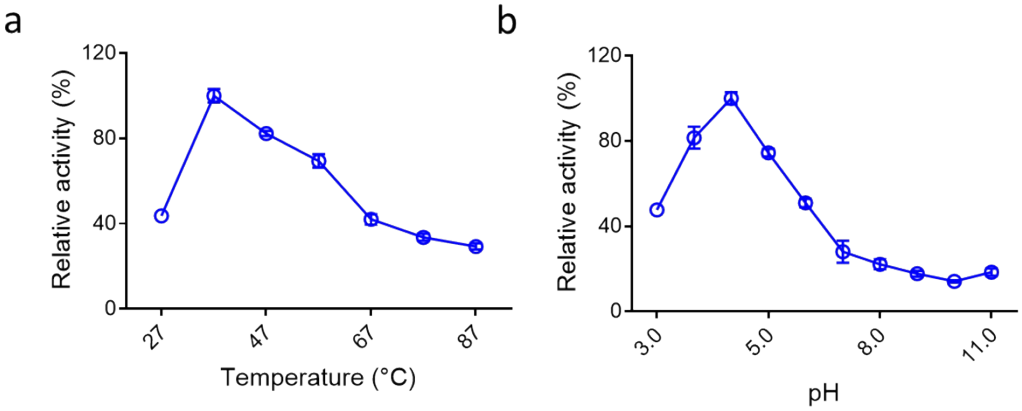

Fig. S10 The POD-like activity of CuS NPs is dependent on temperature (a) and pH (b). [CuS] = 20 μg/mL; [H_2O_2] = 0.5 M; [TMB] = 1.25 mM.

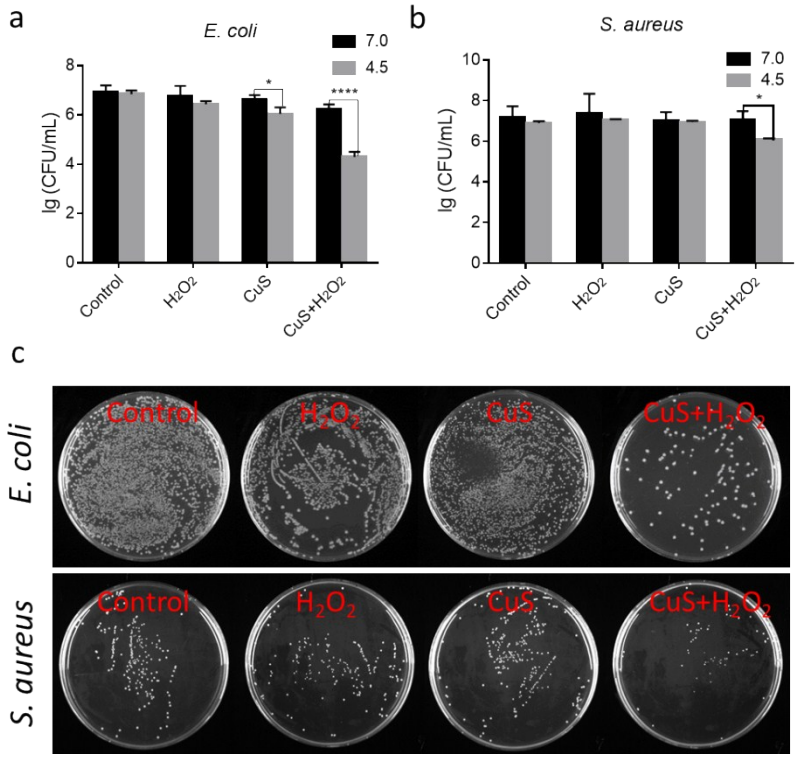

Fig. S11 Bacteria viabilities of (a) *E. coli* and (b) *S. aureus* after being cultured with CuS NPs and/or H_2O_2 at different pH. (c) Photos of bacterial colonies of *E. coli* and *S. aureus* treated with CuS NPs and/or H_2O_2 at pH 4.5. [CuS] = 20 μg/mL; Concentration of H_2O_2 was fixed at 1.0 mM and 10.0 mM for *E. coli* and *S. aureus*, respectively.

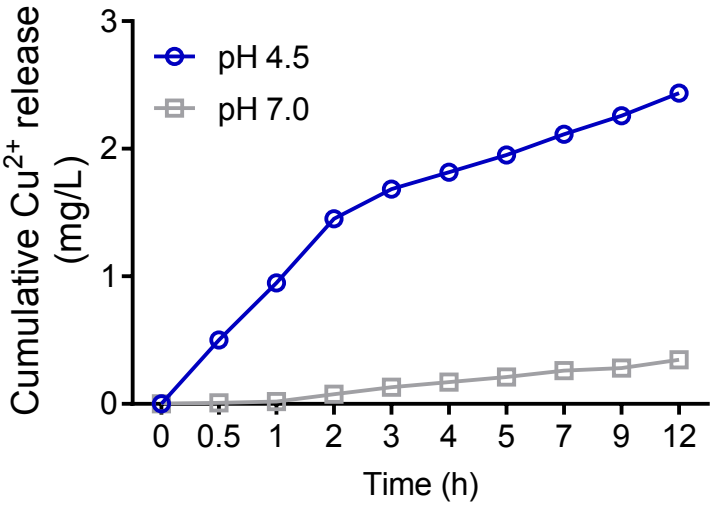

Fig. S12 Release profile of Cu^{2+} from CuS NPs at different pH.

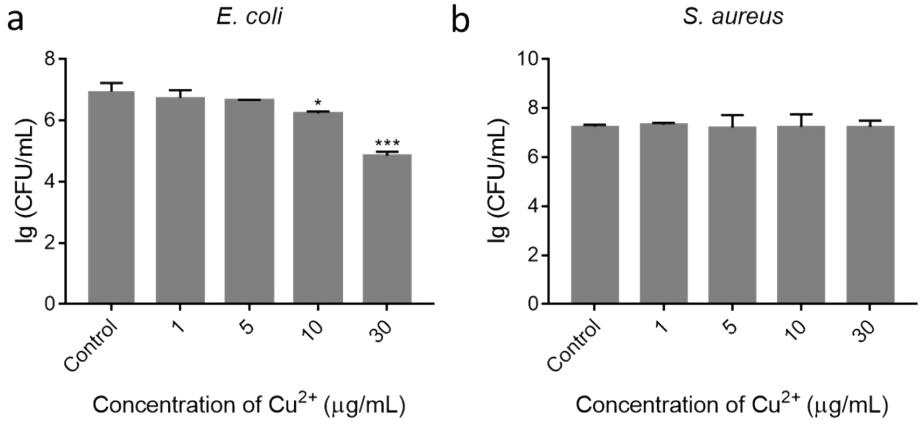

Fig. S13 Bacteria viabilities of (a) *E. coli* and (b) *S. aureus* after being cultured with $CuCl_2$.

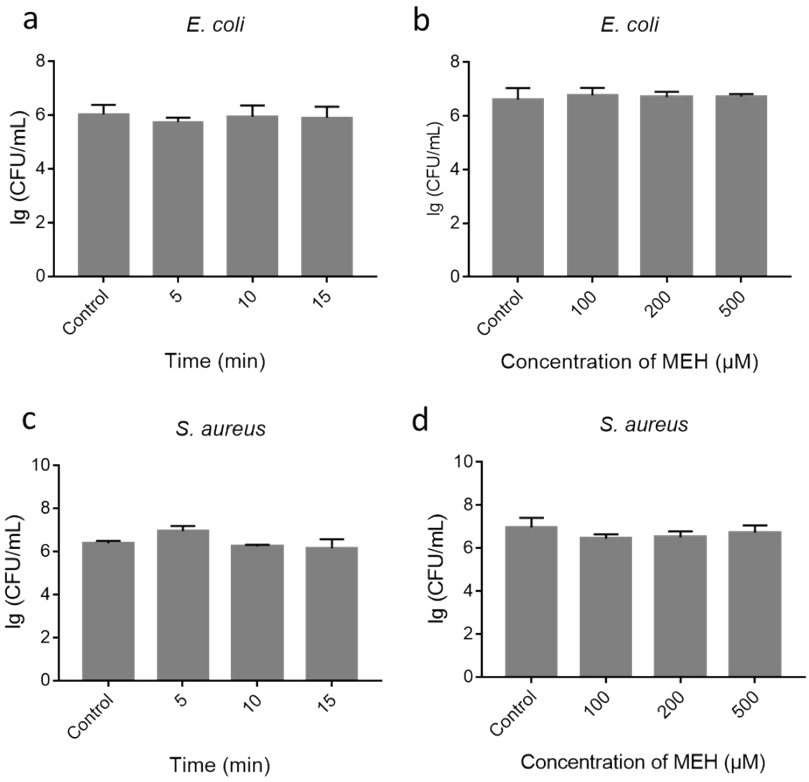

Fig. S14 Effects of illumination time and MEH concentration on the bacteria viabilities. The power density was 375 mW/cm.

Fig. S15 SEM images (a) *E. coli* and (b) *S. aureus* after being cultured with CuS/H_2O_2/MEH/Light. [CuS] = 500 μg/mL; [MEH] = 500 μM. The concentration of H_2O_2 was fixed at 1.0 mM and 10.0 mM for *E. coli* and *S. aureus*, respectively. Illumination conditions: P = 375 mW/cm, 15 min.

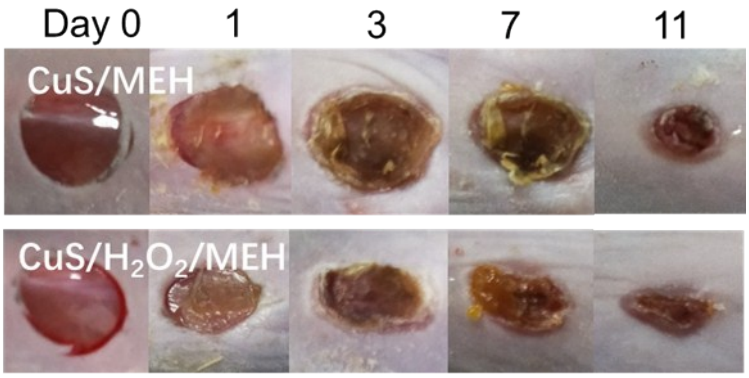

Fig. S16 Photographic images of wound healing degree in the CuS/MEH and CuS/H_2O_2/MEH groups. [CuS] = 500 μg/mL; [MEH] = 500 μM; [H_2O_2] = 10.0 mM.

Table S1 The apparent Michaelis-Menten constant (K_m) and maximum reaction rate (V_{max}) of CuS NPs.

Catalyst	[E] (μg/mL)	substrate	K_m (mM)	V_{max} (nM/s)
CuS NPs	20	TMB	1.25	63.65
CuS NPs	20	H_2O_2	0.058	31.52

References

[1] Z. Shi, P. Peng, D. Strohecker and Y. Liao, *J. Am. Chem. Soc.*, 2011, **133**, 14699-14703.

[2] Y. Qiao, Y. Ping, H. Zhang, B. Zhou, F. Liu, Y. Yu, T. Xie, W. Li, D. Zhong, Y. Zhang, K. Yao, H. A. Santos and M. Zhou, *ACS Appl. Mater. Interfaces*, 2019, **11**, 3809-3822.