COMMUNICATION

Supporting Information

Second Near-Infrared Photodynamic Therapy and Chemotherapy of Orthotopic Malignant glioblastoma with Ultra-small $Cu_{2-x}Se$ Nanoparticles

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EXPERIMENTAL SECTION

Materials

$CuCl_2 \cdot 2H_2O$ (≥ 99%), Se powder (−100 mesh, ≥ 99.5%), sodium borohydride ($NaBH_4$, 99%), and mercaptosuccinic acid (MSA, 99%) were purchased from Sigma-Aldrich. Dimercapto poly(ethylene glycol) (HS-PEG-SH, MW = 5000) was purchased from J&K Scientific LTD. Mono-(6-mercapto-6-deoxy)-β-cyclodextrin (CD-SH) was purchased from Shandong Binzhou Zhiyuan Biotechnology Co.,Ltd. Terephthalic acid (TA) was purchased from Sinopharm Chemical Reagent Co., Ltd. 9,10-Anthracenediyl-bis(methylene)dimalonic acid (ABDA) was purchased from APExBIO. 2,7-dichlorofluorescein diacetate (DCFH-DA) and the Cell Meter Mitochondrial Hydroxyl Radical Detection Kit (MHRD, red fluorescence) were purchased from AAT Bioquest Inc. Single Oxygen Sensor Green Reagent (SOSG) was purchased from Thermo Fisher Scientific. Milli-Q water (> 18 MΩ•cm) was used in the experiments. All chemicals and reagents were used as received without any further purification.

Characterization

TEM images were captured using a FEI Tecnai G20 transmission electron microscope operating at an acceleration voltage of 200 kV. Dynamic light scattering (DLS) was conducted at 25 ℃ on a Malvern Zetasizer Nano ZS90 equipped with a solid state He–Ne laser (λ = 633 nm). The crystal structure of the $Cu_{2-x}Se$ NPs was characterized with a Shimadzu XRD-6000 X-ray diffractometer equipped with Cu $K\alpha_1$ radiation (λ = 0.15406 nm). Ultraviolet–visible–near-infrared (UV–Vis–NIR) spectra were collected on a PerkinElmer Lambda 750 UV–Vis–NIR spectrophotometer. The fluorescence spectra (FL) were recorded on a FLS980 spectrometer (Edinburgh Instruments, UK). Thermogravimetric analysis (TGA) was performed to analyse the contents of surface ligands at a heating rate of 10 ℃ min^{-1} from room temperature to 800 ℃ under nitrogen atmosphere.

Synthesis of cyclodextrin modified ultra-small $Cu_{2-x}Se$ NPs

In a typical synthesis, Se powder (0.5 mmol) was reduced by $NaBH_4$ (1.5 mmol) in 50 mL of H_2O under magnetic stirring at room temperature under nitrogen protection. Then, 5 mL aqueous solution of $CuCl_2 \cdot 2H_2O$ (1 mmol) and MSA (6.66 mmol) was added into the selenium precursor solution under magnetic stirring, and the reaction mixture was kept under stirring for 2 h.

The $Cu_{2-x}Se$ NP solution was centrifuged with a 30 kDa ultrafiltration tube at 4000 rpm to remove the excessive MSA, then the black solution was diluted to 3 μmol mL^{-1} (Cu concentration), and CD-SH (3 mg

mL^{-1}) was added, the reaction mixture was kept under stirring for 8 h.

Synthesis of PEGylated ultra-small $Cu_{2-x}Se$ NPs

The cycodextrin modified $Cu_{2-x}Se$ NP solution was centrifuged with a 30 kDa ultrafiltration tube at 4000 rpm to remove the excessive mono-(6-mercapto-6-deoxy)-β-cyclodextrin. HS-PEG-SH (5mg mL^{-1}) was added to modify the surfaces of the $Cu_{2-x}Se$ NPs at room temperature. The obtained ultra-small $Cu_{2-x}Se$ NPs were purified by a similar ultrafiltration method to remove the free HS-PEG-SH. The purification process was typically repeated three times using Milli-Q water as eluent. The purified $Cu_{2-x}Se$ NPs are denoted to as CS NPs.

DOX loading and release

In a dark room, CS NPs (200 μg mL^{-1}) were mixed with a DOX solution (20 μg mL^{-1}) under magnetic stirring for 6 h. Thereafter, the CS-DOX nanoparticles (denoted to as CS-D NPs) were centrifuged with a 30 kDa ultrafiltration tube at 4000 rpm to remove the excessive DOX, and the ultrafiltrate solution was collected for UV−Vis measurements. The DOX content in the ultrafiltrate solution was determined by UV−Vis spectrscopy at 495 nm.

To study the release of DOX from CS-D NPs, 1 mL CS-D NPs (500 μg mL^{-1}) solution was loaded into a dialysis tube (cut-off molecule weight of 7000 D), and then the dialysis tube was dipped into 20 mL PBS solutions with different pH values (*i.e.* 7.4, 6.5, and 5.6) and set in a water bath at 37 ℃ under magnetic stirring. The dialysis solution was collected for further FL analysis at varied time intervals.

***In vitro* penetration efficiency of 808 and 1064 nm lasers**

For the penetration experiment, 808 and 1064 nm lasers at different power densities (0.25, 0.5, 0.75, 1, 1.5, and 2 W cm^{-2}) were used to irradiate the scalp and skull of a mouse. A photometer was located under the scalp and skull to test the penetration efficiency of the light.

808 and 1064 nm lasers at the same power (0.75 W cm^{-2}) were used to irradiate chicken tissue. A photometer was located under the different thicknesses chicken tissue to test the penetration efficiency of the light.

***In vitro* photothermal and photodynamic performance of CS NPs**

1 mL of CS NPs solution (12.5 μg mL^{-1}) was loaded into a cuvette covered with/without 2.5 mm chicken tissue and irradiated by a 1064 or 808 nm laser at the power density of 0.75 W cm^{-2}. The solution

temperature was monitored by an infrared (IR) thermal camera during the photothermal heating process (0-5 min).

1 mL of CS NPs (12.5 μg mL^{-1}) and H_2O_2 (400 μM) solution were loaded in a cuvette covered with 2.5 mm chicken tissue and irradiated by a 1064 or 808 nm laser at the power density of 0.75 W cm^{-2}. H_2O_2 degradation was detected by measuring the UV-Vis absorbance of the mixture (H_2SO_4/$TiSO_4$) at 405 nm. The generation of ROS was detected by measuring the fluorescence of 2,7-dichlorofluorescein (DCF), which was oxidized from DCFH-DA (10 μM, 1 mL) by ROS radicals.

Detection of •OH radicals

Electron spin resonance (ESR) measurements was performed to monitor the generation of •OH using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) as a spin-trap. ESR spectra of spin trapped •OH were obtained by mixing CS NPs (12.5 μg mL^{-1}), H_2O_2 (400 μM), and DMPO (100 mM) in water. The samples were directly irradiated by a 1064 nm laser at 0.75 W cm^{-2} for 5 min.

Terephthalic acid (TA) was selected as the fluorescence probe for specifically detecting •OH radicals. CS NPs (12.5 μg mL^{-1}), H_2O_2 (400 μM), and TA (6 mM) were mixed in water, and then the solution was directly irradiated by a 1064 nm laser at 0.75 W cm^{-2} for different times. The generation of •OH was detected by measuring the fluorescence of the TA solution under excitation at 315 nm.

Detection of $^1O_2$ radicals

Electron spin resonance (ESR) analysis was performed to monitor the generation of $^1O_2$ radicals using 2,2,6,6-tetramethylpiperidine (TEMP) as the spin-trap. ESR spectra of spin trapped $^1O_2$ were obtained by mixing CS NPs (12.5 μg mL^{-1}), H_2O_2 (400 μM), and TEMP (35 mM) in water. The samples were directly irradiated by a 1064 nm laser at 0.75 W cm^{-2} for 5 min.

9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA) was selected for specifically detecting $^1O_2$. CS NPs (12.5 μg mL^{-1}), H_2O_2 (400 μM), and ABDA (100 μM) were mixed in water, and then the solution was directly irradiated by a 1064 nm laser at 0.75 W cm^{-2} for different times. The generation of $^1O_2$ radicals was detected by measuring the UV-Vis spectra.

Monitoring of total ROS, •OH, and $^1O_2$ radicals in U87 cells

U87 cells were seeded on glass-bottom dishes at 1.0×10^5 cells/well, pre-incubated for 24 h, and then treated with CS NPs (25 μg mL^{-1}), while the control groups were treated with fresh DMEM. After

incubation for 2 h, the cells were washed two times with PBS, and then incubated with different reagents (MHRD for •OH, SOSG for $^1O_2$, and DCFH-DA for total ROS radicals) at 37 ℃ under 5% CO_2 for 0.5 h. They were then irradiated with/without a 1064 nm laser (0.75 W cm^{-2}) for 5 min, and then incubated for 2 h. Subsequently, the culture medium was removed, and then the cells were stained with Hoechst 33342 for 15 min and characterized by confocal laser scanning microscopy.

In vitro cytotoxicity

U87 cells were seeded in a 96-well plate at 0.8-1 $\times 10^4$ cells/well, pre-incubated for 24 h, and CS-D NPs in DMEM culture medium at different concentrations (0, 3.125, 6.25, 12.5, and 25 μg mL^{-1}) were added. After 4 h, the culture medium was removed and washed with PBS for two times, 100 μL fresh DMEM was added to each well, and the cells were irradiated with/without a 1064 nm laser (0.75 W cm^{-2}) for 5 min, then incubated for 20 h. Finally, the PDT and chemotherapy induced cytotoxicity was characterized according to an MTT assay.

Orthotopic malignant glioblastoma model

All animal experiments were carried out accordance with guidelines approved by the ethics committee of Soochow University (Soochow, China). Female athymic nude mice, aged 6–8 weeks, were supplied by laboratory animal center of Soochow University and housed in a temperature- and humidity-controlled room (23 ℃ and 50% humidity) under pathogen-free barrier conditions. The mice were randomly divided into four groups, each group contains ten mice.

For orthotopic malignant glioblastoma implantation, a mixture of U87-Luciferase cells (5×10^5) and PBS (5 μL) were injected into the striatum of mice in the target position, where the bregma was + 1.0 mm, the right lateral was 2.0 mm, and the depth was 3.0 mm. To monitor the tumor growth, the mice were intraperitoneally injected with D-Luciferin potassium salt (75 mg kg^{-1}) and imaged with an IVIS Lumina XRMS Series Imaging system. The orthotopic malignant glioblastoma bearing mice were treated with different therapy methods 7 days after tumor cell inoculation.

BBB opening

A US transducer (0.5 MHz and 30 mm diameter) was used to open the BBB of mice bearing orthotopic malignant glioblastoma, driven by a function generator connected to a power amplifier. A removable cone filled with degassed water was employed to hold the transducer and guide the US beam into the brain. The acoustic parameters used were 0.6 MPa acoustic pressure, 0.5 MHz frequency, 1 ms pulse interval, and 90

s sonication duration. 50 μL of microbubbles (mean diameter of about 2 μm and concentration of about 1 × 10^9 bubbles mL^{-1}) were intravenously injected into the mice before sonication. The mice were administrated with EB dye (30 mg kg^{-1}) *via* a tail vein and then sacrificed 2 h after EB injection.

In-vivo PA imaging

PA imaging was performed with a Multispectral Optoacoustic Tomography scanner (MSOT, iThera Medical). For *in-vivo* PA imaging, nude mice were anesthetized with 1.5 % isoflurane delivered *via* a nose cone. Then, the CS-D NPs (dose: 5 mg kg^{-1}) were intravenously injected into the orthotopic malignant glioblastoma bearing mice after treatment with/without focused ultrasound (sonication: 90 s, microbubbles: 50 μL). The PA images of the mice were captured at different time points.

In vivo PDT for orthotopic malignant glioblastoma

The orthotopic malignant glioblastoma bearing mice were classified into four groups with each group containing ten mice. The four groups included 1) the US + PBS + 1064 nm group, 2) the US + CS-D group, 3) the CS-D + 1064 nm group, and 4) the US + CS-D + 1064 nm group. The CS-D NPs injection dose was 5 mg kg^{-1}, and the power density of the 1064 nm laser was 1 W cm^{-2}. At 10 h after the PBS or CS-D NPs injection, the mice were irradiated with 1064 nm laser irradiation (1W cm^{-2}) for 5 min on the tumor site.

The treatment effects were monitored by bioluminescence through the IVIS Lumina XRMS Series Imaging System. After treatment on Day 20, the brains of different groups were collected for H&E staining to examine the antitumor efficacy.

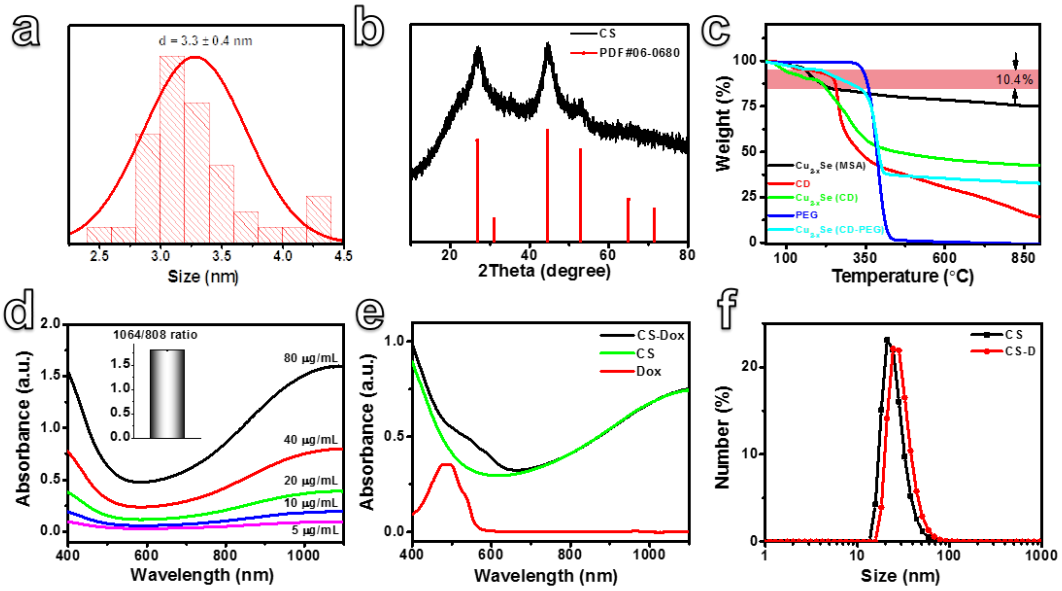

Fig. S1 a) Histogram of size distribution of CS NPs. b) XRD pattern in comparison with the standard peaks of cubic berzelianite (JCPDS Card No. 06–0680). c) TGA curves of $Cu_{2-x}Se$, cyclodextrin (CD), $Cu_{2-x}Se$-CD, polyethylene glycol (PEG), and $Cu_{2-x}Se$-CD-PEG NPs. d) UV-visible near-infrared (UV-Vis-NIR) spectra of the CS NP solutions with different Cu concentrations. e) UV-Vis-NIR spectra of the CS, DOX, and $Cu_{2-x}Se$-DOX NPs (CS-D NPs) solutions. f) Hydrodynamic size of CS and CS-D NPs.

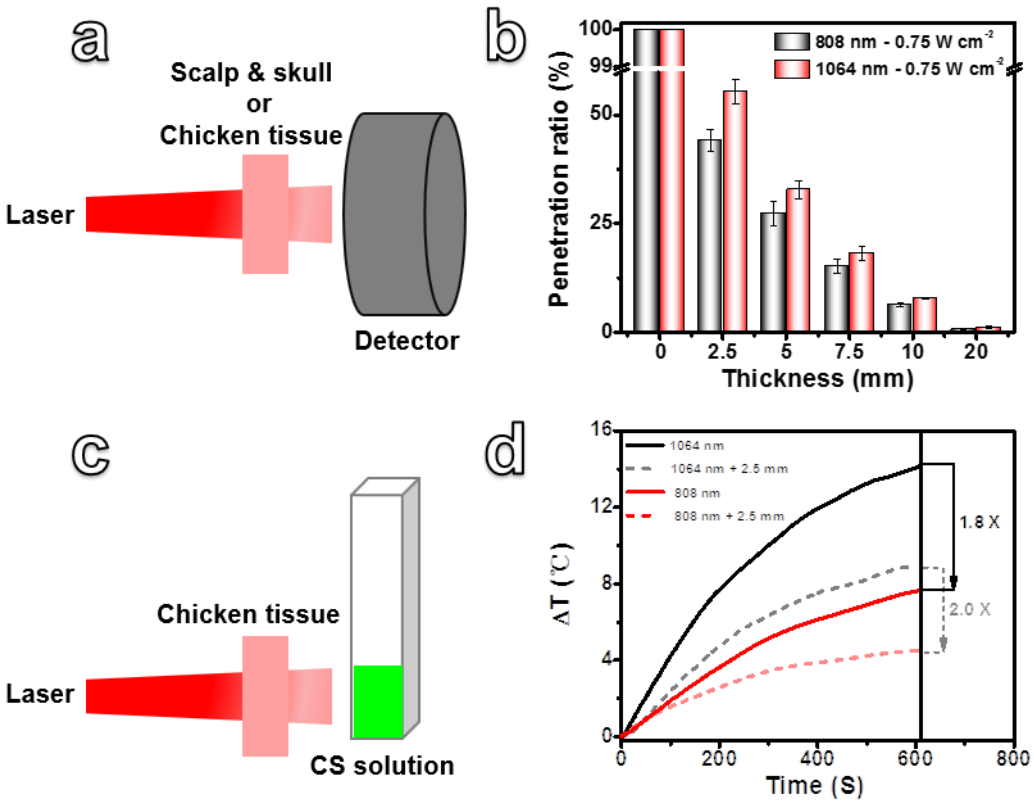

Fig. S2 a) Schematic illustration of 808 nm and 1064 nm laser penetration. b) Penetration ratios of 808 nm and 1064 nm laser (0.75 W cm^{-2}) through scalp and skull or chicken tissue with different thickness. c) Schematic illustration of the 808 nm and 1064 nm laser (0.75 W cm^{-2}) penetration experimental setup. d) Photothermal heating curves of CS NPs (12.5 μg mL^{-1}) under continuous irradiation by 808 nm and 1064 nm lasers (0.75 W cm^{-2}) penetrating through 2.5 mm chicken tissue.

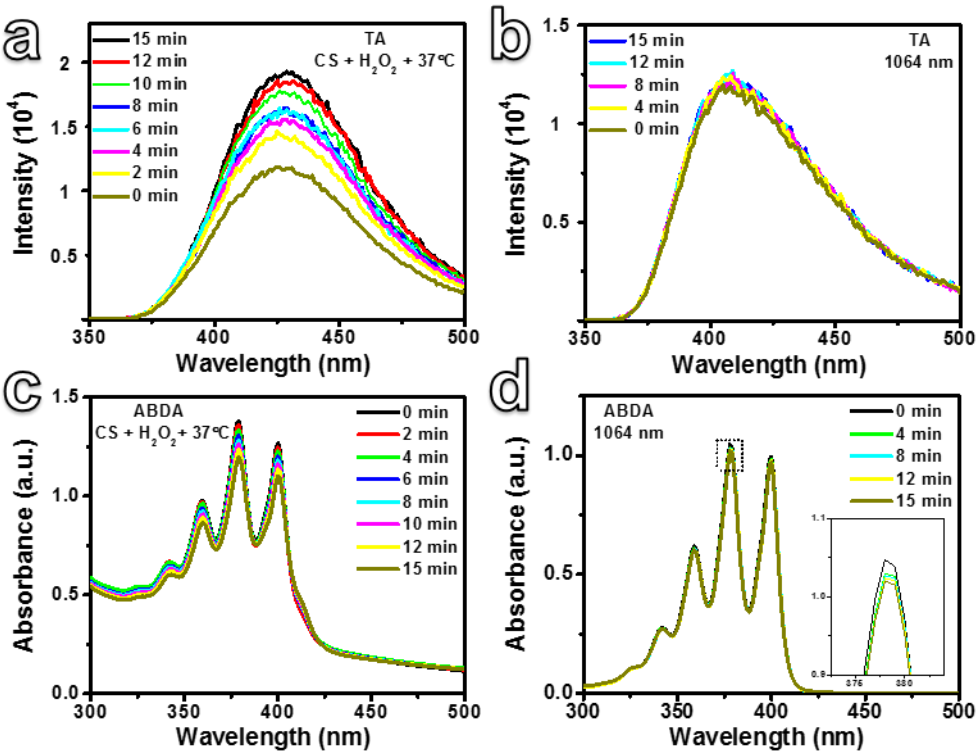

Fig. S3 Fluorescence spectra of terephthalic acid (TA) induced by •OH generation in the a) presence of CS (12.5 μg mL^{-1}) and H_2O_2 (400 μM) at 37 ℃ or b) in the absence of CS and H_2O_2 under irradiation by a 1064 nm laser (0.75 W cm^{-2}). UV-Vis spectra of ABDA induced by $^1O_2$ generation in the c) presence of CS (12.5 μg mL^{-1}) and H_2O_2 (400 μM) at 37 ℃ or d) in the absence of CS and H_2O_2 under irradiation by a1064 nm laser (0.75 W cm^{-2}), with the inset showing an enlargement of the indicated peak.

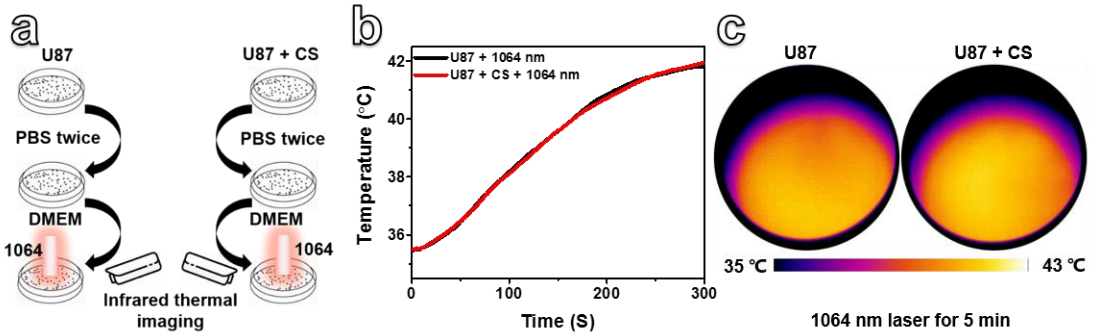

Fig. S4 a) Schematic illustration of the *in vitro* photodynamic therapy performance of CS-D NPs under 1064 nm laser irradiation (0.75 W cm^{-2}) for 5 min. b) Photothermal heating curves and c) thermal images of *in vitro* U87 cells under continuous 1064 nm laser (0.75 W cm^{-2}) irradiation for 5 min. DMEM: Dulbecco's Modified Eagle's medium.

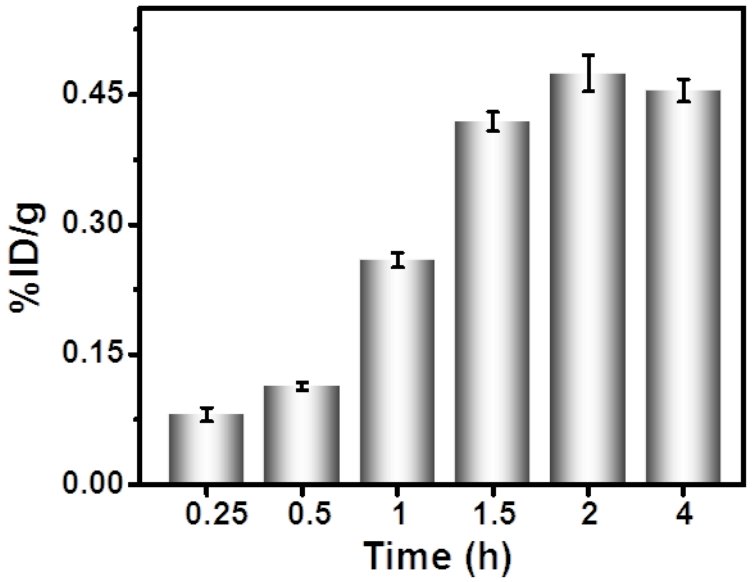

Fig. S5 Time-dependent cellular uptake of CS-D NPs (25 μg mL^{-1}) determined by inductively coupled plasma - mass spectrometry (ICP-MS).

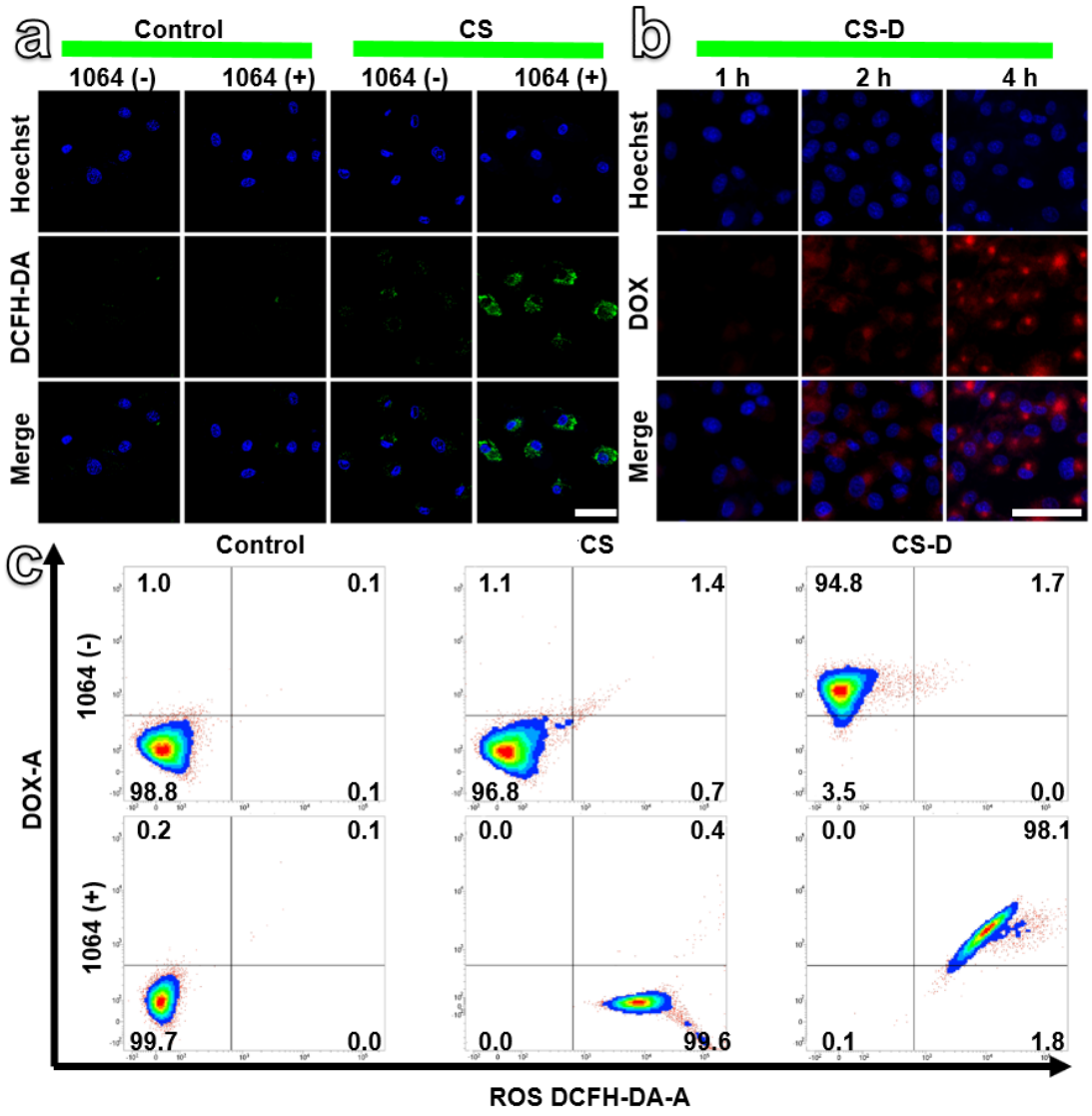

Fig. S6 Confocal laser scanning microscopy (CLSM) images of U87 cells cultured with/without CS NPs (25 μg mL^{-1}) and irradiated with/without a 1064 nm laser (0.75 W cm^{-2}): a) after staining with DCFH-DA for ROS radical detection (scale bar = 50 μm). b) CLSM images of U87 cells cultured with CS-D NPs (25 μg mL^{-1}) and intracellular DOX release at different times (scale bar = 50 μm). c) Flow cytometry analysis of ROS radical generation and DOX release under different conditions (CS NPs, 25 μg mL^{-1}; CS-D NPs, 25 μg mL^{-1}; 1064 nm laser, 0.75 W cm^{-2}; irradiation time: 5 min). The top left quadrant indicates the DOX release; the top right quadrant indicates the ROS radical generation and DOX release; the bottom right quadrant indicates ROS radical generation.

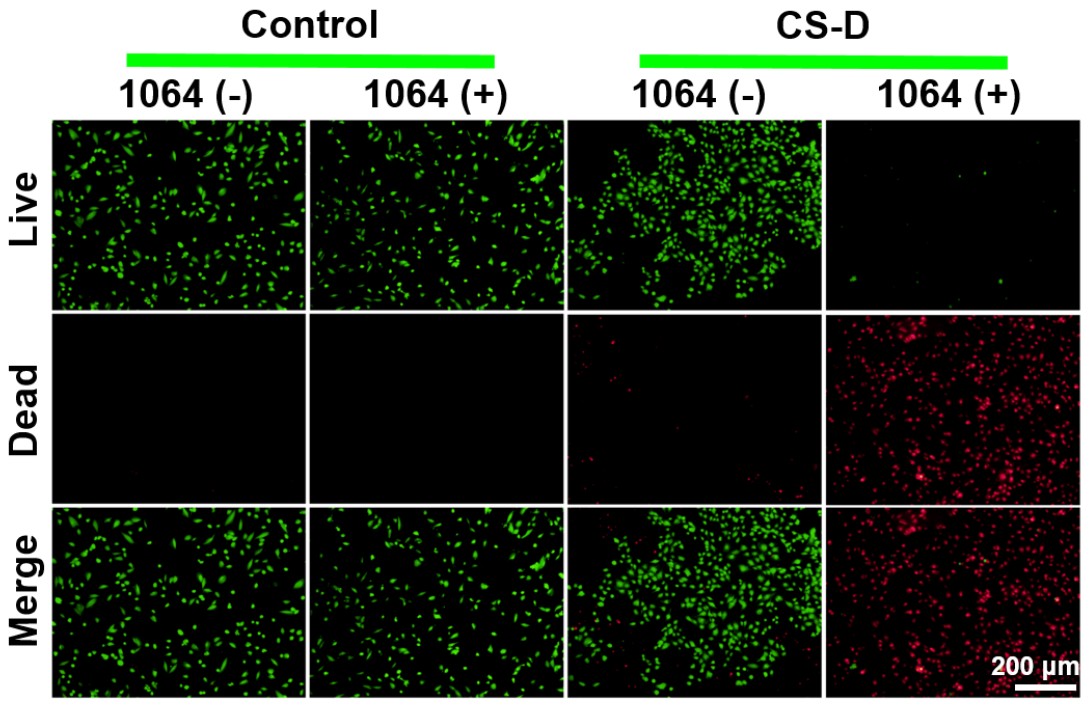

Fig. S7 Fluorescence images of U87 cells stained with a live/dead kit after different treatments (CS-D NPs, 25 μg mL^{-1}; 1064 nm, 0.75 W cm^{-2}, irradiation time: 5 min).

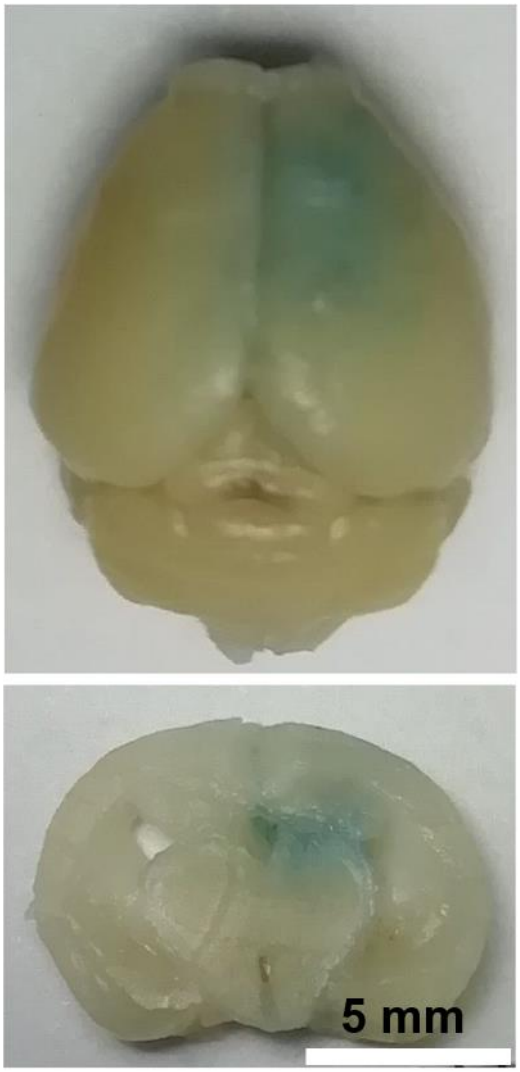

Fig. S8 Evans Blue (EB) staining of mouse brains after focused ultrasound induced opening of the BBB (EB injection dose: 30 mg kg^{-1}).

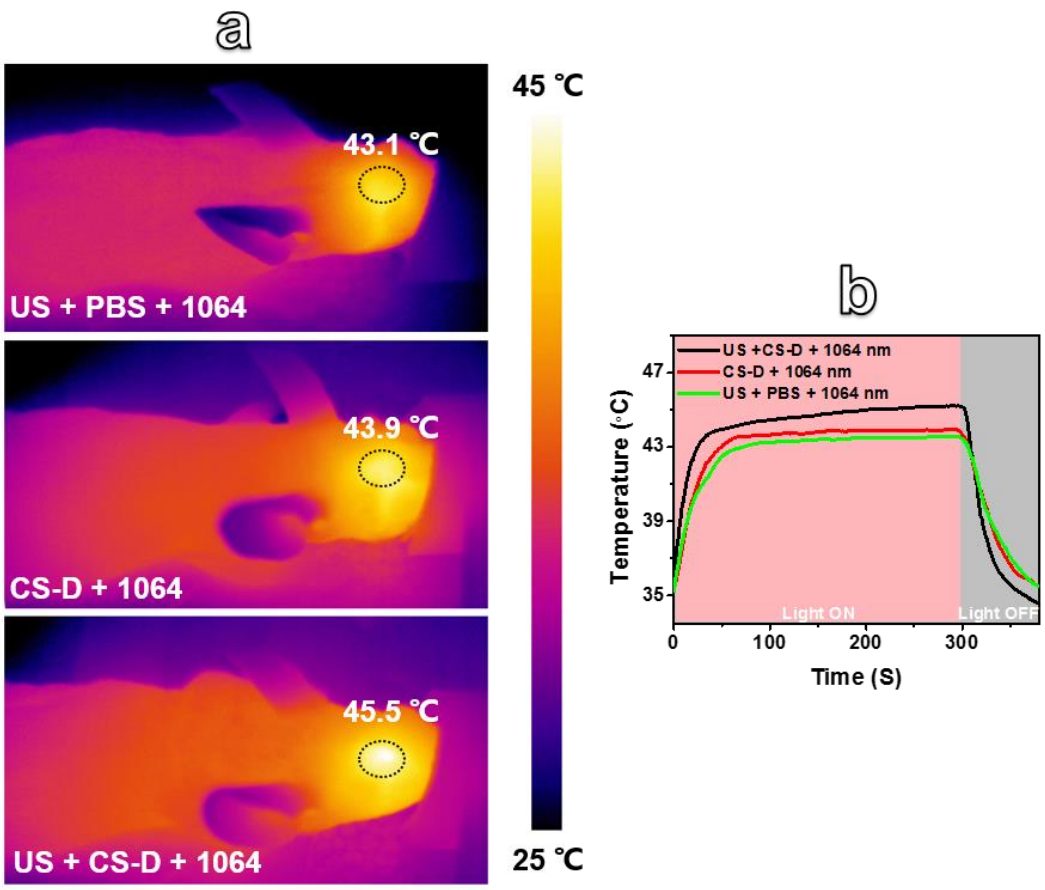

Fig. S9 a) Thermal images and b) Photothermal heating curves of the US + PBS + 1064, CS-D + 1064, and US + CS-D +1064 groups (CS-D NPs injection dose: 5 mg kg-1), (1064 nm, 1 W cm^{-2}, irradiation time: 5 min).

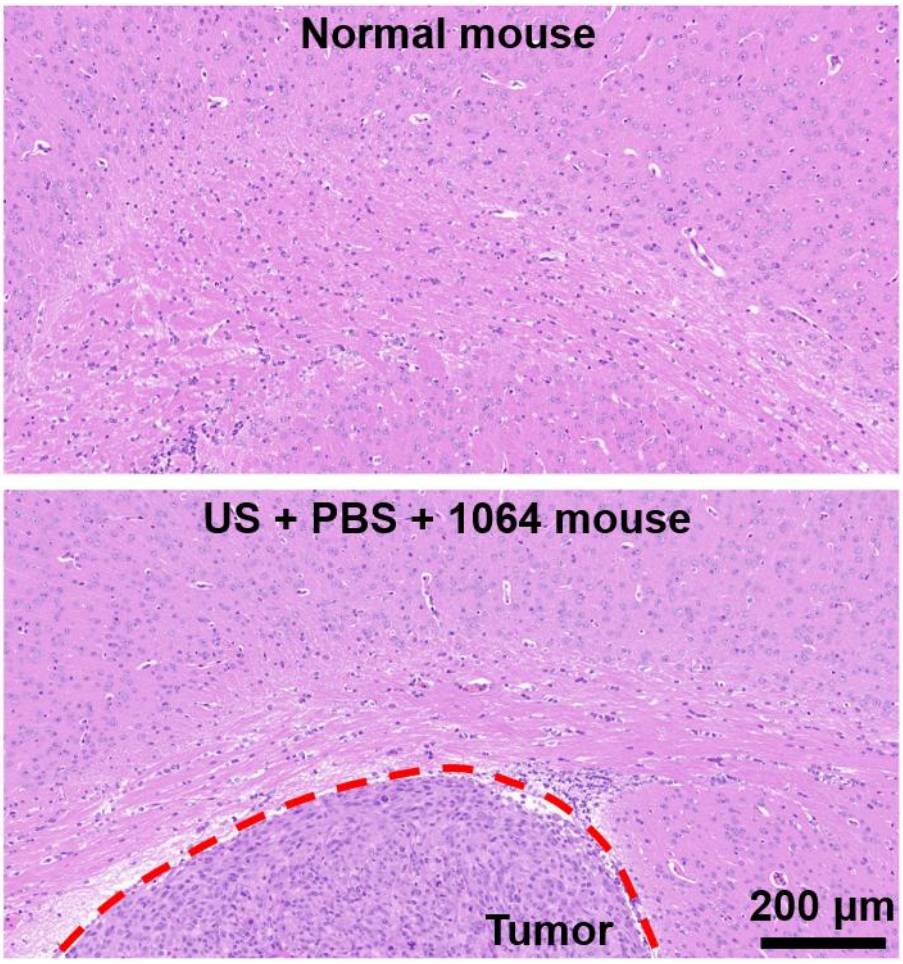

Fig. S10 H&E stained staining of brain slices from normal mouse and the mouse of US + PBS + 1064 group (1064 nm, 1 W cm^{-2}, irradiation time: 5 min).

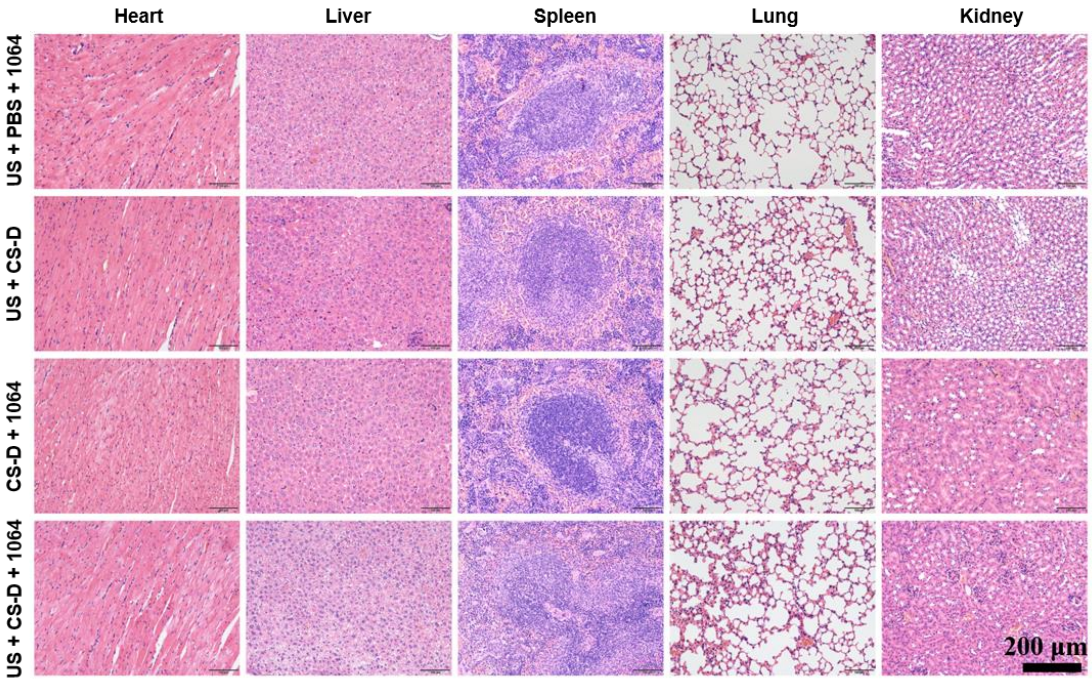

Fig. S11 H&E stained images of major organs, including the heart, liver, spleen, lung, and kidney, which were collected from the mice sacrificed at day 16 after the different treatments.