Supporting Information

Multifunctional $Cu_{39}S_{28}$ Hollow Nanopeanuts for *In Vivo* Targeted Photothermal Chemotherapy

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Materials and Methods

Materials and Reagents: All the chemicals used were analytical reagent (AR) degrade. Copper nitrate ($Cu(NO_3)_2$), L-cysteine (Cy), sodium sulfide, folic acid (FA), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), N-Hydroxysuccinimide (NHS), doxorubicin (DOX), 3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide (MTT), and anhydrous ethanol are analytically pure and were purchased from Aladdin Chemical Reagent Co. (Shanghai, China). NHS-Flurosecein, dimethyl sulfoxide (DMSO), 3′,6′-Di(O-acetyl)-4′,5′-bis[N,N-bis(carboxymethyl) aminomethyl] fluorescein (Calcein AM), propidium iodide (PI) and 2-(N-Morpholino)ethanesulfonic acid (MES) were purchased from Sigma (St Louis, USA). Other reagent are all analytical pure from Aladdin Chemical Reagent Co. (Shanghai, China).

Synthesis of $Cu_{39}S_{28}$ HNPs: $Cu_{39}S_{28}$ HNPs were synthesized as below: 2, 1, 0.75, 0.5 mmol L-cysteine dissolved in 150 mL of deionized water, and 50 mL of 1 mmol $Cu(NO_3)_2$ was added to the above solution under constant stirring. After stirring for 30 min in room temperature, 0.84 mL sodium thiosulfate solution (2, 1, 0.75, 0.5 mmol) was added by drop in 2 min, separately. The solutions were heated to 90 °C soon and the temperature was maintained for 90 min. The solutions

turned to black during the reaction. The synthesized products were centrifuged (10, 000 rpm, 10 min) and then washed with deionized water and ethanol each for three times.

Preparation of $Cu_{39}S_{28}$-FA: Premade $Cu_{39}S_{28}$ with amino group was further conjugated to FA, utilizing standard EDC/NHS-mediated method. In a typical method, FA (44.1 mg) was dissolved in 20 mL anhydrous DMSO. EDC (21 mg) and NHS (13 mg) were added, and the mixture was then stirred in the dark for 2 h. 20 mL $Cu_{39}S_{28}$ NPs solution (1 mg/mL) was added and allowed to react for 12 h under room temperature. The FA-conjugated $Cu_{39}S_{28}$ nanocomposites were separated by centrifugation at 10 000 rpm for 10 min and washed with DMSO and deionized water three times separately. Then the nanocomposites were stored in 4 ^{o}C for consequent use.

Drug loading and release test: 5 mg $Cu_{39}S_{28}$ NPs was added to 5 mL PBS (pH 7.4) and dispersed by ultrasonication. After that, 5 mg DOX was added into the solution with slow stirring at room temperature for 24 h. The as-prepared mixture was centrifugally separated at 8, 000 rpm for 5min and washed 3 times with PBS solution to remove the free drug, the supernatant solution containing DOX was measured at 482 nm by a UV-VIS spectrophotometer. Encapsulation efficiency % = Weight of drug loaded into the NPs/ Initial weight of drug; Loading content % = (Weight of drug loaded into the NPs) / (Weight of NPs + Weight of drug loaded into the NPs).

The DOX release from $Cu_{39}S_{28}$ HNPs was performed by dispersing 5 mg $Cu_{39}S_{28}$/DOX nanocomposites in 5 mL PBS solution (pH 5, 7) with stirring in 37 ^{o}C and room temperature respectively. The $Cu_{39}S_{28}$/DOX solutions were centrifuged at every pointed time, and the supernatant solutions were collected. The process was repeated and the pointed time set as 1, 2, 4, 8, 12, 24, 36 and 48 h, separately. With the same process, the DOX release in pH=5 PBS was tested under 808 nm irradiation for 10 min and the laser off in 10-100 min, then the laser was turned on in 100-110 min for another 10 min. The release percentage of DOX in the solution was analyzed at the wavelength of 482 nm by spectrophotometer.

Characterization: Optical absorption spectra of the samples were measured with a spectrophotometer (Perkin Elmer, USA) working in a spectral range from 200 to 1000 nm. The crystalline structure, size and shape of the nanoparticles were observed by X-ray diffraction (XRD) and high-resolution transmission electron microscopy (HRTEM). XRD was measured using a Siemens Kristalloflex 810 D-500 X-ray diffractometer (Karslruhe, Germany) under an operating mode of 40 kV and 30 mA, with $\lambda = 1.5406$ Å radiation. $Cu_{39}S_{28}$ HNPs in solution were placed onto holey carbon-covered copper grids for HRTEM observation. The HRTEM images of the particles were obtained with a Hitachi 2100 electron microscope with accelerating voltage of 300 KV. Fourier Transform Infrared Spectroscopy (FTIR) spectra were measured on a IR spectrophotometer (Vertex 70, German) using the KBr pellet technique. N_2 adsorption/desorption isotherms were obtained on a Micromeritics ASAP Tristar II 3020 apparatus. Zeta potential measurements were determined with the Zetasizer Nano Z (Malvern, UK). UV-Vis absorption spectra were measured by the multifunctional microplate reader (Thermo,USA). Pore size distribution was calculated by the Barrete-Jonere-Halenda (BJH) method. The X-ray photoelectron spectroscopy (XPS) experiments

were carried out on a Thermao Escalab 250 system using Al Kα radiation, the binding energies were calibrated by referencing the C 1s peak (284.6 eV) to reduce the sample charge effect. The samples used for analysis were washed with distilled water and ethanol each for three times, respectively, and then dried under dynamic vacuum at 60 ^{o}C for 3h.

Proliferation: The viability of $Cu_{39}S_{28}$ and $Cu_{39}S_{28}$-FA nanoparticles on MCF-7 cells (purchased from the Type Culture Collection of the Chinese Academy of Sciences) was characterized by using MTT assay. Briefly, cells were seeded onto the substrates at a density of 1×10^4 cells per cm^2 and cultured in 37 ^{o}C for 12 h. Then gradient concentrations of 0, 15.6, 31.2, 62.5, 125, 250, 500, 1000 μg/mL $Cu_{39}S_{28}$ HNPs and $Cu_{39}S_{28}$-FA were added to the plates, after co-cultured for 24 h, 10 μL of MTT solution (5 mg/mL in PBS) was added to 100 μL culture medium and incubated for 4 h at 37 ^{o}C. Subsequently, the medium was removed, the formazan reaction products were dissolved in 100 μL DMSO for another 5 min. The optical density of the formazan solution was read using a microplate reader (Thermo, Multiskn Go) at 490 nm.

Chemo-photothermal therapy *in vitro*: MCF-7 cells (1×10^4/mL, 100 μL) were plated into a 96-well plate in a complete medium for 24 h before the experiments. The culture medium was discarded and replaced with a complete medium containing DOX, $Cu_{39}S_{28}$/DOX NPs, $Cu_{39}S_{28}$-FA/DOX NPs and cultured for another 6 h. After the incubation, the cells were washed with PBS and replaced with fresh medium. The cells were irradiated with or without 808 nm laser (1W/cm^2) for 10 min, and were then incubated for a further 24 h. The standard MTT assay was carried out to determine the cell viabilities relative to the control untreated cells.

Hemocompatibility Tests: The hemocompatibility was evaluated by hemolysis ratio. Red blood from healthy human containing sodium citrate (3.8 wt. %) was diluted to 4/5 with PBS (pH=7.4). Diluted blood cells (0.5 mL) were then mixed with 4.5 mL deionized water as a positive control; 4.5 mL PBS as a negative control; 4.5 mL $Cu_{39}S_{28}$ materials suspensions with varying concentrations (400, 100, 25,6.25 μg/mL). Then the samples were shaken and kept stable in 37 ^{o}C for 2 h, the tubes were centrifuged at 3000 rpm for 5 min, and the optical density (OD) values of the supernatant placed in a 96-well plate was determined on an ELISA plate reader (Thermo, USA) at a wavelength of 545 nm. The hemolysis rate (HR) was calculated as follows by Eq. based on the average of three replicates:

HR= (O.D.(Samples)-O.D.(negative))/(O.D.(positive)-O.D.(negative))

Live/Dead staining: For viability staining studies, Cells were seeded on 96-well plates at a concentration of 5×10^4 cells/cm^2 at 37 °C with 5 % CO_2 for 24 h. 100 μL optimum concentration of nanoparticles ($Cu_{39}S_{28}$, $Cu_{39}S_{28}$-FA, $Cu_{39}S_{28}$/DOX, $Cu_{39}S_{28}$-FA/DOX, free DOX) with 25μg/mL DOX were added to the plate, then irradiated with or without 808 nm laser at density of 1 W/cm^2 for 10 min, the medium was removed and the adherent cells were subjected to Live/Dead staining following the manufacturer's protocol (Sigma, USA). Briefly, after the removal of the culture medium, the cells were rinsed once in PBS, followed by addition of 100 μL PBS containing 1 μM

calcein AM and 2 μM PI. Cells were then photographed using a fluorescence microscope (Olympus, BX51, Japan).

Cell apoptosis and necrosis: The MCF-7 cells suspension (400 μL) was seeded on a 24-well cell culture plate, at a density of 1 $\times 10^5$ cells/mL. After 24 hours, $Cu_{39}S_{28}$, $Cu_{39}S_{28}$-FA, $Cu_{39}S_{28}$/DOX, $Cu_{39}S_{28}$-FA/DOX, free DOX were added to the plate, then treated with (or without) laser for 10 min. To analyze the changes in the nuclear morphology, MCF-7 cells were tested using Guava Nexin Reagent (Millipore, USA). Brefily: Cells on the substrates were washed with PBS and centrifuged at 1000 rpm for 5 min. The cell pellets were resuspended in 100 μL DMEM medium supplemented with 1% FBS, and then incubated with 100 μL of Annexin V-PE and 7-AAD labelling solution for 20 min at room temperature. Cells were finally analyzed with a Guava EasyCyte 5HT Fow cytometer (Millipore, USA) by using 488 nm excitation and a 575 nm bandpass filter for Annexin V-PE detection and using 546 nm excitation and a 647 nm filter for 7-AAD detection. The data were analyzed using Guava Nexin Software v2.2.2

In vitro $Cu_{39}S_{28}$-FA targeting: The *in vitro* $Cu_{39}S_{28}$-FA targeting capability was studied by flow cytometry through NHS-Fluorescein conjugating. Briefly, 2 mg $Cu_{39}S_{28}$ and $Cu_{39}S_{28}$-FA nanoparticles were dissolved in 5 mL MES solution (1 mmol/L) separately, then 5 mg NHS-Fluorescein (dissolved in DMSO) was added to the $Cu_{39}S_{28}$ and $Cu_{39}S_{28}$-FA solution, respectively. The reaction was kept for 2 h in dark. After that, the samples were collected by centrifugation (12, 000 rpm, 10 min), washed with DMSO and double distilled H_2O each for 3 times. All of the above steps need to avoid light.

For flow cytometry analysis, MCF-7 cells were first cultured for 12 h with serum free medium, then the fluorescein $Cu_{39}S_{28}$ HNPs and $Cu_{39}S_{28}$-FA nanocomposites were added and co-cultured for 6 h. Then the cells were collected and analyzed with a Guava EasyCyte 5HT flow cytometer (Millipore, USA) which equipped with 488 and 633 nm lasers.

Animals: The nude mice (6~8 weeks old female Balb/c) were purchased from Medical Experimental Animal Center of Guangdong Province. All animal experiments were performed in compliance with the local ethics committee and Guangzhou General Hospital of Guangzhou Military Command institutional guidelines.

***In vivo* targeting and biodistribution :** A suspension of 1×10^7 MCF-7 cells was injected subcutaneously into the bakc of nude mice. When tumors grew to 5~7 mm in diameter, the MCF-7 bearing nude mice were randomly divided into 3 groups with each of 5 mice: 1) PBS (50 μL), 2) $Cu_{39}S_{28}$ nanocomposites (50 μL, 20 mg/kg) 3) $Cu_{39}S_{28}$-FA nanocomposites (50 μL, the same amount of Cu^{2+} with group 2) through intravenous (*i.v.*) injection. After 24 h, mice were killed and the distribution of Cu^{2+} in brain, heart, liver, spleen, lung, kidney, muscles and tumor were tested using ICP-MS method . The main organs were weighed, then 5 mL HCl: HNO_3 (3:1) added to the each sample and digested at 60 ^oC for 30 min, then tranfered to the flask for testing, a series of standard Cu^{2+} standard solutions (0, 0.5, 1, 5, 10, 20, 50, and 100 ppm) were prepared. Both standard and test solutions were measured by ICP-MS (Thermal Fisher Scientific Inc., USA). And the amount of Cu^{2+} in main organs, muscles and tumor site were compared.

Evaluation of the Chemo/photothermal Effects in Mice: Mice bearing breast tumors were randomly divided into seven treatment groups with each of 5 mice and injected *via* tail vein with: 1) PBS (50 μL); 2) PBS+NIR ; 3) DOX (50 μL, 2 mg/kg); 4) $Cu_{39}S_{28}$-FA (50 μL, 2 mg/kg); 5) $Cu_{39}S_{28}$-FA/DOX (50 μL, 2mg/kg); 6) $Cu_{39}S_{28}$-FA (50 μL, 2 mg/kg) + NIR, 7) $Cu_{39}S_{28}$-FA/DOX (50 μL, 2 mg/kg) +NIR . After 120 min, the tumors were irradiated with or without NIR light (808 nm, 1 W/cm^2, 10 min). Tumor size and body weight were recorded with a caliper and electronic scales every other day for two weeks, separately. The tumor volume was calculated by the following formula: $V = (L \times W^2)/2$, where L and W are the longest and shortest tumor dimensions respectively.

Hematoxylin and eosin (H&E) Staining: After the treatments, mouse organs including heart, liver, spleen, lung, kidney, and tumor were collected and fixed in 4% paraformaldehyde overnight in 4 °C. The H&E staining was performed following a standard protocol. Briefly, tissue samples were embedded in paraffin blocks, sectioned into 5 μm slices, mounted onto the glass slides, and stained with hematoxylin and eosin stains. All slices from different treatment groups were blindly processed. Images of the main organs sections stained by H&E were visualized with a fluorescence microscope.

Statistical analysis：All statistical analysis was performed by analysis of variance (ANOVA) using SPSS 13.0. The results were considered statistically significant if the p-value was <0.05

Photothermal Conversion: Following a previous method[1] the photothermal conversion efficiency (η) of the $Cu_{39}S_{28}$ HNPs was measured. The aqueous solution of the $Cu_{39}S_{28}$ HNPs (250 ppm) was under continuous irradiation of the laser (808 nm, 1 W/ cm^{-2}) until a steady state temperature was reached. Subsequently, the laser was shut off, and the temperature decrease of the aqueous solution was recorded to measure the rate of heat transfer from the $Cu_{39}S_{28}$ HNPs solution system to the environment (Figure 3a). The η value was calculated as follows:

$$\eta = \frac{hS\Delta T\max - Q_{DIS}}{I(1-10^{-A808})} \quad (1)$$

where h is the heat transfer coefficient, S is the surface area of the container, and the value of hS was gained from Figure S4. $T_{max} - T_{Surr}$ is the temperature change of the $Cu_{39}S_{28}$ HNPs solution at the maximum steady-environmental temperature, I is the power of the laser, A808 is the absorbance of $Cu_{39}S_{28}$ HNPs at 808 nm, and Q_{Dis} expresses heat dissipated from light absorbed by the solvent and the container.

The maximum steady temperature (T_{max}) of the solution of the $Cu_{39}S_{28}$ HNPs was 42.3 °C and environmental temperature (T_{Surr}) was 22.3 °C. So, the temperature change (T_{max}-T_{Surr}) of the solution of the $Cu_{39}S_{28}$ was 20 °C. The laser power *I* is 1 W. The absorbance of the $Cu_{39}S_{28}$ HNPs at 808 nm A_{808} is 0.7051. Q_{Dis} expresses heat dissipated from the light absorbed by the solvent and container.

In order to gain *hS*, a dimensionless parameter θ is introduced as followed:

$$\theta = \frac{\Delta T}{\Delta T \max} \qquad (2)$$

Substituting Eq.2 into Eq.1 and rearranging Eq.1:

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

The light source was shut off, the $Q_{NP}+Q_{Dis} = 0$, reducing the Eq. 3

$$hs = \frac{m_D C_D}{\tau_s} \qquad (4)$$

Thus, *hS* can be determined by applying the linear time data from the cooling period vs $-ln\theta$. The τ_s was determined to be 139.91, and m_D=0.6g, C_D=4.2 J/g· °C, *hS*= 18.01 mW/°C. Qdis expresses heat dissipated from the light absorbed by the quartz sample cell itself, and it was measured independently to be 30.01 mW using a quartz cuvette cell containing pure water, substituting *hS* value into Eq.1, the heat conversion efficiency (η) of $Cu_{39}S_{28}$ HNPs can be calculated to be 41.13 %

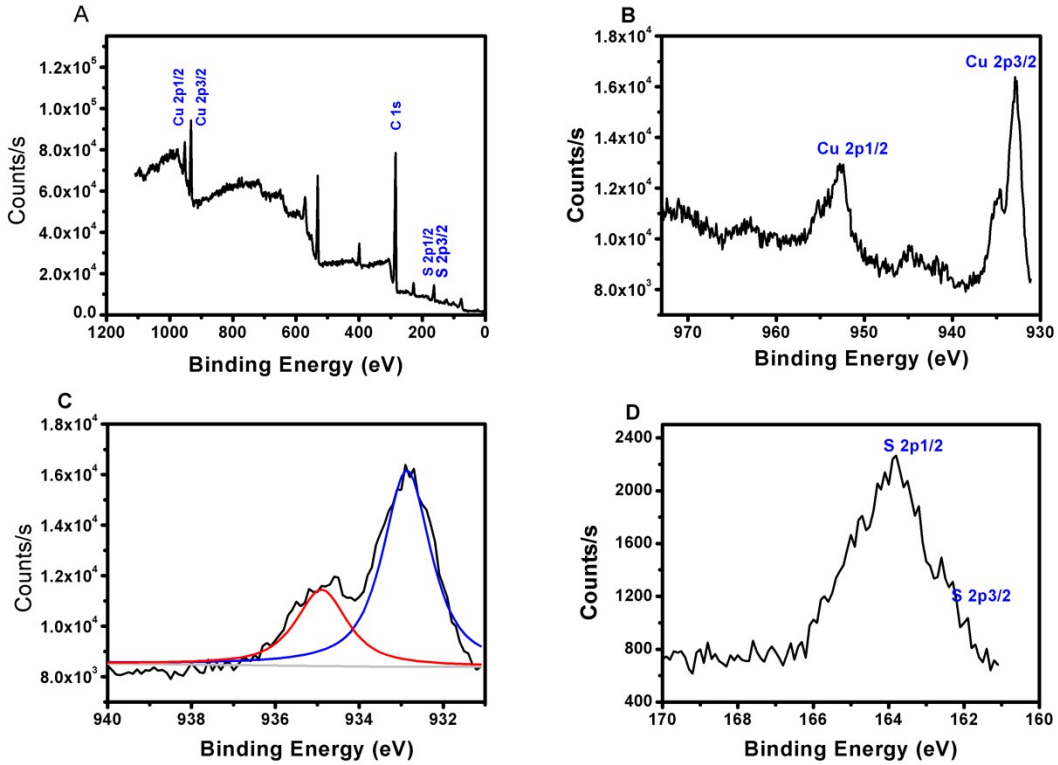

Figure S1. XPS spectra of $Cu_{39}S_{28}$ sample (90 min). (A) XPS spectra of survey; (B) XPS spectra of Cu 2p; (C) Cu $2p_{2/3}$ peak fit. The Cu $2p_{2/3}$ peak fit reveals a 2 peaks at 932.2 eV (corresponded to Cu^+ in Cu(I)cysteine) and 933.5 eV (assigned to Cu^{2+} in CuS), corresponding well with the XRD results ($Cu_{39}S_{28}$); (D) XPS spectra of S 2p;

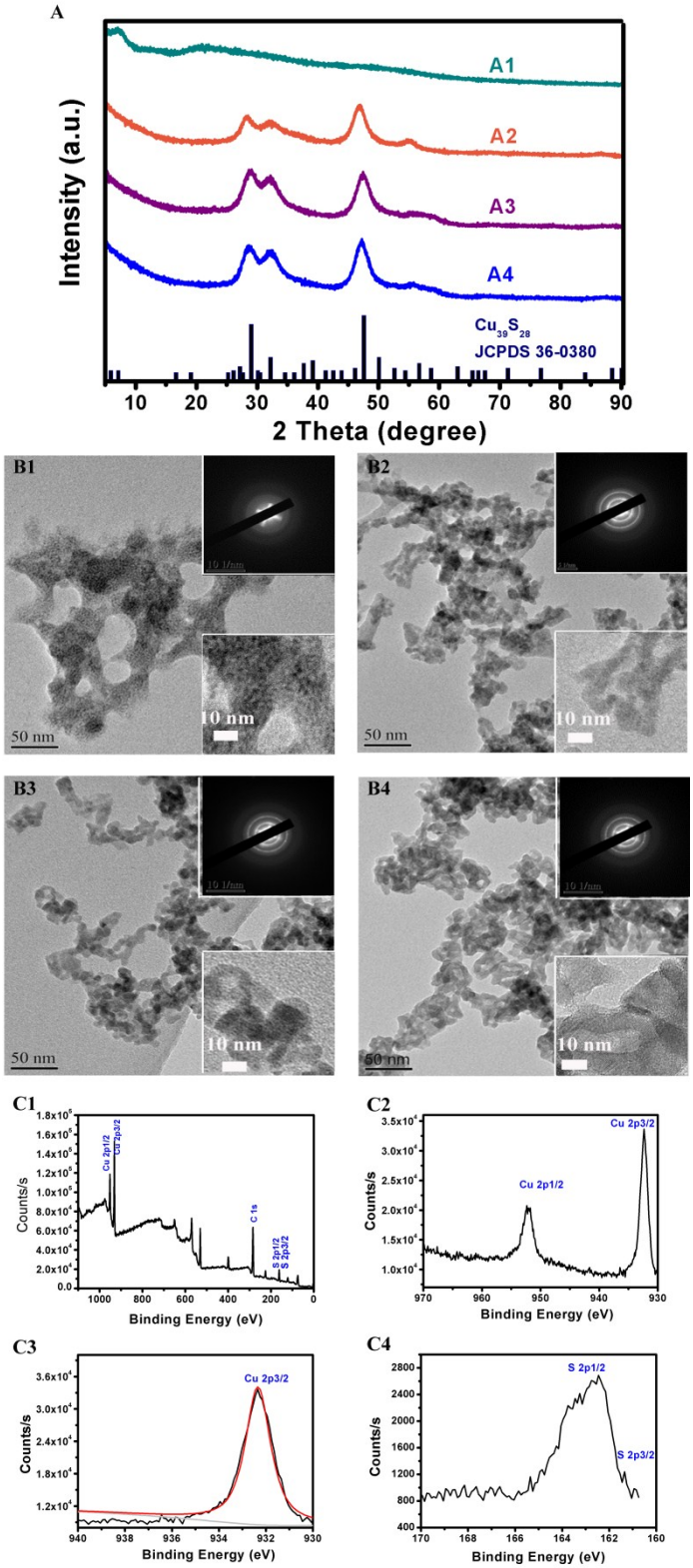

Figure S2. (A)XRD patterens of $Cu_{39}S_{28}$ samples with different reaction times. (A1) Cu-Cy precusors (without addition of Na_2S), (A2)$Cu_{39}S_{28}$ sample reacted with Na_2S for 30 min, (A3)$Cu_{39}S_{28}$ sample reacted with Na_2S for 60 min, (A4)$Cu_{39}S_{28}$ sample reacted with Na_2S for 90 min; (B)TEM images of $Cu_{39}S_{28}$ samples with different reaction times; Inset: image above is the corresponding SAED and image below is the magnified TEM. (B1) Cu-Cy precusors (without addition of Na_2S), (B2) $Cu_{39}S_{28}$ sample reacted with Na_2S for 30 min, (B3) $Cu_{39}S_{28}$ sample reacted with Na_2S for 60 min, (B4) $Cu_{39}S_{28}$ sample reacted with Na_2S for 90 min; (C) XPS spectra of Cu-

Cy prescusor without addition of Na_2S. (C1) XPS spectra of survey; (C2) XPS spectra of Cu 2p; (C3) Cu $2p_{2/3}$ peak fit. The Cu $2p_{2/3}$ peak fit reveals a main peak at 932.2 eV (corresponded to Cu^+ in Cu(I)cysteine), (C4) XPS spectra of S 2p;

There was almost no peaks in the XRD images (Figure S2 A1) of the Cu-Cy prescusor, indicating the non-crystal structure of the precursor. After reacted with Na_2S for 30, 60, 90 min (Figure S2 A2, A3, A4), the samples corresponding well with $Cu_{39}S_{28}$. In Figure S2 A1, first a loose and irregular $Cu_{39}S_{28}$ structure were formed, and amorphous structure were detected by SAED when only with the reaction of $Cu(NO_3)_2$ and L-cysteine. Then after reacted with Na_2S for 30 min, hollow $Cu_{39}S_{28}$ structure were formed. With time increased, more clear hollow nanostructure appeared. The 90 min products (Figure S2 A4) exhibited unique hollow nanopeanuts sctructure.

The XPS results of the former precursors were investigated (without Na_2S, as shown in Figure S2 C). For the reducibility of L-cysteine, the Cu $2P_{3/2}$ showed a main peak at 932.3 eV, which corresponding well with Cu^+ in Cu(I)cysteine, and the reaction between cupric ions and cysteine was as below:[2]

Cu^{2+}+ $HOOCCH(NH_2)CH_2SH \rightarrow Cu^+ + OOCCH(NH_2)CH_2S\text{-}SCH_2CH(NH_2)COOH$

$Cu^+ + HOOCCH(NH_2)CH_2SH \rightarrow HOOCCH(NH_2)CH_2S\text{-}Cu(I)$

After reacted with Na_2S for 90 min, the superfluous Cu^{2+} ions were reacted with Na_2S to form $Cu_{39}S_{28}$ embracing the initial Cu(I)cysteine, and the final products exhibited 2 peaks at Cu 2p3/2 (as shown in Figure S1), the peak at 932.2 eV corresponding to Cu(I)cysteine and the 933.5 eV peak assigned to Cu^{2+} in $Cu_{39}S_{28}$ (Figure S1), corresponding well with the XRD results ($Cu_{39}S_{28}$).

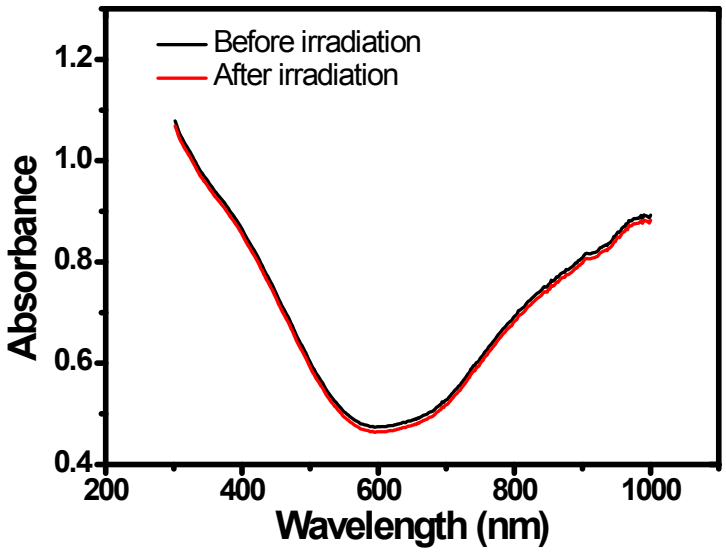

Figure S3. The absorbance curve of $Cu_{39}S_{28}$ HNPs before irradiation and after irradiation (808 nm 1W/cm^2,1h)

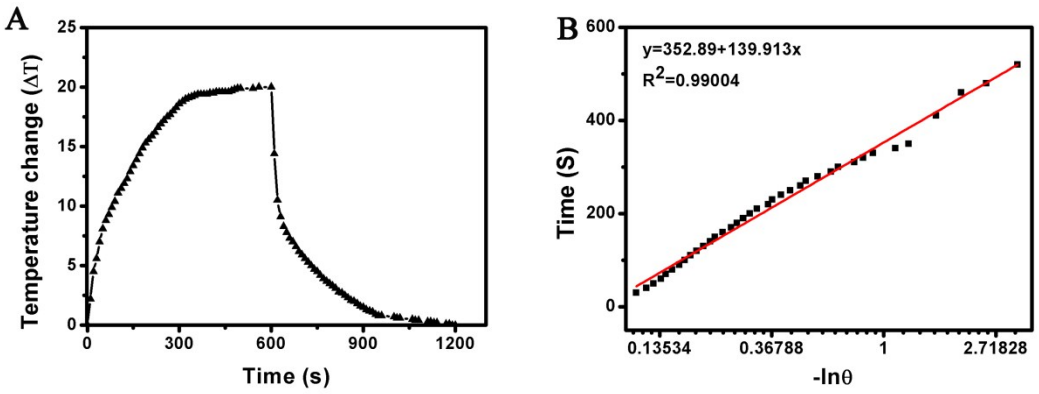

Figure S4. (A) Heating/Cooling experiment of 250 μg/mL $Cu_{39}S_{28}$ NPs aqueous solution under 1 W/cm^2 808 nm laser irradiation. The continuous wave laser was switched off after 10 min and the cooling rate was recorded; (B) Linear time data *versus* –ln(θ) obtained from the cooling period of (A).

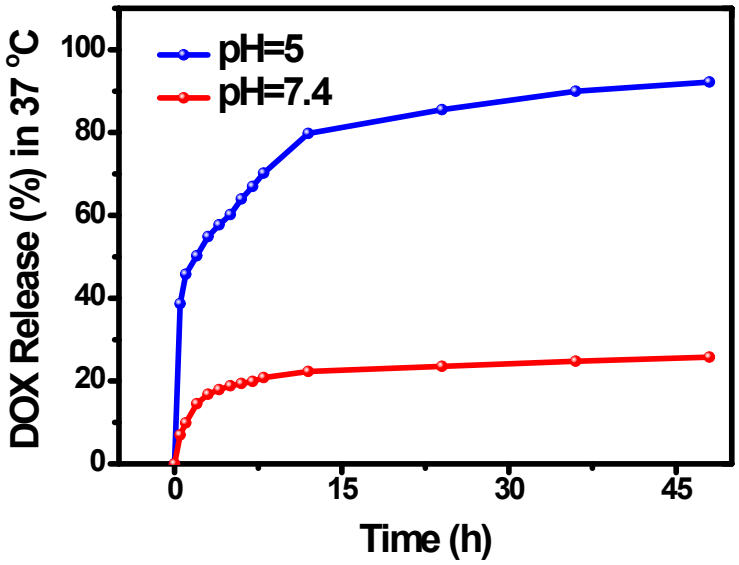

Figure S5. DOX-release profiles of DOX-loaded $Cu_{39}S_{28}$-FA nanoparticles measured at pH 5.0 and pH 7.4 in PBS buffer at 37 °C .

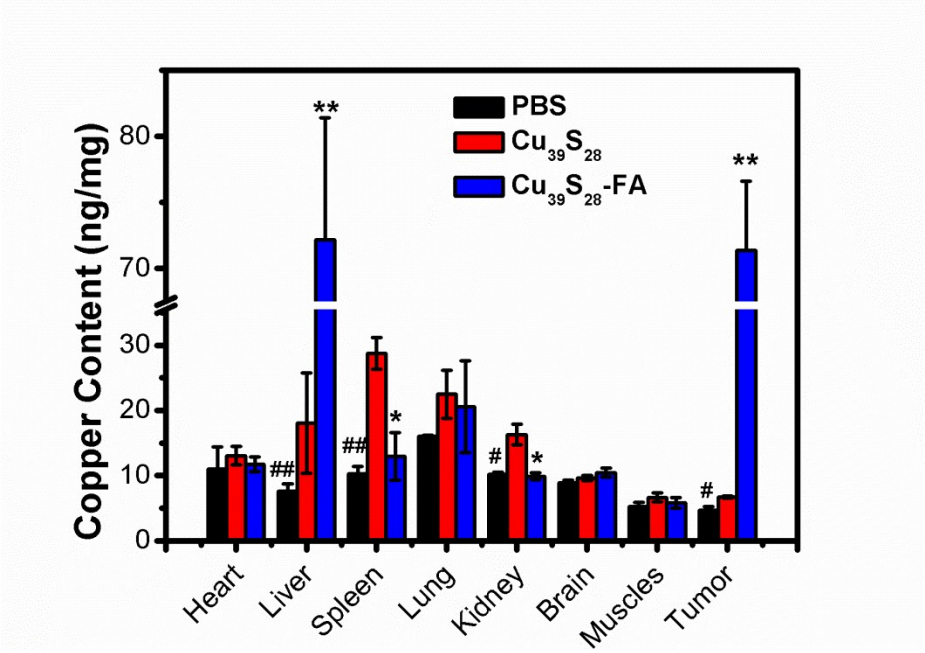

Figure S6. Biodistribution of $Cu_{39}S_{28}$ and $Cu_{39}S_{28}$-FA nanoparticles in the main organs and tumors of nude mice. ($^{\#}$,*p<0.05; $^{\#\#}$,**p<0.01 with $Cu_{39}S_{28}$ group).

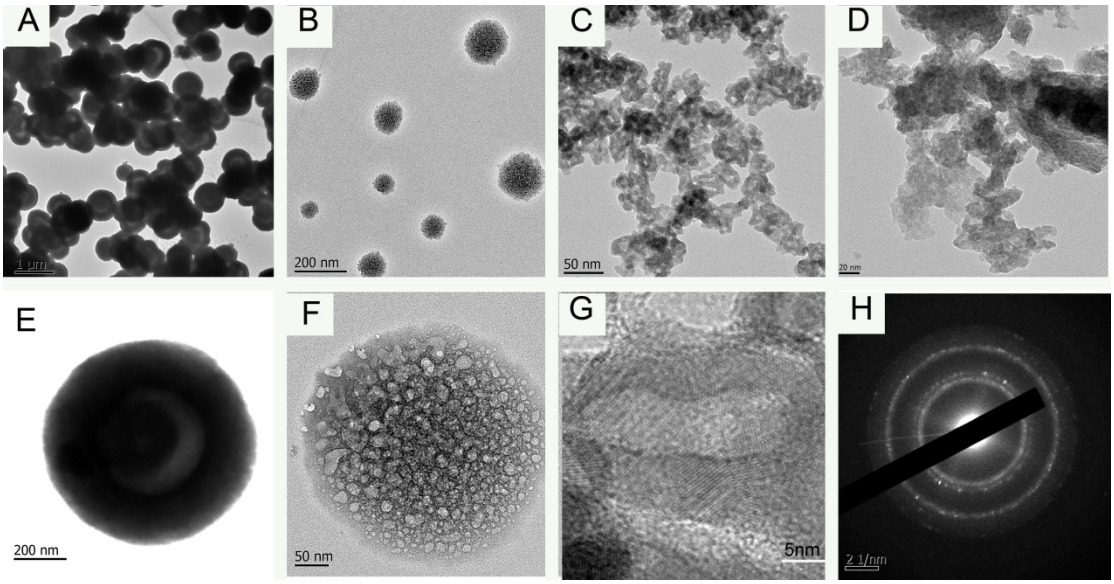

Figure S7. The HRTEM images of CuS nanstructures with different ratios. (A,E) CuS NPs with ratio of $Cu(NO_3)_2$: L-cysteine: Na_2S is 1: 2: 2; (B, F) CuS NPs with ratio of $Cu(NO_3)_2$: L-cysteine: Na_2S is 1: 1: 1; (C, G) CuS NPs with ratio of $Cu(NO_3)_2$: L-cysteine: Na_2S is 1: 0.75: 0.75; (D) CuS NPs with ratio of $Cu(NO_3)_2$: L-cysteine:Na_2S is 1: 0.5: 0.5; (H) SAED of CuS with ratio 1:1:1.

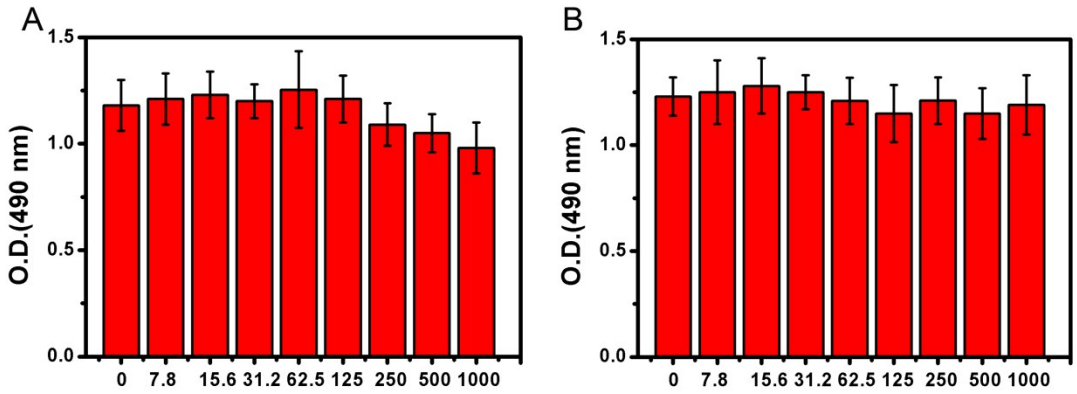

Figure S8. (A) Cell Viability of gradient concentrations of $Cu_{39}S_{28}$ HNPs cocultured with L929 for 24 h. (B) Cell Viability of gradient concentrations of $Cu_{39}S_{28}$-FA HNPs cocultured with L929 for 24 h.

References:

1. D. K. Roper, W. Ahn and M. Hoepfner, *J. Phys. Chem. C*, 2007, **111**, 3636-3641.
2. B. Li, Y. Xie and Y. Xue, *J. Phys. Chem. C*, 2007, **111**, 12181-12187.