Electronic Supplementary Information (ESI)

Multifunctional Cu-Ag_2S nanoparticles with high photothermal conversion efficiency for photoacoustic imaging guided photothermal therapy *in vivo*

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1. Experimental

1.1 Materials and characterization

Copper (II) acetylacetonate (98%), oleylamine (C18 content 80−90%), and polyvinylpyrrolidone (PVP 58000) were purchased from Aladdin Industrial Corporation (Shanghai, China). Silver nitrate ($AgNO_3$, 99.8%), sulfur powder (S, 99.5%), chloroform (99.0%), ethanol (99.7%), dichloromethane (99.0%), ether (99.0%), and cyclohexane (99.0%) were purchased from Beijing Chemical Regents. All the reagents were analytical grade and used directly without further purification. Deionized water was used throughout the experiments.

Low-/high-resolution transmission electron microscopy **(**TEM) images were obtained with a FEI TECNAI G2 high-resolution transmission electron microscope operating with a field-emission gun operating at 200 kV. Powder X-ray diffraction (XRD) patterns were obtained on a D8 ADVANCE X-ray diffractometer with Cu Kα radiation (λ = 1.5418 Å) with an operation voltage and current maintained at 40 kV and 40 mA. Energy-dispersive X-ray (EDX) spectrometry was obtained on field-emission scanning electron microscope (FE - SEM, S-4800, Hitachi). X-ray photoelectron spectroscopy (XPS) was obtained on a VG ESCALAB MKII spectrometer. Inductively couple plasma mass spectrometry (ICP-MS) was carried out on an ELAN 9000/DRC. Inductively couple plasma-optical emission spectrometer (ICP-OES) was taken on a PerkinElmer ICP instrument. Fourier transform infrared spectroscopy (FT-IR) was recorded on a PerkinElmer 580B IR spectrophotometer using the KBr pellet technique. The absorption spectrum was recorded on a Shimadzu UV-3600 UV-VIS-NIR spectrophotometer.

1.2 Synthesis of $Cu_{2-x}S$-1 NPs

The as-prepared $Cu_{2-x}S$-1 NPs were synthesized by a modified literature procedure. Briefly, 2 mmol of sulfur was dissolved in 12 mL of oleylamine, and stirred at 70 °C for 0.5 h in an oil bath. Subsequently, this solution was added dropwise into 20 mL chloroform solution containing 5 mL oleylamine and 2 mmol Cu $(acac)_2$. After the mixture was further stirred for another 0.5 h. and the $Cu_{2-x}S$-1 NPs were isolated by

centrifugation, washed two times with ethanol and chloroform, and re-dispersed in cyclohexane (40 mL) for later use.

1.3 Synthesis of Ag_2S product

4 mL chloroform solution containing 1 mL oleylamine and 0.5 mmol $AgNO_3$ was added dropwise into 3 mL oleylamine and 10 mL cyclohexane solution of the $Cu_{2-x}S$-1 NPs and stirred at 70 °C for 0.5 h. The Ag_2S NPs were obtained by centrifugation, washed with ethanol and cyclohexane, and re-dispersed in cyclohexane (10 mL) for later use.

1.4 Synthesis of Cu-Ag_2S NPs

1 mmol of sulfur was dissolved in 3 mL of oleylamine, and rapidly injected into a 10 mL cyclohexane solution of the as-synthesized $Cu_{2-x}S$-1 NPs at 70 °C and stirred at this temperature for 10 min in an oil bath. Then 4 mL chloroform solution containing 1 mL oleylamine and 0.5 mmol $AgNO_3$ was added dropwise into the above solution and stirred at 70 °C for 0.5 h. The Cu-Ag_2S NPs were obtained by centrifugation, washed with ethanol and cyclohexane, and re-dispersed in cyclohexane (10 mL) for later use.

1.5 Surface modification of Cu-Ag_2S NPs

150 mg of Cu-Ag_2S NPs were first dispersed in 100 mL of dichloromethane. Then, 1 g of PVP was added and the mixture was stirred at room temperature for 10 min. Cu-Ag_2S/PVP NPs were precipitated by adding ether and obtained by centrifugation, and the re-dispersed in PBS solution. The resulting solution was centrifugation at a high speed and then the obtained solution was transferred into dialysis bags and dialyzed against PBS solution for 72 h. Finally, the Cu-Ag_2S/PVP NPs were dispersed in PBS solution and kept at 4 °C for later use.

1.6 Photothermal experiments in PBS solution

1.0 mL of PBS solution containing Cu-Ag_2S/PVP NPs with various Cu concentrations (0, 6.25, 12.5, 25, 50, and 100 ppm) were placed in a quartz cuvette and then irradiated by an 808 nm laser (1.58 W cm^{-2}, 10 min). The solution temperature was measured every 2 s by a thermocouple microprobe.

1.7 *In vitro* cytotoxicity assessment

In vitro cytotoxicity of Cu-Ag_2S/PVP NPs was evaluated by MTT assay of 4T1 murine breast tumor cells. Cells were seeded into 96-well cell culture plates (10^4 per well) at 37 °C and 5% CO_2 for 24 h. After 24 h of incubation, the DMEM was taken out from the wells, and the cells were washed for three times with PBS solution. The PBS solution of Cu-Ag_2S/PVP NPs with various Cu concentrations (0, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 and 200 ppm) were added into the medium. Then, the cells were incubated at 37 °C and 5% CO_2 for 24 h. Thereafter, MTT (10 μL, 5 mg mL^{-1}) solutions was added into each well and the plate was incubated for 4 h at 37 °C and 5% CO_2 in order to form a purple formazan dye, which was dissolved in DMSO for 15 min after removing the medium. Finally, the enzyme-linked immunosorbent assay reader was used to measure the optical absorbance of the colored solution at 570 nm. Each experiment was repeated three.

1.8 *In vitro* cellular uptake assay

4T1 murine breast tumor cells were seeded in 1 mL of medium in each well (3.5 cm in diameter) of a 6-well plate at the density of 10^5 cells per well cells. After 24 h of incubation at 37 °C, the medium was replaced with fresh medium containing Cu-Ag_2S/PVP NPs with Cu concentration of 25, 50 and 100 ppm. After another 24 h of incubation at 37 °C, the medium was removed; the cells were washed three times with PBS (pH = 7.4) and trypsinized. Then, the cell suspensions were digested using 1 mL of concentrated $HNO_3$10% (*v/v*) and the intracellular Cu concentration used for ICP-MS measurement.

1.9 *In vitro* photothermal ablation of cancer cells

4T1 murine breast tumor cells were seeded into 96-well cell culture plates (10^4 per well) at 37 °C and 5% CO_2 for 24 h. Then the cells were treated with PBS solution containing Cu-Ag_2S/PVP NPs with various Cu concentrations (0, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 and 200 ppm). Then, the cells were irradiated by an 808-nm laser with a power density of 1.58 W cm^{-2} for 10 min. The cell viabilities were measured by MTT assays.

1.10 Live/dead staining kit

4T1 murine breast tumor cells (4×10^4 cells per well) were placed in a 24-well plate. After overnight incubation, culture medium was removed and cells were washed three times with PBS. Then cells were treated with PBS solution containing only or Cu-Ag_2S/PVP NPs with various Cu concentrations (0, 3.12, 6.25, 12.5, 25, 50, 100 and 200 ppm) incubated for 6 h. Then they were irradiated under 808 nm laser irradiation (1.58 W cm^{-2}) for 10 min. After 18 h incubation, 4T1 murine breast tumor cells were washed with PBS and stained with calcein AM (2 μM) and propidium iodide (PI, 4 μM) for 2 hours and observed using NikonTi-S fluorescence microscope (Tokyo, Japan), live cells showed green color and dead ones exhibited red color.

1.11 Animal Experiments

Female Kunming mice were obtained from the Laboratory Animal Center of Jilin University (China). All *in vivo* experiments were conducted in strict adherence the criteria of the Regional Ethics Committee for Animal Experiments. The tumor models were established by subcutaneous injection of H22 cells in the left axilla of each Kunming mouse.

1.12 *In vitro* and *in vivo* PA imaging

1.0 mL of PBS solution containing Cu-Ag_2S/PVP NPs with various Cu concentrations (12.5, 25, 50, and 100 ppm) embedded in agar gel cylinders with a diameter of 1 cm were performed quantitatively to investigate the PA signal. The excitation wavelength of 808 nm was adopted. The tumor-bearing mice were used for the *in vivo* PA imaging. 100 μL of PBS solution containing Cu-Ag_2S/PVP NPs with Cu concentration of 200 ppm were injected intravenously into the tumor-bearing mice, the PA images were performed at different time intervals (pre, 2 h, 6 h and 24 h).

1.13 *In vivo* photothermal effect

100 μL of PBS solution containing Cu-Ag_2S/PVP NPs with Cu concentration of 200 ppm were intratumoral injected into the tumor-bearing mice. Then, the tumor was exposed to an 808 nm laser (1.58 W cm^{-2}) for 10 min and imaged by an IR thermal imaging camera at different time intervals (pre, 1, 3, 5, and 10 min). The tumor-bearing mice were also intravenous injected with PBS solution containing Cu-Ag_2S/PVP NPs at the same dose (100 μL, 200 ppm). Then, the tumor was also

exposed to an 808 nm laser (1.58 W cm^{-2}) for 10 min and imaged by an IR thermal imaging camera at different time intervals (pre, 0.5 h, 2 h, 6 h, and 24 h).

1.14 *In vivo* biodistribution of Cu-Ag_2S/PVP NPs

100 μL of PBS solution containing Cu-Ag_2S/PVP NPs with Cu concentration of 200 ppm were intravenous injected into the tumor-bearing mice**.** At 0 h, 2 h, 6 h, 1d, 3d and 7d after administration, the tumor-bearing mice were euthanized for analysis of the systemic distribution of Cu-Ag_2S/PVP NPs (n = 3 at each time point). After digesting the major organs (heart, liver, spleen, lung, kidney, and tumor) and feces with aqua regia solution for 6 h . The Ag and Cu amount per unit mass was quantified by ICP-MS.

1.15 *In vivo* photothermal therapy and histology examinations

The tumor-bearing mice (the tumors volume were about 200 mm^3) were randomly allocated into four treatment groups (three animals per treatment group): 100 μL of PBS solution containing Cu-Ag_2S/PVP NPs with Cu concentration of 200 ppm were intravenous injected into the tumor-bearing mice and treated with 808 nm irradiation (1.58 W cm^{-2}, 10 min) after 24 h as group I (Cu-Ag_2S/PVP + NIR); 100 μL of PBS solution were intravenous injected into the tumor-bearing mice and treated with 808 nm irradiation (1.58 W cm^{-2}, 10 min) after 24 h as group II (NIR); 100 μL of PBS solution containing Cu-Ag_2S/PVP NPs with Cu concentration of 200 ppm were intravenous injected into the tumor-bearing mice as group III (Cu-Ag_2S/PVP); 100 μL of PBS solution were intravenous injected into the tumor-bearing mice as group IV (PBS). The tumor sizes were measured by a caliper every day after photothermal treatment and calculated according to the formulation $V = Dd^2/2$ (D and d represent length and width of the tumor, respectively). Relative tumor volumes were calculated as V/V_0 , where V_0 was the tumor volume when the treatment was initiated. We also collected the body weight data of the mice on a daily basis for 14 d. The tumor-bearing mice were killed 14 day after the laser treatment. Then, the organs (heart, liver, spleen, lung, and kidney) obtained from group I, group IV and healthy mice and the tumors obtained from group I and group IV were stained with H&E for histological analysis.

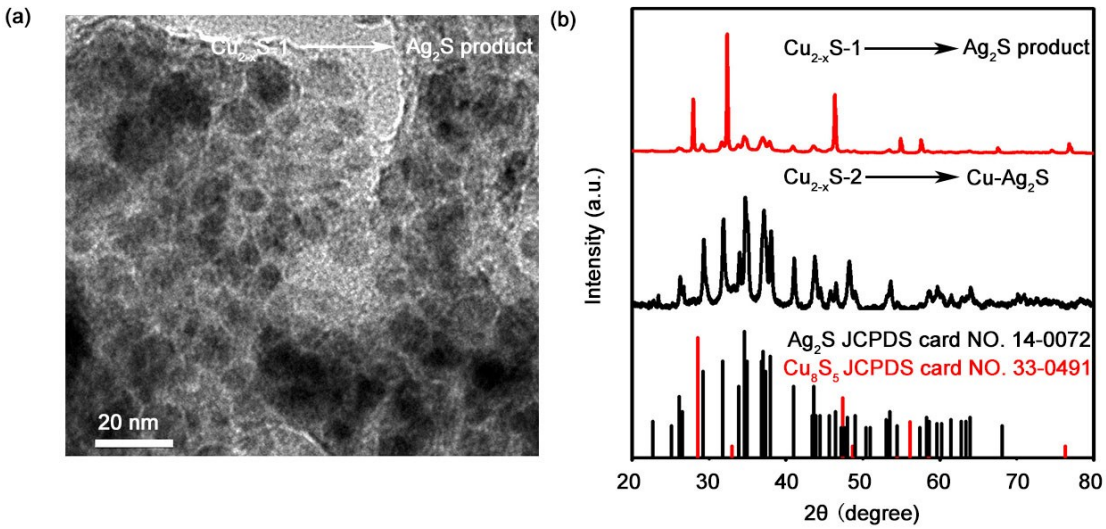

Fig. S1 (a) TEM images of as-synthesized Ag_2S product using $Cu_{2-x}S$-1 as template; (b) the corresponding XRD patterns of as-synthesized Ag_2S product and Cu-Ag_2S NPs.

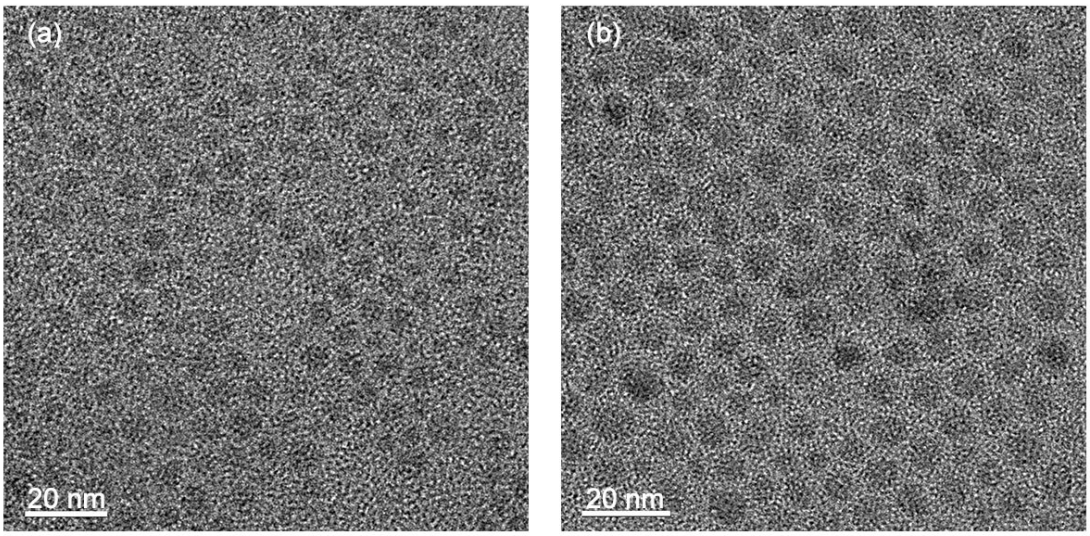

Fig. S2 TEM images of $Cu_{2-x}S$-1 NPs (a) and $Cu_{2-x}S$-2 NPs (b).

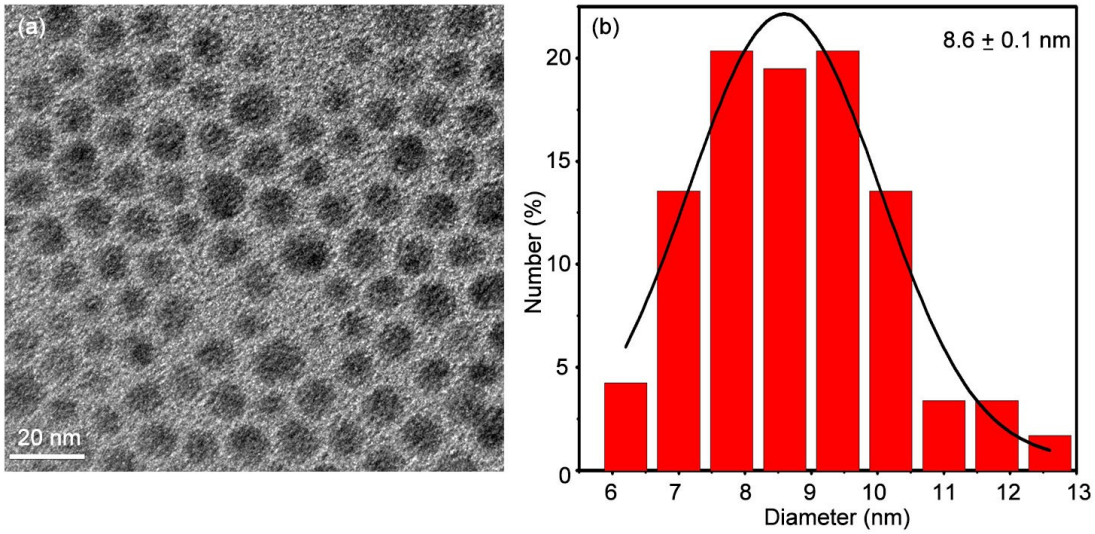

Fig. S3 TEM images of Cu-Ag_2S NPs (a) and the corresponding size distribution histograms (b).

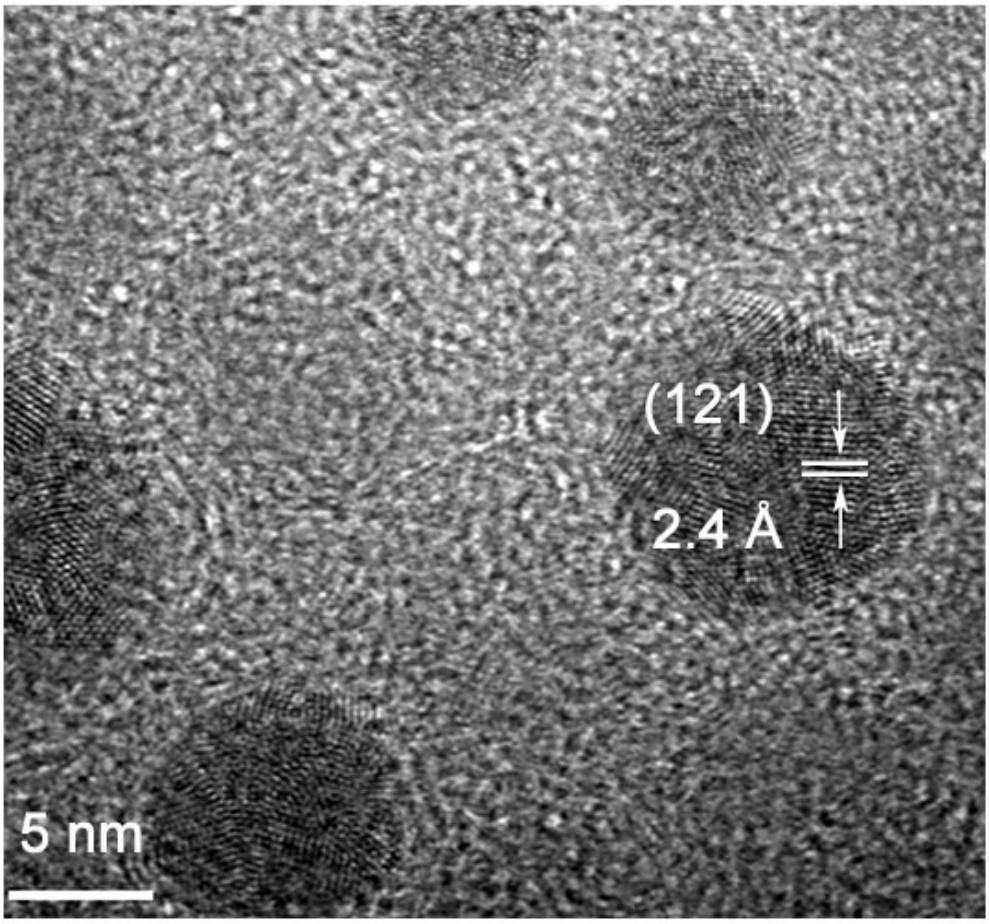

Fig. S4 High-resolution transmission electron microscopy (HRTEM) image of Cu-Ag_2S NPs.

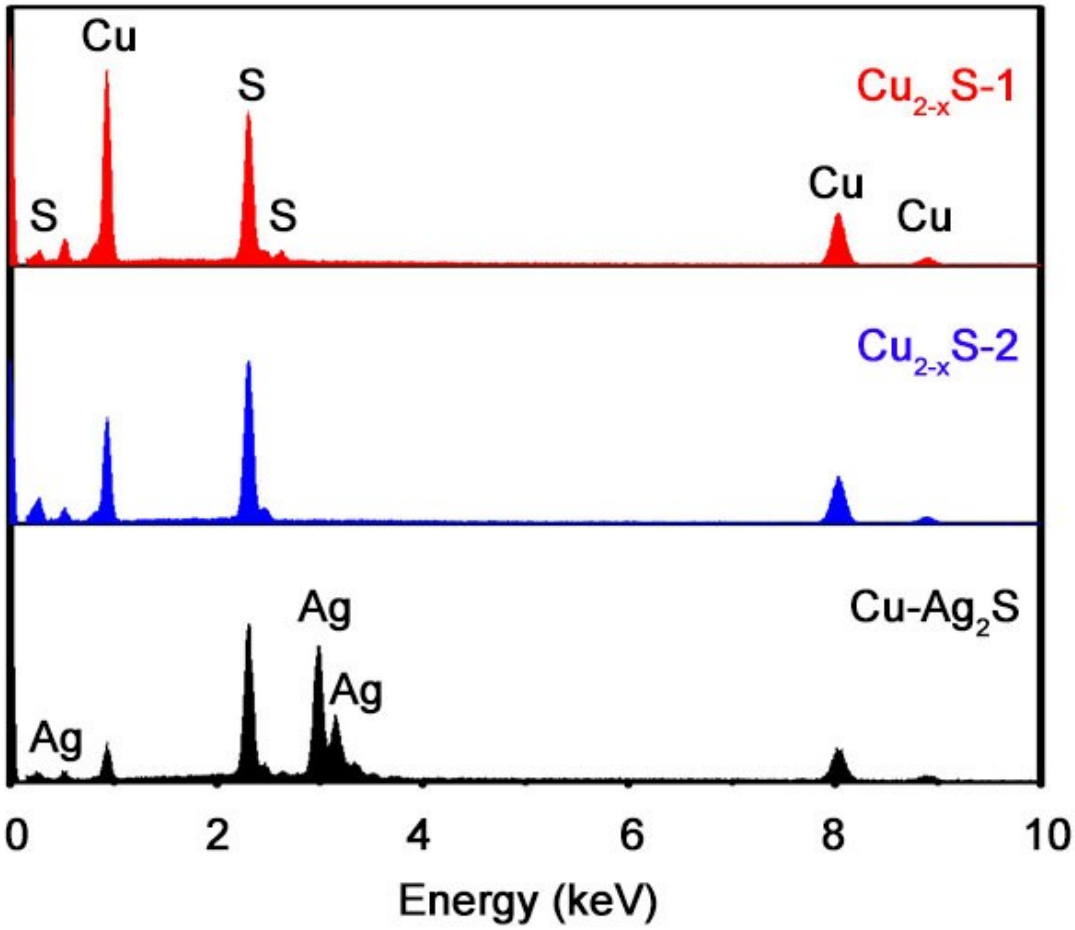

Fig. S5 EDX spectra of $Cu_{2-x}S$-1 NPs, $Cu_{2-x}S$-2 NPs, and Cu-Ag_2S NPs.

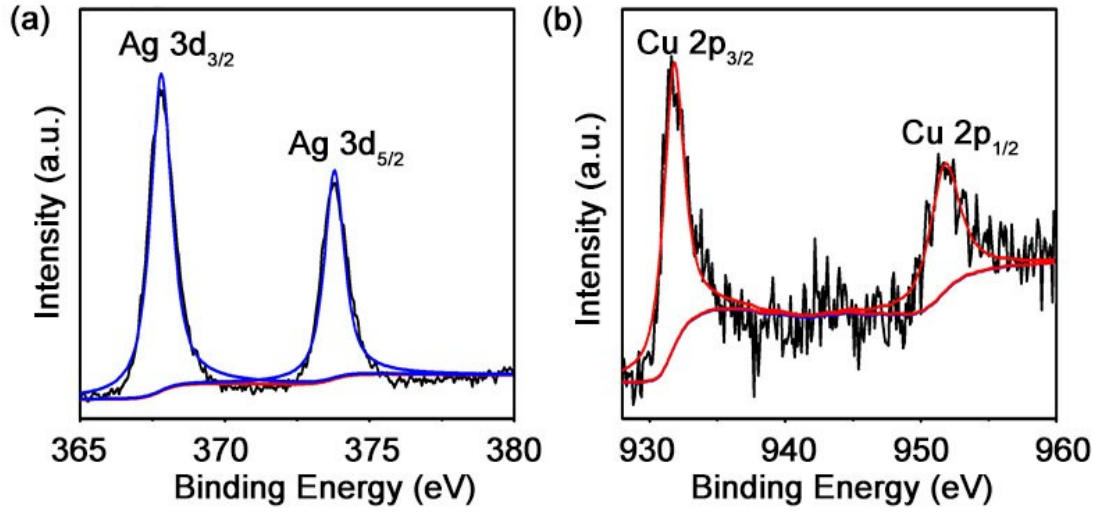

Fig. S6 XPS spectra of Ag 3d (a) and Cu 2p (b) in Cu-Ag_2S NPs.

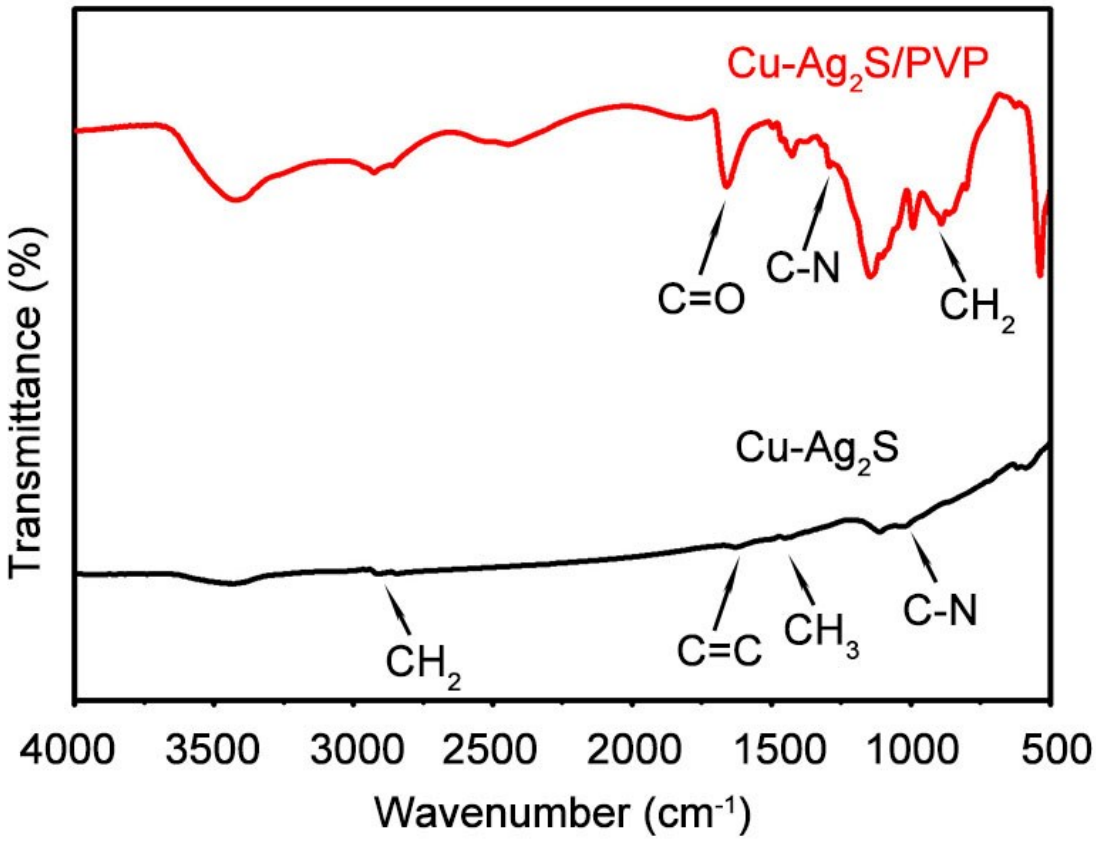

Fig. S7 FTIR spectra of Cu-Ag_2S and Cu-Ag_2S/PVP NPs.

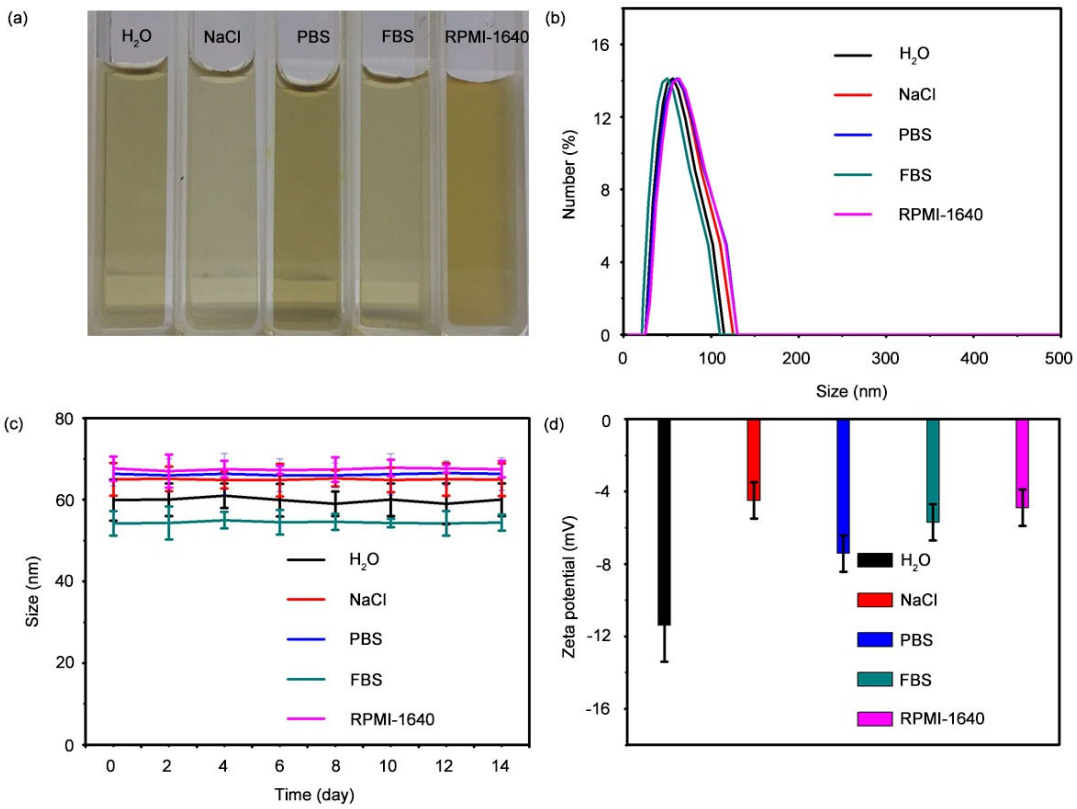

Fig. S8 (a) The photographs of Cu-Ag_2S/PVP NPs dispersed in water, 0.9% NaCl solution, PBS solution, FBS solution and RPMI-1640; (d) Hydrodynamic sizes of Cu-Ag_2S/PVP NPs dispersed in water, 0.9% NaCl solution, PBS solution, FBS solution and RPMI-1640; (c) Hydrodynamic size changes of Cu-Ag_2S/PVP NPs dispersed in water, 0.9% NaCl solution, PBS solution, FBS solution and RPMI-1640 for 15 d; (e) Zeta potentials of Cu-Ag_2S/PVP NPs dispersed in water, in water, 0.9% NaCl solution, PBS solution, FBS solution and RPMI-1640.

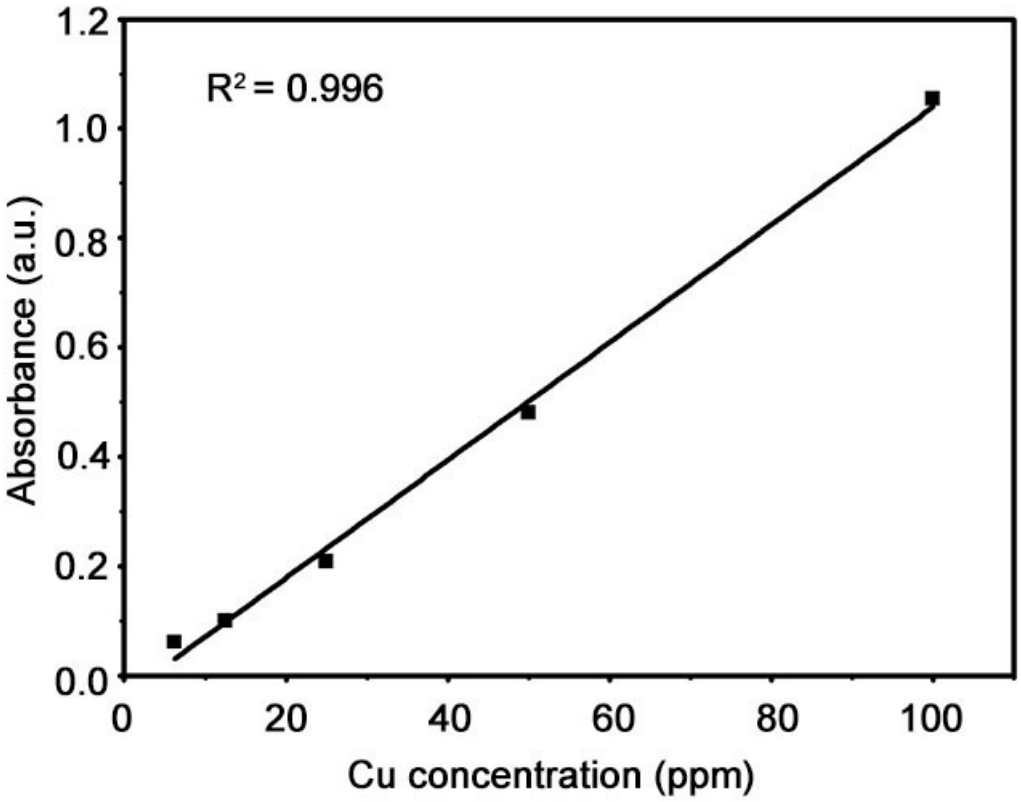

Fig. S9 Linear fitting plot of NIR absorbance at 808 nm versus Cu concentrations of Cu-Ag_2S/PVP NPs PBS solution.

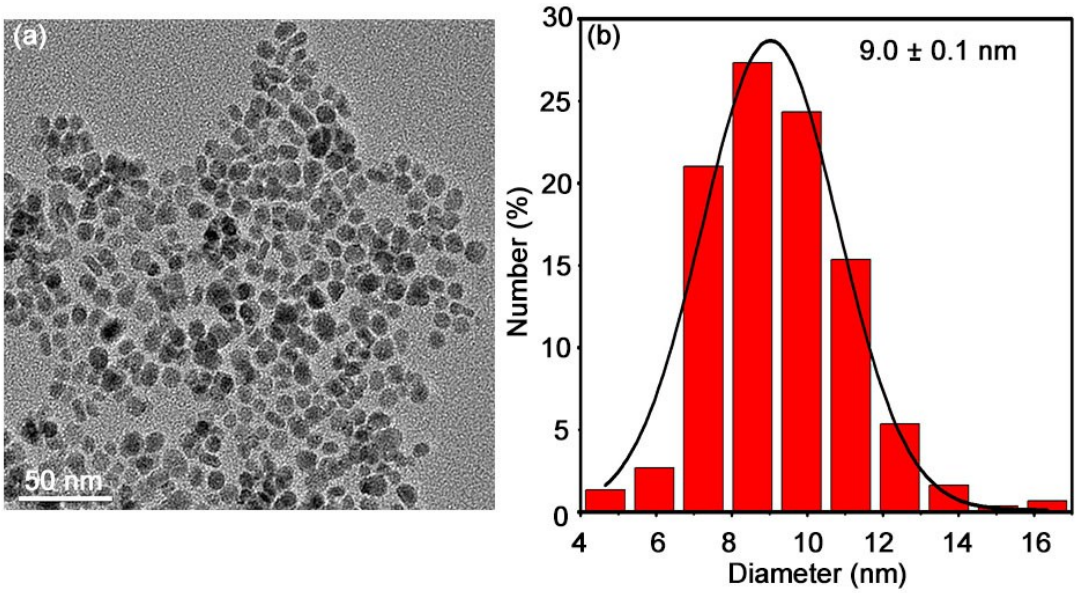

Fig. S10 TEM images of Cu-Ag_2S/PVP NPs (a) and the corresponding size distribution histograms (b).

SI-1 Calculation for molar extinction coefficient:

The molar extinction coefficient (ε) of Cu-Ag_2S/PVP NPs at 808 nm cloud be calcuted by the following equations:

$$\varepsilon = \left(A \frac{\pi}{6} d^3 \rho N_A \right) / (LC_{wt})$$

where A, d, ρ, N_A, L and C_{wt} are the absorbance at 808 nm, the average diameter of nanospheres, the density of nanospheres, Avogadro's constant, the pathlength through the sample and the weight concentratio1n of the nanospheres, respectively. The absorbance value is 1.055 at 808 nm when Cu-Ag_2S/PVP NPs with Cu concentration of 100 ppm (Figure S7). The average diameter of nanospheres is 9.0 nm (Figure S8). The density ρ for bulk Ag_2S is about 7.23 g cm^{-3}. The density of Cu-Ag_2S/PVP NPs was approximately replaced by the bulk one. L is equal to 1 cm. Therefore the molar extinction coefficient is about 1.8×10^7 M^{-1} cm^{-1}. In comparison with Cu-Ag_2S/PVP NPs, the molar extinction coefficient of $Cu_{2-x}S$/PVP NPs in this work is only about 2.6×10^6 M^{-1} cm^{-1}.

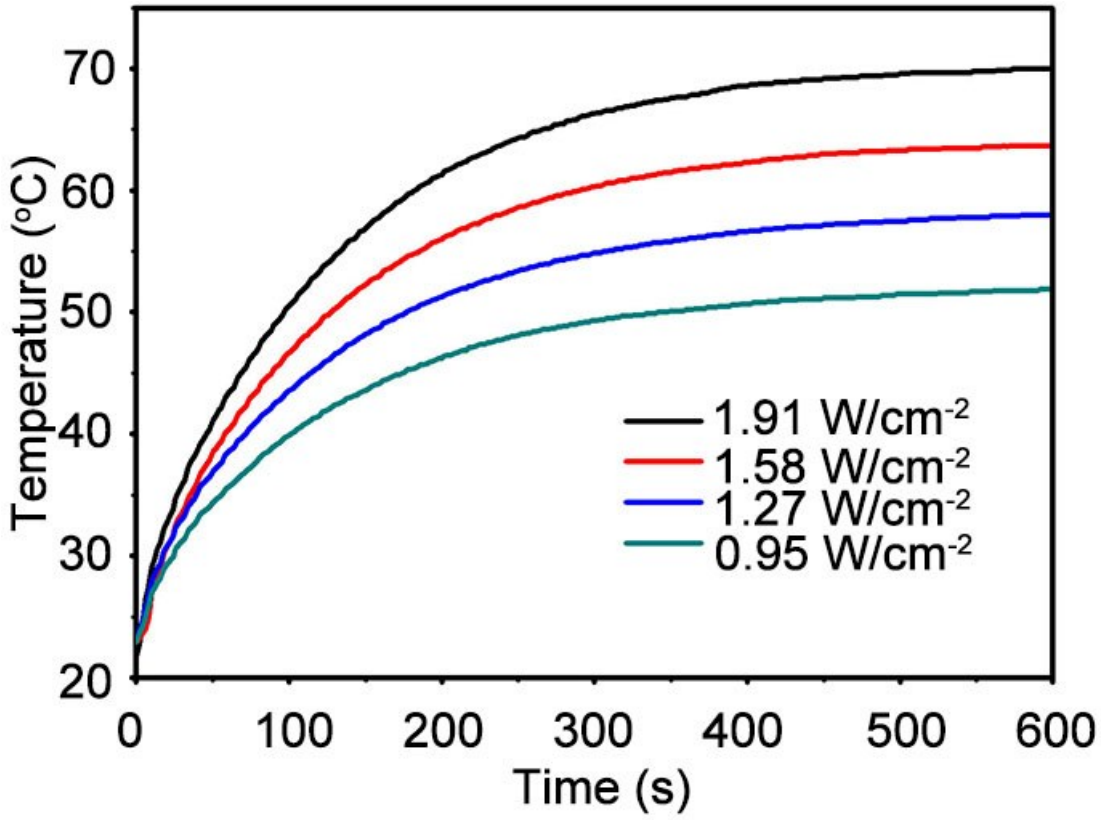

Fig. S11 Temperature elevation profiles of PBS solution containing Cu-Ag_2S/PVP NPs (Cu concentration: 100 ppm) upon 808 nm laser irradiation with different power density.

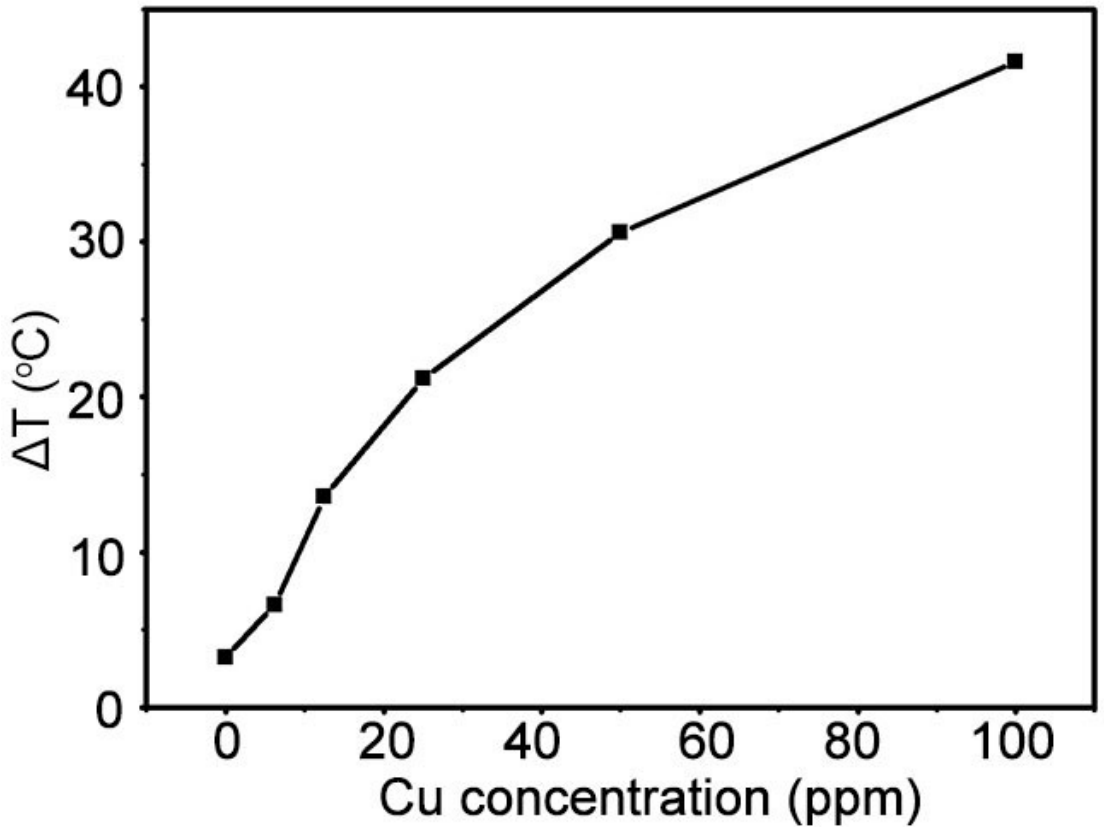

Fig. S12 The temperature increase (ΔT) of PBS solution containing Cu-Ag_2S/PVP NPs with various Cu concentrations upon 808 nm laser irradiation (1.58 W cm^{-2}, 10 min).

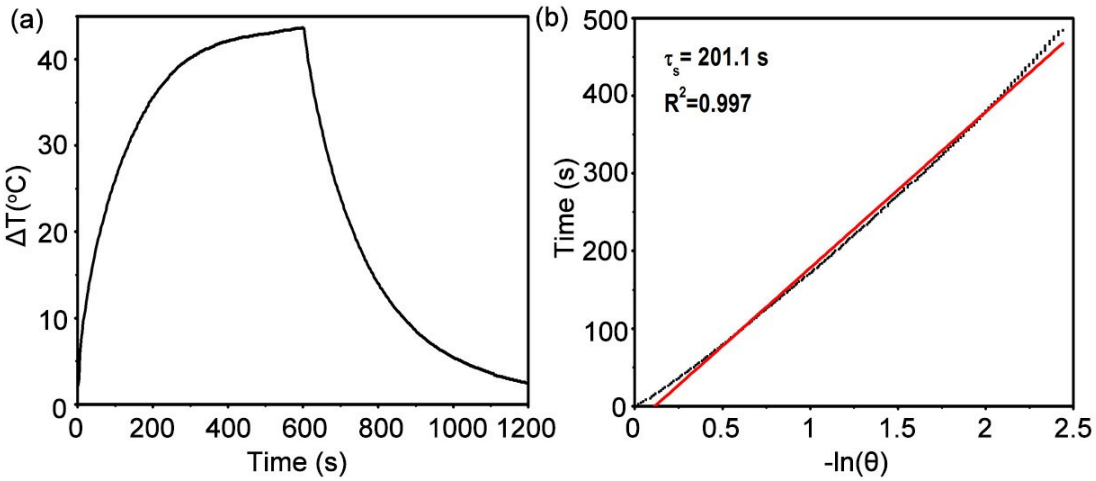

Fig. S13 (a) Temperature elevation profiles of PBS solution containing Cu-Ag_2S/PVP NPs (Cu concentration: 100 ppm) upon 808 nm laser irradiation (1.58 W cm^{-2}, 10 min) and then the laser was shut off. (b) Plot of the cooling time versus –ln(θ) obtained from the cooling stage as shown in (a).

SI-2 Calculation for photothermal conversion efficiency:

The photothermal conversion efficiency (η) colud be calcuted by the following equations:

$$\eta = \frac{hS(T_{\max} - T_{surr}) - Q_{dis}}{I(1-10^{-A_{808\,nm}})} (1)$$

where h, S, T_{max}, T_{surr}, Q_{dis}, I, and A are heat transfer coefficient, irradiated area, the equilibrium temperature, the ambient temperature of the surroundings, the heat dissipation from the light absorbed by the quartzsample cell, the laser power density, and absorption of Cu-Ag_2S/PVP NPs at 808 nm, respectively. The value of hS is calculated by using the following equation (2) to (4):

$$hS = \frac{\sum m_i C_{p,i}}{\tau_S} \quad (2)$$

$$t = -\tau_s \ln\theta \quad (3)$$

$$\theta = \frac{T - T_{surr}}{T_{\max} - T_{surr}} \quad (4)$$

where m, C_p, t, τ_s are the mass of sample, the thermal capacity of sample, cooling time after irradiation, and the sample system time constant, respectively. The Q_{dis} was measured independently using a quartz cuvette cell containing PBS without Cu-Ag_2S/PVP NPs. The value of Q_{dis} is calculated by using the following equation:

$Q_{dis}=hS(T_{max,PBS,}-T_{surr,PBS})$

The T_{max}, T_{surr}, and τ_s was obtained from Figure S11 to be 64.8 ^oC, 21.2 ^oC, and 201.1s, The value of m and C_p are 1g and 4.2 J/(g•^oC). Therefore, hS is calculated to be 20.89 mW/^oC. The value of Tmax-Tsurr equals 43.6 ^oC, Q_{dis} and A_{808nm} are measured independently as 66.83 mW with power density (I) 1.58 W/cm^{-2} and 1.055, respectively. Substituting all of value to parameters into the equation (1), photothermal conversion efficiency (η) of Cu-Ag_2S/PVP NPs can be calculated to be 58.2%. In comparison with Cu-Ag_2S/PVP NPs, the photothermal conversion efficiency of $Cu_{2-x}S$/PVP NPs is about 27.1%.

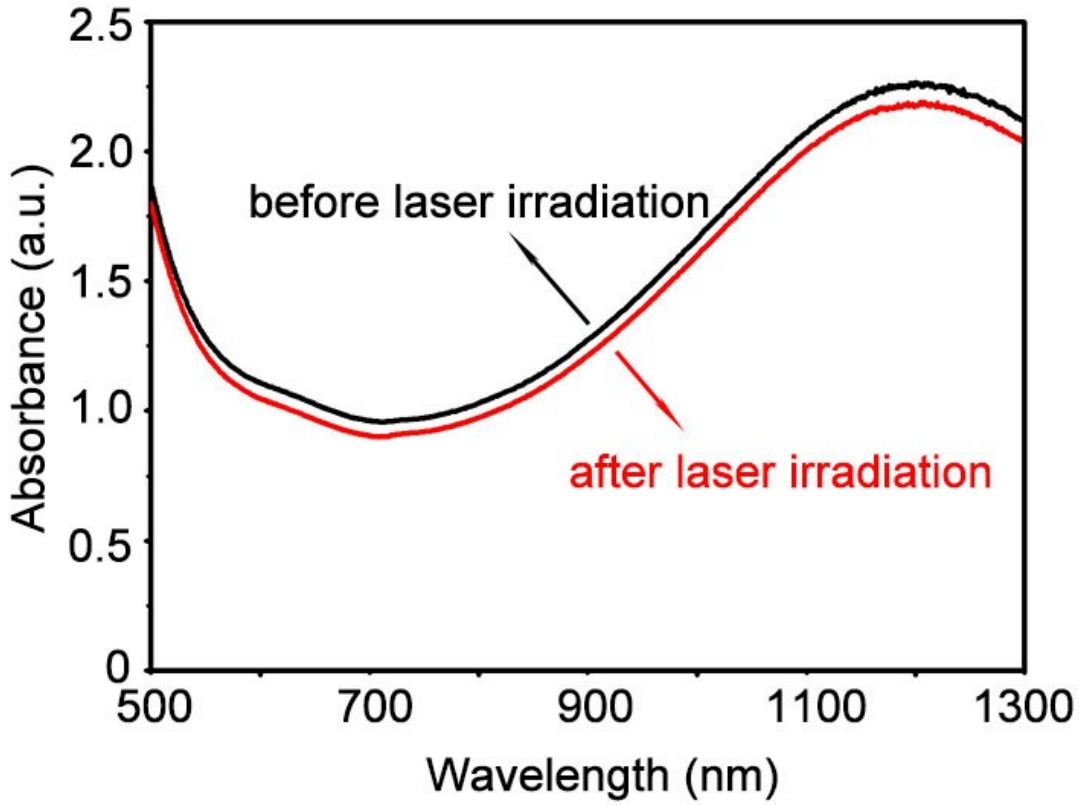

Fig. S14 The UV–vis–NIR absorption spectrum of PBS solution containing Cu-Ag_2S/PVP NPs (Cu concentration: 100 ppm) before and after 808 nm laser irradiation (1.58 W cm^{-2}, 60 min).

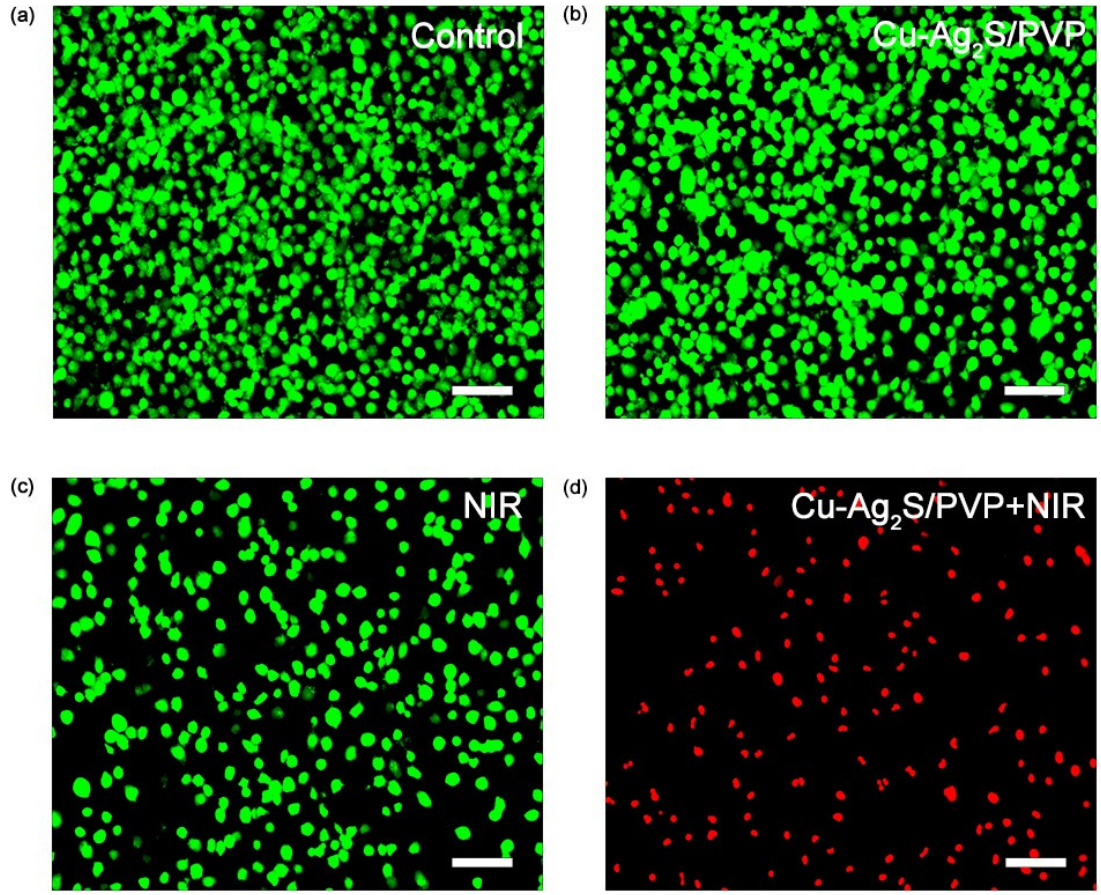

Fig. S15 Live−dead staining images of 4T1 murine breast tumor cells incubated with or without PBS solution containing Cu-Ag_2S/PVP NPs (Cu concentration: 200 ppm) upon 808 nm laser irradiation (1.58 W cm^{-2}, 10 min). All the scale bars are 200 μm.

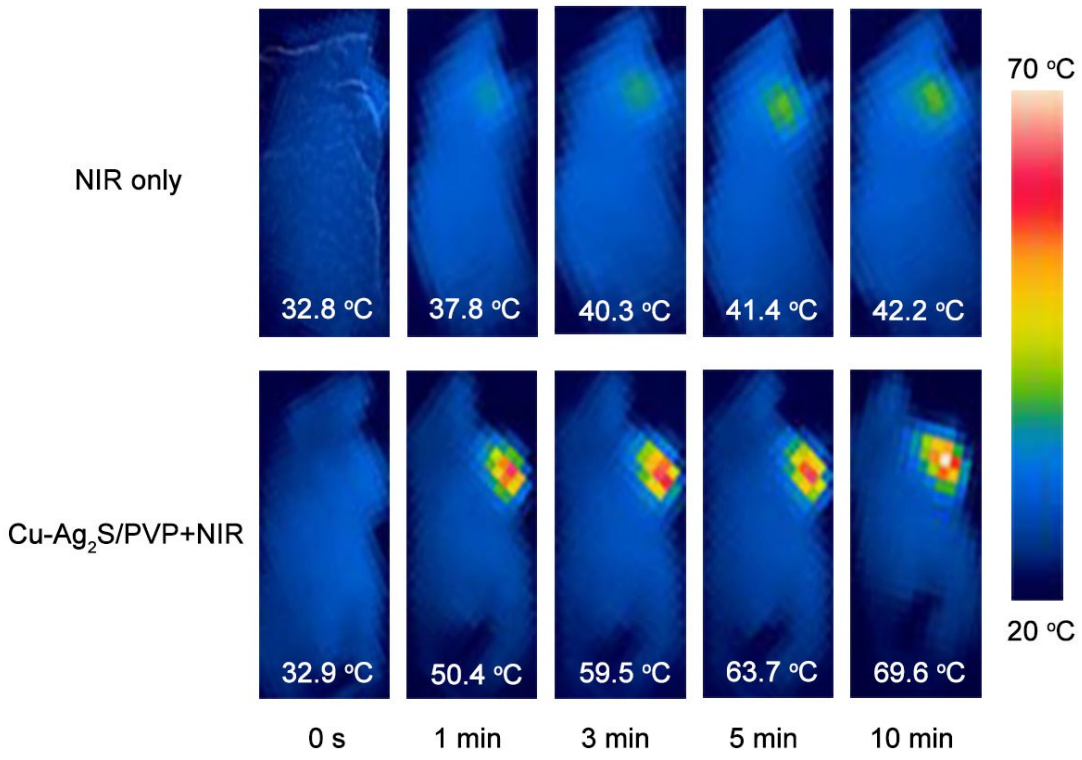

Fig. S16 Photothermal images of tumor-bearing mice recorded during the laser irradiation after intratumoral injection of PBS or PBS solution containing Cu-Ag_2S/PVP NPs (100 μL, 200 ppm).

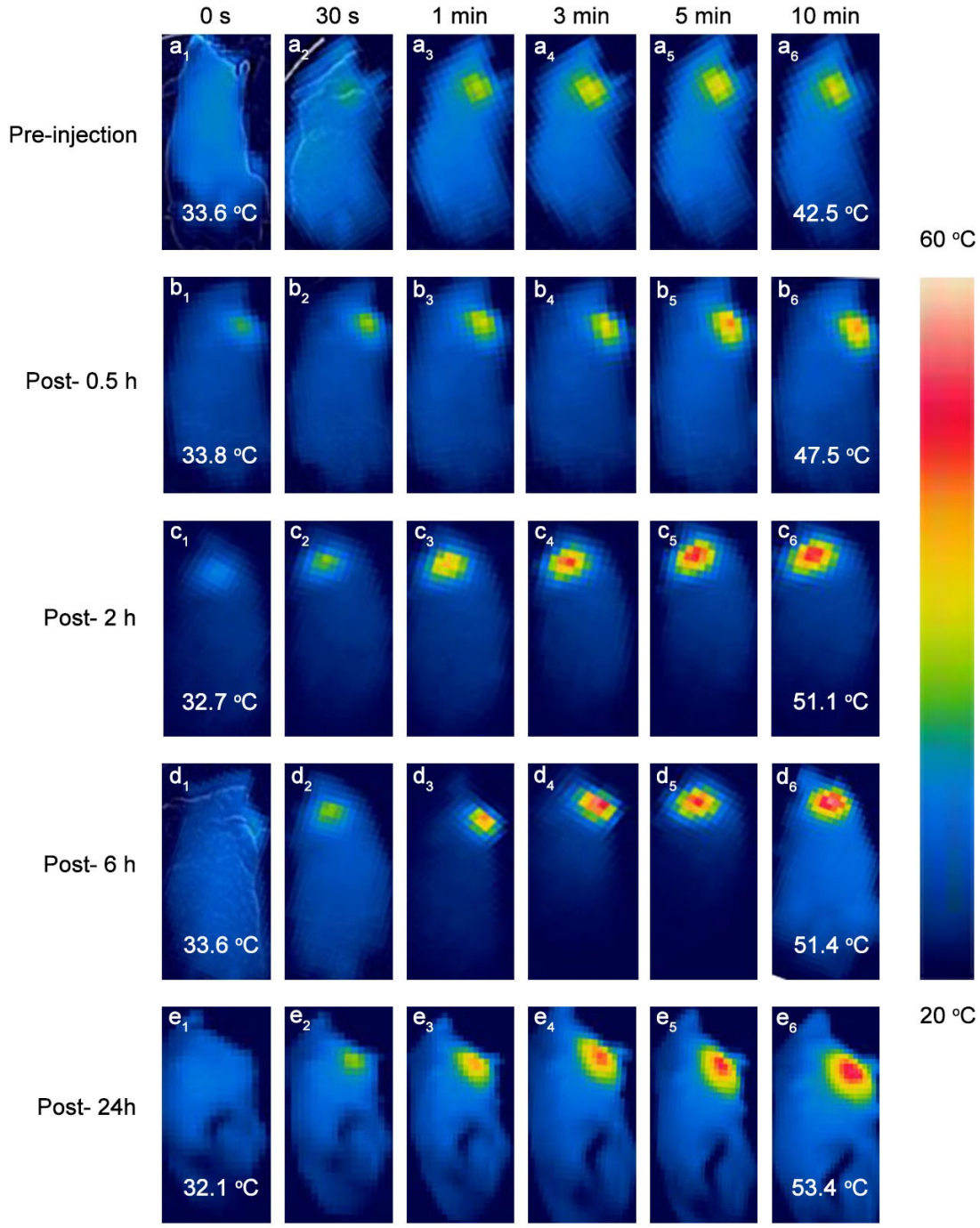

Fig. S17 Photothermal images of tumor-bearing mice recorded during the laser irradiation after intravenous injection of PBS solution containing Cu-Ag_2S/PVP NPs (100 μL, 200 ppm) taken at different time intervals.

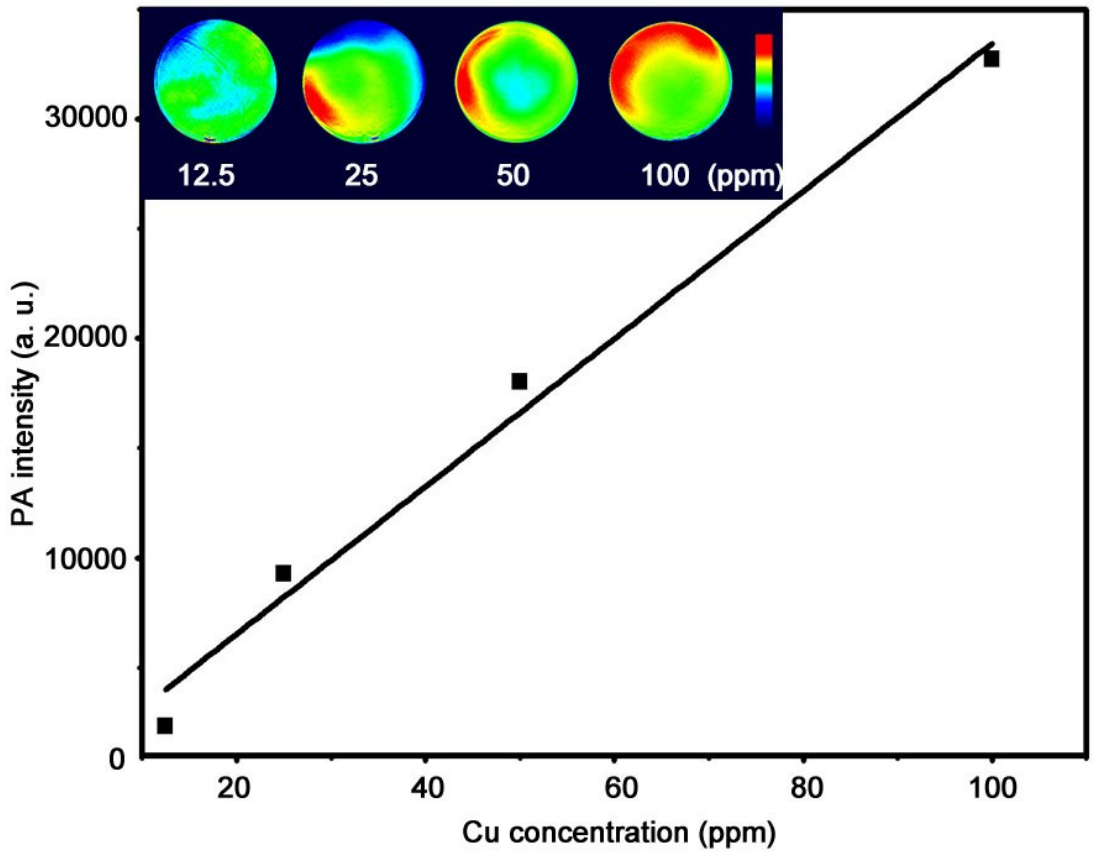

Fig. S18 Linear fitting plots of PA amplitudes versus Cu concentrations of Cu-Ag_2S/PVP NPs PBS solution under 808 nm laser irradiation. The inset is the PA image of agar gel cylinder at versus Cu concentrations of Cu-Ag_2S/PVP NPs PBS solution.

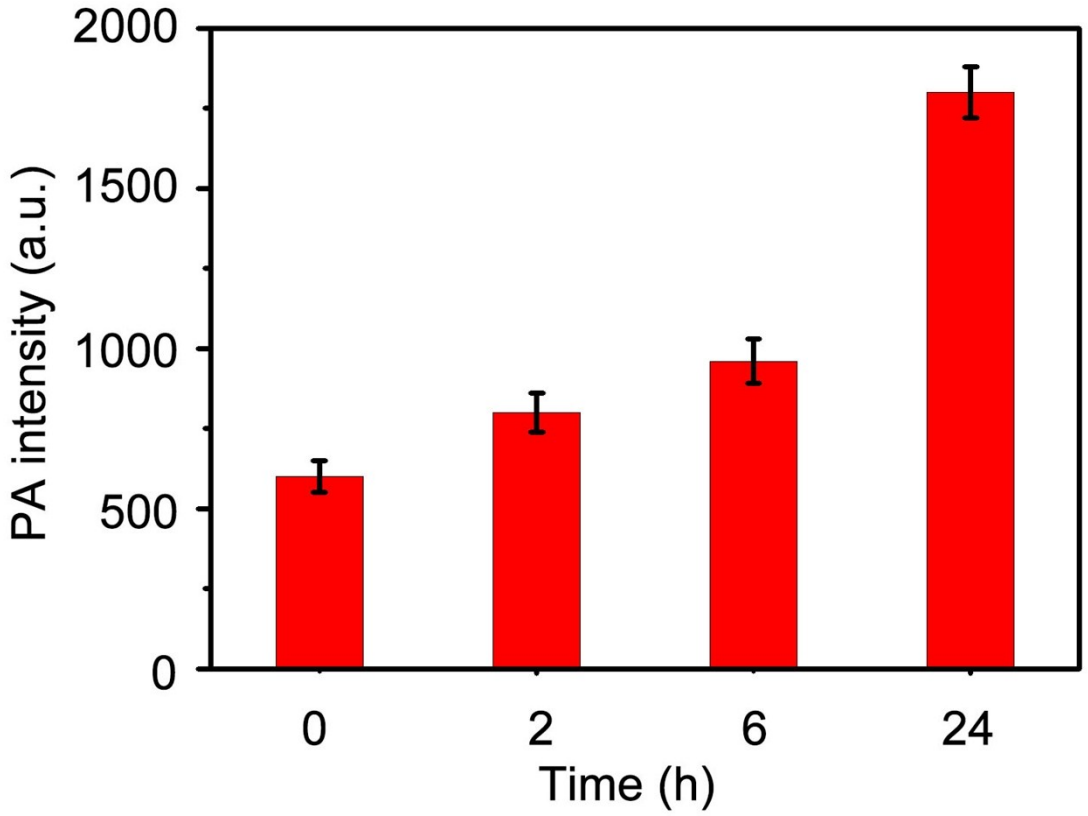

Fig. S19 The quantitative PA intensity of the tumor corresponding to the given time points (0, 2, 6, and 24 h).

Table S1. The content of Cu and Ag in feces or urine at different time points (2 h, 6 h, 1d, 3 d, and 7 d) after intravenous injection of PBS solution containing Cu-Ag_2S/PVP NPs.

Post-injection	0 h	2 h	6 h	1 d	3 d	7 d
Content of Cu in Feces (μg/g)	5.2	9.93	13.13	20.76	17.84	9.91
Content of Cu in Urine (μg/mL)	0.53	0.63	0.92	2.26	1.36	0.44
Content of Ag in Feces (μg/g)	0.08	0.08	0.09	0.53	0.35	0.11
Content of Ag in Urine (μg/mL)	0.01	0.02	0.02	0.09	0.04	0.02

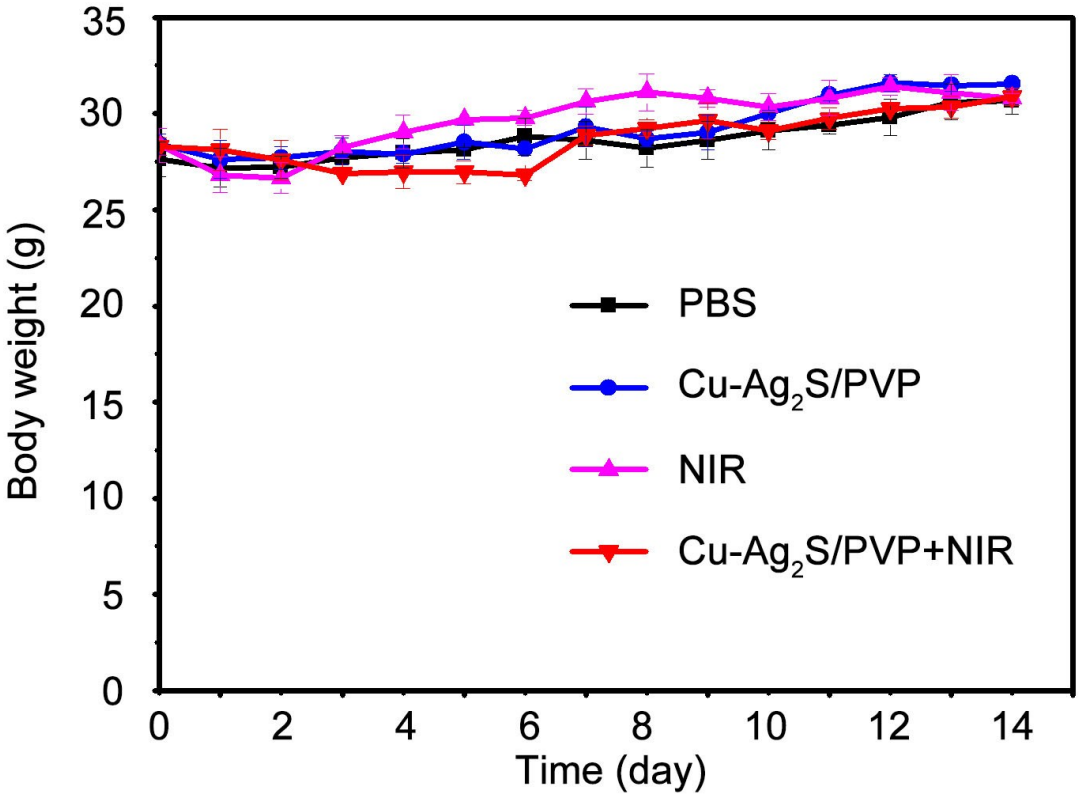

Fig. S20 Body weight of tumor-bearing mice after treatment with PBS, Cu-Ag_2S/PVP, NIR, and Cu-Ag_2S/PVP+NIR, respectively.

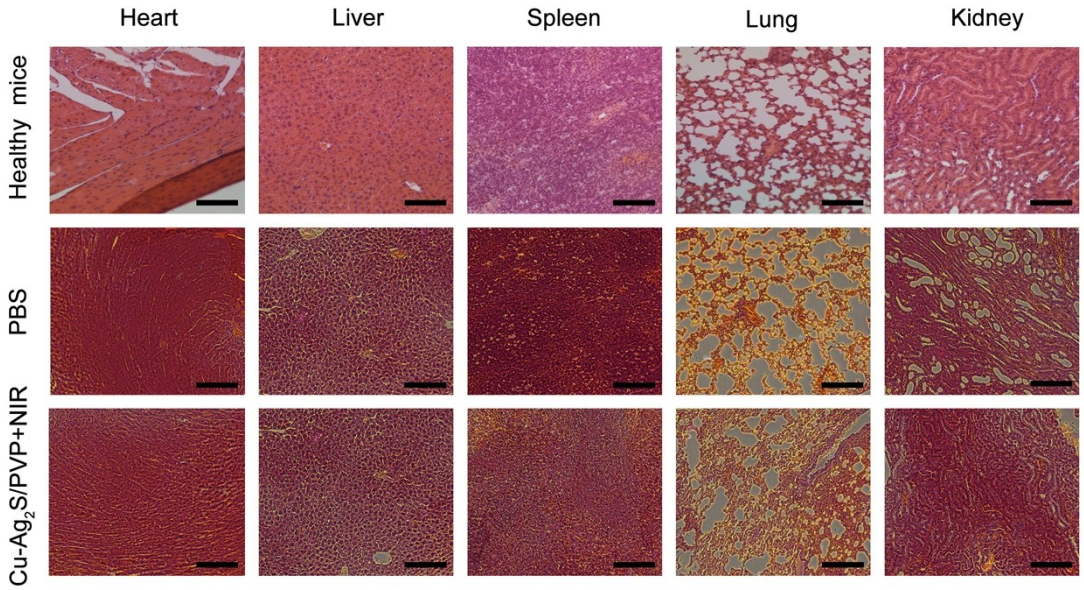

Fig. 21 Histological analysis of the heart, liver, spleen, lung, and kidney obtained from group I, group IV, and healthy mice. All the scale bars are 100 μm.

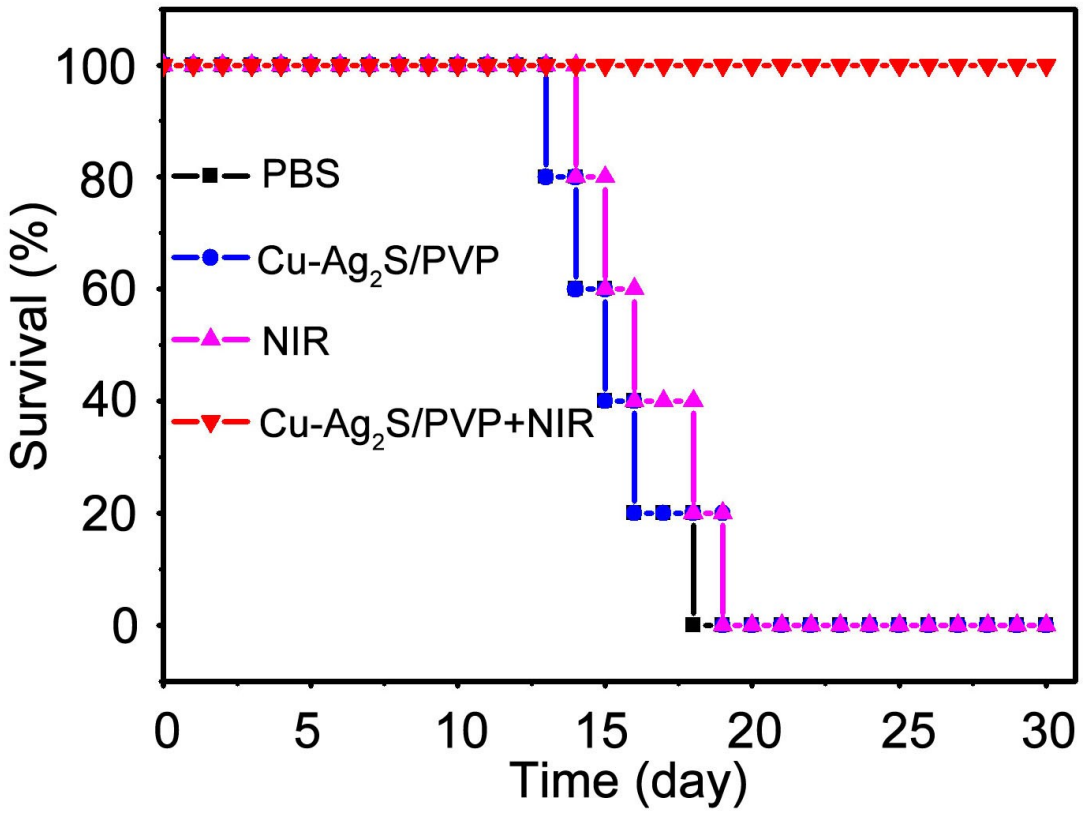

Fig. 22 Survival rates of four groups of tumor-bearing mice (n = 5) as a function of time post-treatments.