Supplementary Information

Cuprous oxide-demethyleneberberine nanospheres for single near-infrared light-triggered photoresponsive-enhanced enzymatic synergistic antibacterial therapy

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1 Materials

Phosphoric acid (85%), hydrogen peroxide (30%), dimethylbenzene, acetic acid (CH_3COOH), methanol (CH_3OH), ethanol (C_2H_5OH), berberine, hydrochloric acid (HCl), 5,5′-dithiobis(2-nitrobenzoic acid (DTNB), *N,N*-dimethylformamide (DMF), and pyrogallol were purchased from Sinopharm Chemical Reagent Co. Trifluoromethanesulfonic acid (CF_3SO_3H), Acetate monohydrate [$Cu(OAc)_2$], 3-propyl-2-[5-(3-propyl-2(3H)-benzothiazolylidene)-1,3-pentadien-1-yl]-iodide(1:1) [diSC3(5)], crystal violet (CV), amplex red (AR) and xylene were obtained from Aladdin Reagent Co. Phosphate buffered saline (PBS) was obtained from Servicebio. 3,3′5,5′-tetramethylbenzidine (TMB), O-phenylenediamine (OPD), tetramethylpiperidine (TEMP), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) and 1,3-diphenylisobenzofuran (DPBF) were obtained from Sigma-Aldrich. All other chemicals were reagent grade or better. All other reagents and solvents were used as received. The deionized water of resistivity 18.2 MΩ cm^{-1} was used in all experiments. *MRSA* and *AREC* were provided by Sichuan Provincial People's Hospital, Chengdu, China.

2 Characterizations

Scanning electron microscopy (SEM) images were obtained on a HITACHI Regulus 8100 at a working voltage of 15 kV after platinum coating for 45 s. Transmission electron microscopy (TEM), energy dispersive X-ray spectroscopy (EDS) and EDS element mapping were acquired on the JEOL JEM-F200. XPS (X-ray photoelectron spectrometry, AXIS SUPRA) and XRD (X-ray diffraction, ULTIMALV) were employed to evaluate the phase composition of the samples on an X-ray diffractometer (Cu K_α radiation, k = 0.15406 nm). Fourier transform-infrared (FT-IR) spectra were conducted on a Thermo Fisher Nicolet iS50 (KBr pellet technique ranging from 4000 to 400 cm^{-1} with a 2.0 cm^{-1} resolution). The concentration of the released Cu^{2+} ions was detected using inductively coupled plasma-atomic emission spectrometry (ICP-AES, Atomscan Advantage). UV-Vis absorbance measurements were carried out on a

PEERSEE TU-1810 UV-Vis spectrophotometer with a Peltier temperature control accessory and CARY5000 UV-Vis spectrophotometer with an integrating sphere. The fluorescence was tested by F-4700. The ζ-potential and size of the nanoparticles were measured in a Zetasizer 3000HS analyzer. All electron spin resonance (ESR) measurements were carried out on a JEOL JES FA200 spectrometer at ambient temperature.

3 GSH-oxidase-like catalytic activity of Cu_2O-DMB and kinetic assay

The GSH-oxidase (GSH-OXD)-like activity assays of Cu_2O-DMB was detected by Ellman's assay. All experiments were carried out in the dark. Ellman reagent DTNB reacted with thiol groups (-SH) in GSH to obtain a yellow product (TNB). Cu_2O-DMB was treated with GSH (100 μL, 10 mM) in PBS (1.9 mL, pH 8.0) under 20 ℃ and 50 ℃ water bath environment. DTNB (10 μL, 5 mM) was added at different time respectively for mixing. Next, the mixture solution was centrifuged and the supernatant was required to measure GSH consumption through UV-vis absorption (at 412 nm for TNB) spectrum to study the concentration dependent and time-dependent consumption of GSH. The loss of GSH was calculated as follows:

$$Loss\ of\ GSH\ (\%) = (A_n - A_s)/A_n \times 100 \quad \text{(S1)}$$

where A_s is the absorbance of the sample and A_n is the absorbance of the negative control. All assays were performed as triplicates. To test whether O_2 is required for the reaction, the mixture reacted in air, O_2, and a N_2 atmosphere, respectively.

For kinetic assay, the Michaelis constant (K_M) is defined as the substrate concentration at half the maximum reaction rate. K_M reflects the affinity of Cu_2O-DMB for its substrate. Maximal reaction velocity (V_{max}) is the maximal reaction rate that is observed at saturating substrate concentrations. The kinetics constants K_M and V_{max} were calculated through fitting the initial reaction velocity values (V) and the substrate concentrations to equations S2-S4.

$$V=(V_{max}\times[S])/(K_M+[S]) \quad \text{(S2)}$$

where $[S]$ is the concentration of substrate, V is the initial velocity and is calculated using the following equation:

$$V=\Delta A/(\Delta t\times\varepsilon\times l) \quad \text{(S3)}$$

where ΔA is the change of absorbance value, Δt is the initial reaction time (s), ε is the molar absorption coefficient of the colorimetric substrate, and l is the path length of light traveling in the cuvette (cm).

The catalytic constant (k_{cat}) is defined as the maximum number of substrate molecules converted to product per unit of time and is calculated by the following equation:

$$k_{cat}=V_{max}/[E] \quad \text{(S4)}$$

where $[E]$ is the concentration of Cu_2O-DMB (M).

k_{cat}/K_M characterizes both the affinity and catalytic ability of the enzyme to the substrate, reflecting the catalytic efficiency of Cu_2O-DMB. The kinetic assay was performed in the reaction of Cu_2O-DMB (50 μg mL^{-1}) with different concentrations of GSH (0.05, 0.1, 0.2, 0.4, 0.6, 0.8 mM).

4 Detection of H_2O_2

Amplex red (AR) was used as a probe. In brief, Cu_2O-DMB (0, 20, 50 μg mL^{-1}) with GSH (0.5 mM) were mixed with AR (0.1 mg/mL) in 0.2 M PBS (pH 4.0) for 60 min. Fluorescence spectra of the mixture solution excited at 521 nm were detected on a microplate reader.

5 Peroxidase-like catalytic activity of Cu_2O-DMB and kinetic assay

The peroxidase-like activity assays of Cu_2O-DMB were carried out using TMB and OPD as the reagents in the presence of H_2O_2 in 0.12 M acetate buffer solution (pH 4.0). The UV-Vis absorbance of the color reaction (at 652 nm for TMB and at 425 nm for OPD) was recorded at a certain reaction time to express the peroxidase-like activity. The steady-state kinetic assay of Cu_2O-DMB with H_2O_2 as the substrate was performed by adding 10 μg/mL Cu_2O-DMB into 0.2 M HAc-NaAc buffer solution (pH 4.0) containing TMB (1 mM) and different concentrations of H_2O_2 (0.125, 0.25, 0.5, 1, 2, 4, 8 mM).

6 Photodynamic effect of Cu_2O-DMB nanospheres

The photodynamic activity of Cu_2O-DMB was assessed by the degradation of DPBF under 808 nm NIR (1.0 W cm^{-2}). Cu_2O-DMB (2.5 mg) and DPBF (13.5 mg) were added into DMF (50 mL) and stirred in the dark for 10 min. After NIR irradiation, the photoreactive solution (3 mL) was separated by centrifuging for 3000×g for 5 min to remove the particles. Then the absorbance at 425 nm was measured at a UV-Vis spectrophotometer.

7 ROS detection by ESR

5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was used to detect •OH and $O_2^{\bullet-}$, 2,2,6,6-tetramethylpiperidine (TEMP) was used to detect $^1O_2$. 10 μL of DMPO was mixed with 50 μL of Cu_2O-DMB (50 μg mL^{-1}). 10 μL of TEMP was mixed with 100 μL of Cu_2O-DMB (50 μg mL^{-1}). The mixture was placed into a quartz capillary for detection. For the H_2O_2 containing group, 8 mM of H_2O_2 was added in mixture. For the NIR group, 808 nm laser (1.0 W cm^{-2}, 3 min) was used to irradiate the mixture before detection.

8 Calculation of the photothermal conversion efficiency (η) of Cu_2O-DMB nanospheres

Cu_2O-DMB aqueous solutions with different concentrations (0–40 μg mL^{-1}) were exposed to an 808 nm laser irradiation (0.5 W cm^{-2}) for 600 s, and the temperature was recorded every 30 s by a thermocouple probe. Cu_2O-DMB aqueous dispersion (0.2 mL) with different concentrations (0, 10, 20, 30, and 40 μg mL^{-1}) were exposed to an 808 nm laser irradiation (0.5 W cm^{-2}) for 600 s, then shutted off the laser for cooling the solution to room temperature. Heating and cooling temperature patterns of 40 μg mL^{-1} Cu_2O-DMB were recorded. The η of Cu_2O-DMB was calculated according to the following equations:

$$\eta = [hS(T_{max}-T_{surr})-Q_{diss}]/I(1-10^{-A808}) \quad \text{(S5)}$$

$$\tau_s = mC_p/hS \quad \text{(S6)}$$

Where h is the heat-transfer coefficient, S is the surface area of the container, T_{max} is the equilibrium temperature, T_{surr} is the ambient temperature, Q_{diss} is the heat obtained by container under 808 nm laser irradiation, I is the density of laser power, A_{808} is the absorbance of the Cu_2O-DMB suspension at 808 nm, and τ_s is the time constant obtained from Figure S6b.

9 Protein leakage

MRSA and *AREC* cells (10^6 CFU/mL) were treated with increasing concentrations of Cu_2O-DMB for 1 h at 37 °C and then irradiated for 5 min under 808 nm NIR (1.0 W cm^{-2}). Subsequently, the cells were pelleted down at 5000

rpm for 5 min, and the cell-free supernatant was collected. The concentration of leaked proteins in the supernatant was measured using the standard Bradford assay.

10 Cytoplasmic membrance depolarization

The membrane potential-sensitive fluorescent dye, diSC3(5), was employed as an indicator of membrane depolarization. Overnight cultured bacteria were diluted in a fresh LB broth and cultured to the mid log phase. *AREC* and *MRSA* cells were collected by centrifugation and washed with 5 mM PBS and 5 mM glucose. Following that, 1950 μL of the bacterial suspension and 50 μL of 20 μM diSC3(5) were added. The fluorescence of the suspension was monitored at room temperature for 20 min at an excitation wavelength of 622 nm and an emission wavelength of 670 nm. Cu_2O-DMB (0, 1, 2, 4 and 8 μg/mL) was added into the cuvette and the increased potential was monitored after irradiated for 2 min under 808 nm NIR (1.0 W cm^{-2}).

11 Antibiofilm

Overnight cultured bacteria were diluted in a fresh LB broth and cultured to the mid log phase, then resuspended in fresh medium (OD_{600} of approximately 0.1). Aliquots of 100 μL of bacterial suspension and final concentrations of 0, 1, 2, 4, 8 and 16 μg/mL of Cu_2O-DMB were co-stored in a 96-well plate at 37°C for 24 h, and irradiated every 8 h for 5 min under 808 nm NIR (1.0 W cm^{-2}). The medium was removed from the wells and the biofilm was carefully washed twice with PBS to remove planktonic bacteria. The biofilms were fixed with 10% ethanol for 10 min, and then stained with 0.1% CV for 20 min in each well. After discarding CV, the biofilm samples were washed with PBS, and 33% AcOH was added to dissolve the fuel on the biofilm, then the absorbance at 570 nm was measured using a microplate reader. The calculation formula of relative biofilm biomass is calculated as follows:

$$\textit{Relative biofilm biomass (\%)} = (A_1\text{-}A_2)/A_1 \times 100 \quad \text{(S7)}$$

Where A_1 represents positive control and A_2 represents experiment group.

12 Bacterial resistance

The strains of *MRSA* and *AREC* were exposed to Cu_2O-DMB+NIR group for sustained passages, and then the MICs of Cu_2O-DMB were determined against each passage of the strain. The freshly diluted *MRSA* (1.0×10^5 CFU) and *AREC* (1.0×10^5 CFU) in the broth medium were respectively cultured in 1 μg/mL Cu_2O-DMB+NIR (808 nm, 1.0 W cm^{-2}, 5 min) at 37 °C for 12 h on a shaker bed at 90 rpm, and the sensitivity of each strain passage to Cu_2O-DMB was tested. For comparative analysis, ceftizoxime was used as a control.

13 Antibacterial experiments *in vitro*

The antibacterial ability of Cu_2O-DMB was determined by plate counting method. Briefly, bacteria suspensions (1×10^8 CFU mL^{-1}, 100 μL) were incubated with berberine (64 μg mL^{-1}), Cu_2O (1 μg mL^{-1}), and Cu_2O-DMB (1 μg mL^{-1}) in a 96-well plate respectively. PBS was used as the control. An 808nm laser light (1.0 W cm^{-2}) irradiated the NIR group for 5 min. After incubated at 37 °C for 40 min, the bacteria suspension (diluted by 1-10^4 fold, 100 μL) was spread on the LB agar plates. The number of bacteria colonies was counted and recorded after incubation for 24 h at 37 °C.

14 Cytotoxicity measurement

Cytotoxicity evaluation was performed on Human umbilical vein endothelial cells lines (HUVEC). These cells were implanted into 96-well microplates and permitted to adhere overnight. Subsequently, the culture medium was substituted by fresh culture medium including Cu_2O-DMB (0-64×MIC)+NIR (808 nm, 1.0 W cm^{-2}, 5 min). After the co-incubation for 24 h, the culture medium was substituted by MTT (20 μg mL^{-1}) culture solution and incubation for 4 h. Ultimately, DMSO (150 μL) was added to each well. Cell viability was calculated by measuring the absorbance at λ = 490 nm to the control via a microplate reader.

15 Hemolysis

Fresh blood from mice was taken and erythrocytes were isolated by centrifugation (1,000 rpm, 10 min). The obtained erythrocytes were washed three times with saline and then diluted to a final concentration of 5% (v/v). Cu_2O-DMB (500 μL) with erythrocyte solution (500 μL) was added into a 24-well microtiter plate and then shaken at 150 rpm for 1 h in an incubator at 37°C. Afterwards, the microplate contents were centrifuged (1,000 rpm,10 min) and the supernatant (100 μL) was introduced into a 96-well microplate. The absorbance of the solution at 540 nm was determined using a microplate reader. Triton X-100 (0.1%) was used as a positive control. The hemolysis rate is calculated as follows:

$$Hemolysis\ rate\ (\%) = (A_p\text{-}A_b)/(A_t\text{-}A_b)\times 100 \quad \text{(S8)}$$

Where A_p represents the experimental group, A_t represents the positive control and A_b represents blank control.

16 Cu^{2+} release

Cu_2O (2 mL, 1.0 mg/mL^{-1}) and Cu_2O-DMB (2 mL, 1.0 mg/mL^{-1}) solutions were respectively mixed with 0.2 M PBS (pH 3.0, 4.5, 6.0 and 7.5). The mixture was placed on a shaker and taken out at the specified time point, then centrifuged at 8000 rpm for 4 min to obtain the supernatant for ICP-AES test.

17 Mice wound model

All animal experiments in this study were approved and compliant with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Qingdao University of Science and Technology. The wound model was built on the back of male Kunming mice (22–26 g, 4–5 weeks old), which were purchased from the Model Animal Research Center (MARC) of JiNan PengYue Co. Ltd. (JiNan, China). The mice were slashed with 6 mm diameter round wound and *MRSA* cells (1 × 10^8 CFU, 50 μL) were injected into the wound for constructing the infected wound model. After 24 h, the infected mice were respectively treated with PBS, berberine, Cu_2O and Cu_2O-DMB. Meanwhile, photographs of the wounds were taken every day. For histological analysis, the mice were sacrificed with an overdose of pentobarbital (100 mg·kg^{-1}) on day 7. The entire wound with adjacent normal skin was collected and fixed using 4% paraformaldehyde solution for 24 h at 4 °C. The sample of subcutaneous tissue was analyzed using H&E staining method. All sections were observed and photographed with a microscope (BX51, Olympus, Japan), meanwhile, the wound was excised and incubated in sterile saline for 24 h at 37 °C. Then, the culture solution was diluted 10^3-fold and cultured on LB agar plates at 37 °C for 24 h for counting the number of bacterial colonies. The wound healing rate was calculated as follows:

$$Wound\ healing\ rate\ (\%) = (A_{initial} - A_{time})/A_{initial} \times 100 \quad (S9)$$

where $A_{initial}$ is the initial wound area (day 0), and A_{time} is the wound area at different time points.

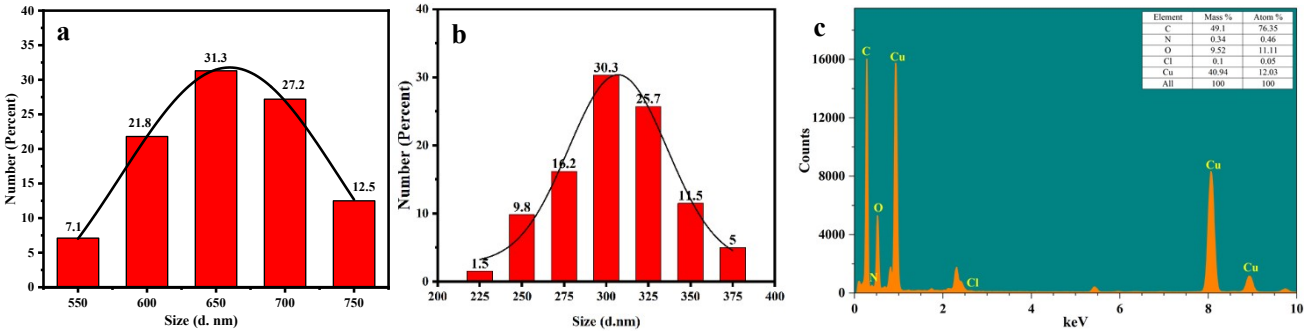

Figure S1. The size distribution histogram of (a) cube Cu_2O and (b) Cu_2O-DMB nanospheres; (c) EDS mapping of Cu_2O-DMB nanospheres.

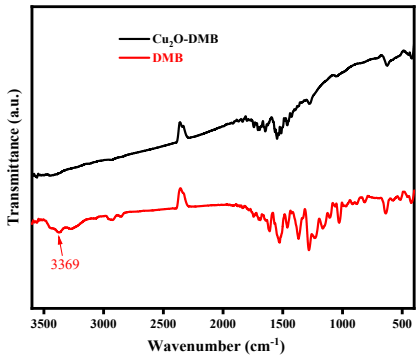

Figure S2. FT-IR spectra of DMB and Cu_2O-DMB nanospheres.

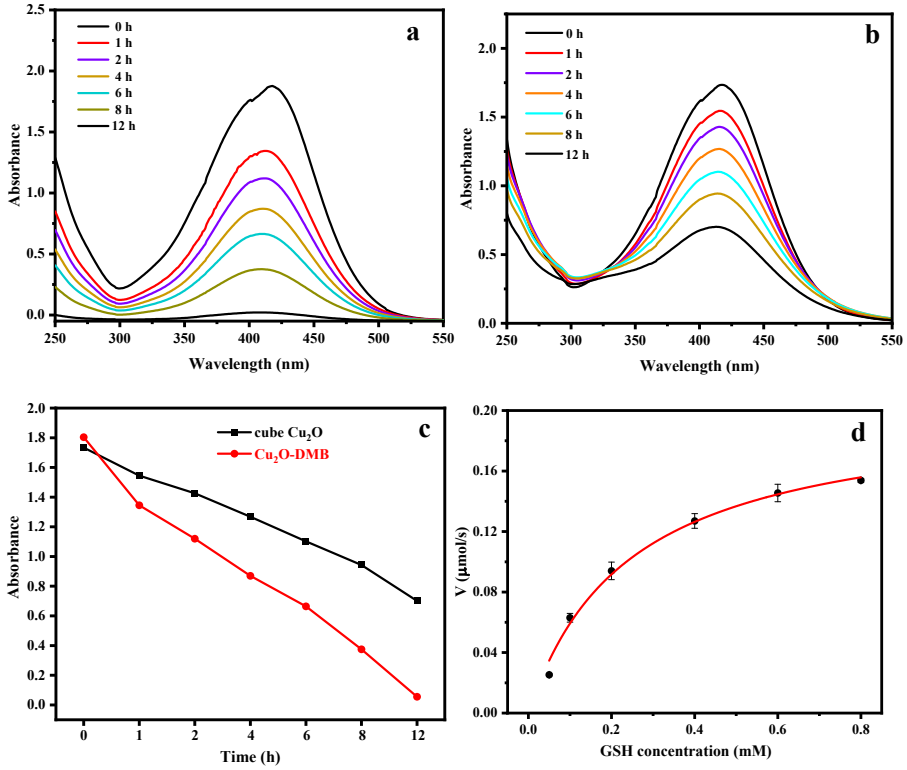

Figure S3. (a) Time-dependent GSH depletion by (a) Cu_2O-DMB nanospheres (50 μg mL^{-1}) and (b) cube Cu_2O (50

μg mL^{-1}); (c) the absorption intensity of TNB at 412 nm in presence of 50 μg mL^{-1} of Cu_2O-DMB nanospheres at different time points; (d) Kinetic assay for the GSH-OXD-like activity of Cu_2O-DMB with GSH as substrate.

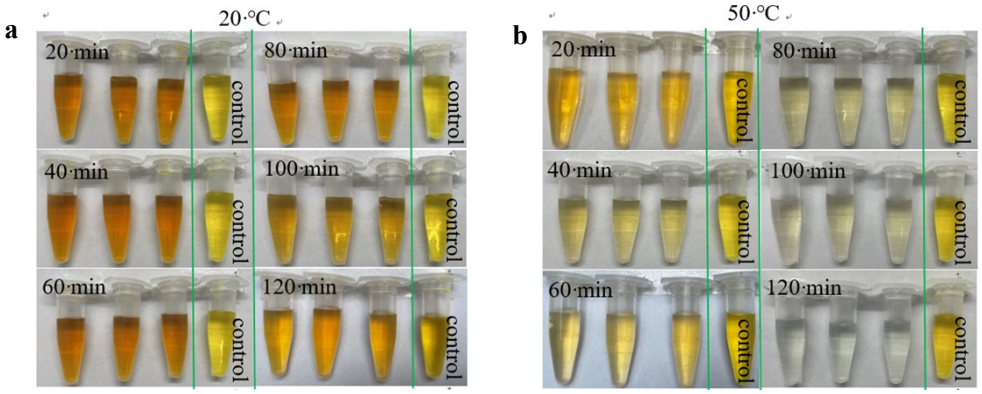

Figure S4. Photographs for the color change after GSH oxidation with Cu_2O-DMB nanospheres (50 μg mL^{-1}) at different time intervals determined by Ellman's assay at (a) 20 °C and (b) 50 °C.

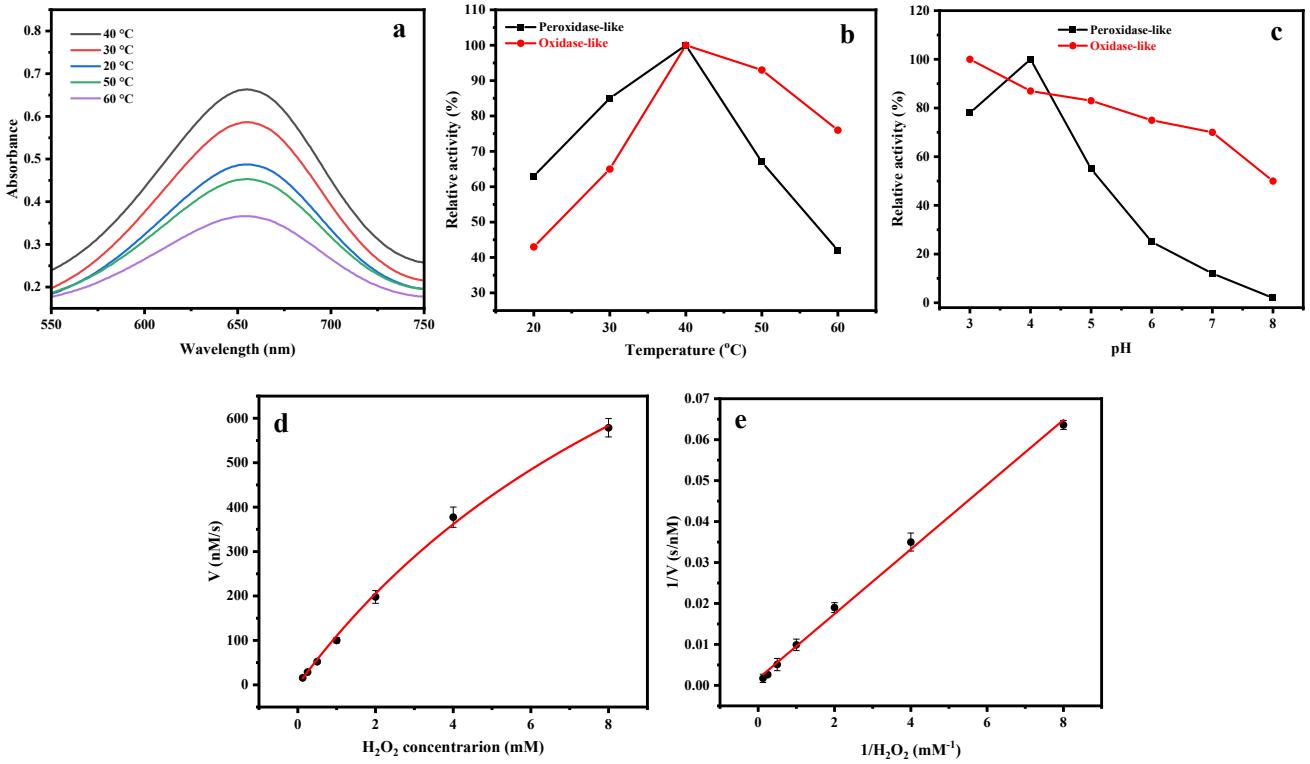

Figure S5. (a) UV-vis absorption spectra of TMB+Cu_2O-DMB+H_2O_2 solution under different temperatures; Effect of (b) temperature and (c) pH on the both peroxidase-like and oxidase-like catalytic performance; (d) Kinetic assay for the peroxidase-like activity of Cu_2O-DMB with H_2O_2 as substrate; (e) Corresponding double-reciprocal plots of peroxidase-like activity of Cu_2O-DMB at a fixed concentration of TMB (1 mM) versus varying concentration of H_2O_2 (0.125, 0.25, 0.5, 1, 2, 4, 8 mM), data presented as mean ± SD (n = 3)

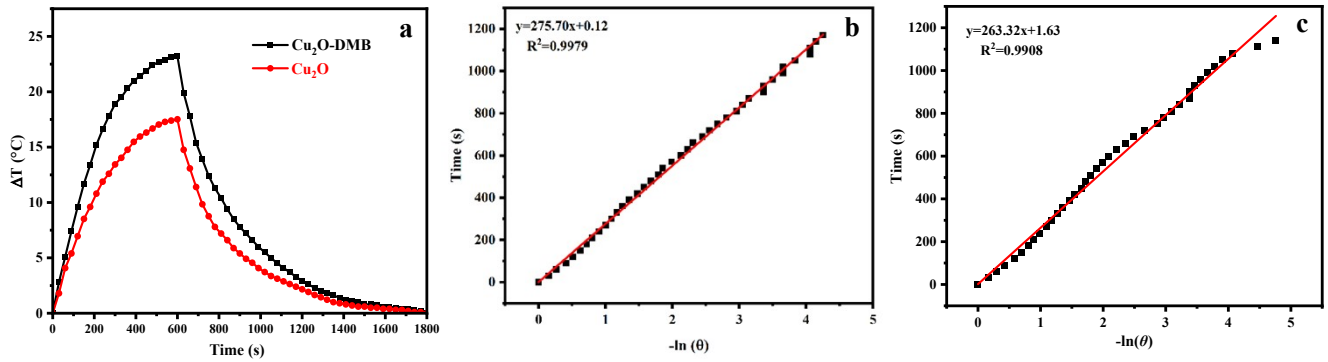

Figure S6. (a) The photothermal effect of Cu_2O and Cu_2O-DMB aqueous solution under 808 nm NIR laser irradiation for 600 s and then turned off the NIR laser for 1200 s; (b) The cooling time plot after 1200 s *vs* the negative natural logarithm of driving force temperature (-lnθ) with slope of (b) 275.70 for 40 μg mL^{-1} of Cu_2O-DMB and (c) 263.32 for 40 μg mL^{-1} of Cu_2O.

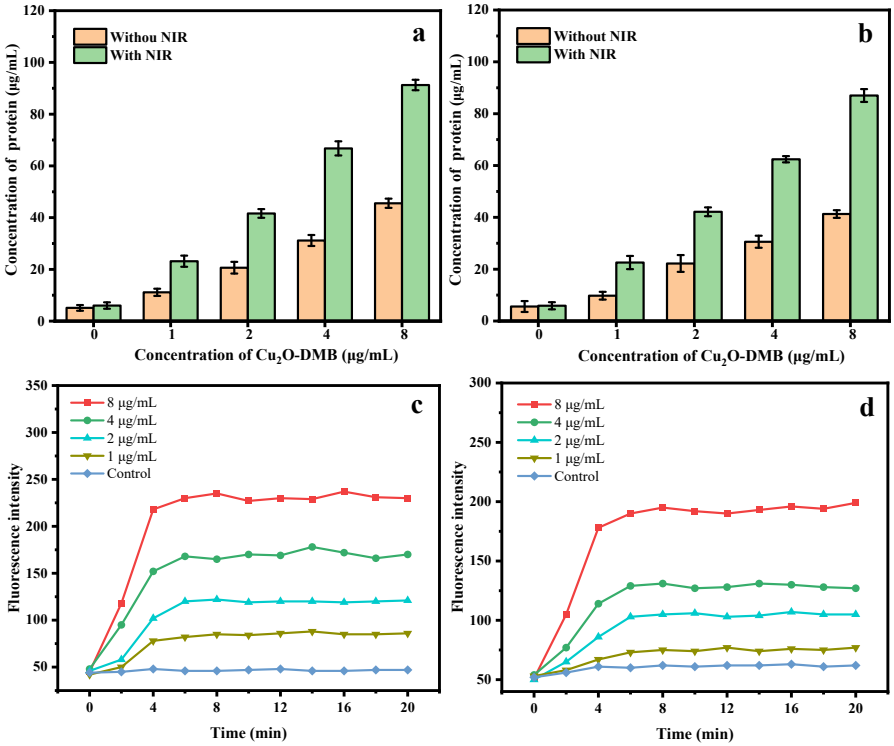

Figure S7. Protein leakages of (a) *MRSA* and (b) *AREC* treated with Cu_2O-DMB nanospheres (NIR, 808 nm, 1.0 W cm^{-2}, 5 min); Membrane depolarization of (c) *MRSA* and (d) *AREC* in the presence of Cu_2O-DMB+NIR.

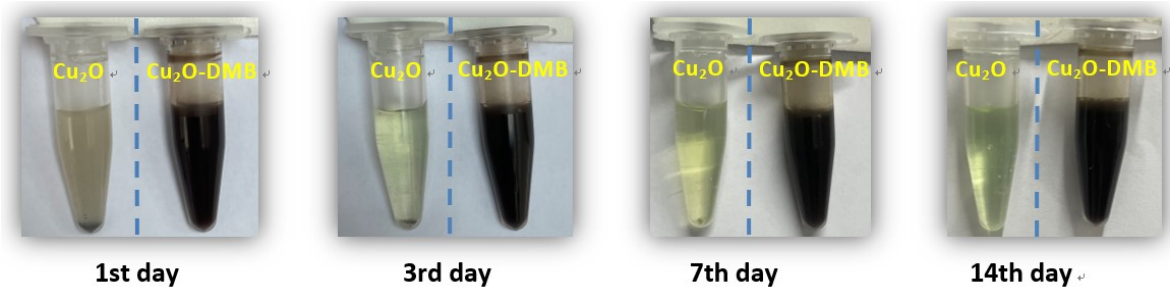

Figure S8. Photographs for indicating the stability of Cu_2O and Cu_2O-DMB solution (pH 4.5) after 1, 3, 7, and 14 days' storage.

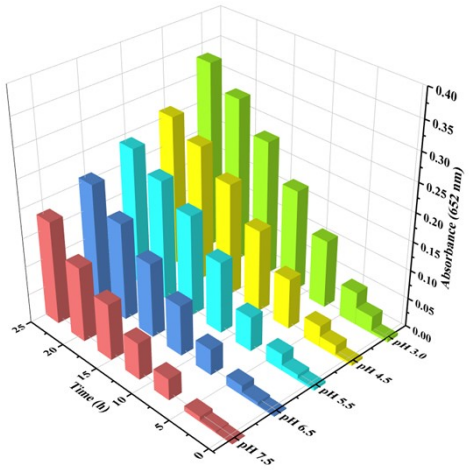

Figure S9. The absorbance at 652 nm of TMB (1 mM)+Cu_2O-DMB (100 μg/mL)+GSH (10 mM) solution with different pH values (3.0, 4.5, 5.5, 6.5, 7.5) for 2 h, 4 h, 8 h, 12 h, 16 h, 20 h and 24 h, respectively, the operating temperature is 40 °C.

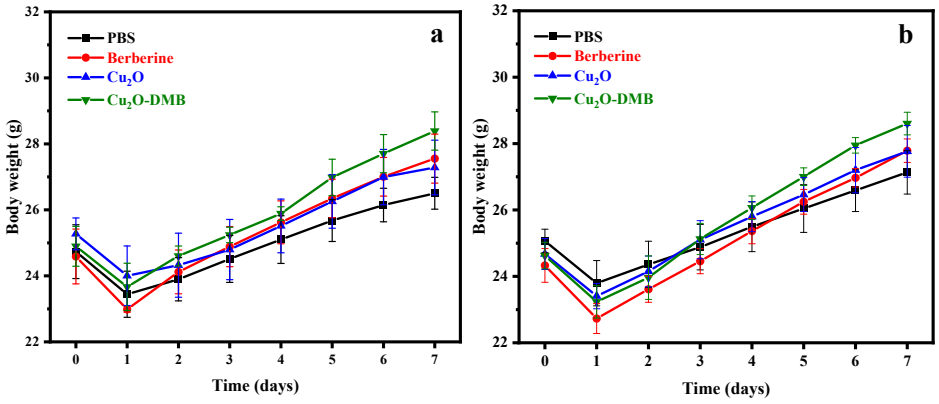

Figure S10. Body weight changes of mice after various treatments (a) without NIR and (b) with NIR (808 nm, 1.0 W cm^{-2}, 5 min). Data presented as mean ± SD (n = 3).