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SUPPORTING INFORMATION

Biodegradable Nano-photosensitizer with Photoactivatable Singlet Oxygen Generation for Synergistic Phototherapy

Jiaxin Shen,[a,b] Dandan Chen,[a] Ye Liu,[a] Guoyang Gao,[b] Zhihe Liu, [a] Guofeng Wang,[b] Changfeng Wu[a] and Xiaofeng Fang*[a]

[a]Department of Biomedical Engineering, Southern University of Science and Technology Shenzhen, Guangdong 518055, China.

[b]Key Laboratory of Functional Inorganic Material Chemistry, Ministry of Education, School of Chemistry and Materials Science, Heilongjiang University, Harbin 150080, China.

* Corresponding to: fangxf@sustech.edu.cn

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Materials

Biodegradable polymer poly(lactic acid) amine terminated (PLA; average Mn 4,000), poly(lactic-co-glycolide acid) acid terminated (PLGA; Mw 7,000-17,000), N,N-dimethylformamide (DMF), and Ce6 were purchased from J&K Chemical Ltd. N-ethyl-N-(3-(dimethylamino)propyl) carbodiimide hydrochloride (EDC·HCl) and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Anagi Co. Ltd. Singlet Oxygen Sensor Green (SOSG) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. Indocyanine Green Carboxylic Acid (ICG-COOH) was purchased from Xi'an Ruixi Biological Technology Co. Ltd.

Preparation of ICG-Ce6-doped NPs, Ce6-doped NPs, and ICG-doped NPs

PLGA, PLA, ICG-COOH and Ce6 were dissolved in DMF to prepare for stock solutions (1 mg/mL). PLA was coupled with Ce6 and ICG-COOH through amide coupling reactions. 0.33 mL PLA and 0.5 mL Ce6 or ICG were mixed and then 500 μL EDC (10 mg/mL) was added; the mixture was stirred at room temperature in the dark for 12 h. Then, the solution mixtures with mass-ratio$_{Ce6:\ ICG}$ = 1:2 and 1:4 were each added into the PLGA solution, making sure that the Ce6 mass accounted for 5% of the whole system. After that, the solution mixture was immediately added to 10 mL of Milli Q water under sonication for 2 min. The hydrophilic nanoparticles were formed by self-assembly of polymer chains. Next, the nanoparticles were dialyzed via dialyzers (Mw 3500) with methanol and water for three times under dark conditions to remove DMF and uncoupled small dye molecules. Finally, the solution was

filtrated by a 0.22 μM filter to prepare for further use. The obtained ICG-Ce6-doped NPs (mass-ratio$_{Ce6:\ ICG}$ = 1:2 was named *NPs 1:2* and mass-ratio$_{Ce6:\ ICG}$ = 1:4 was named *NPs 1:4*) were stored at 4^{o}C.

Characterizations of NPs

Particle sizes and zeta potentials were evaluated by dynamic light scattering (DLS, Malvern Zetasizer Nano ZS). UV-Vis-NIR absorbance spectra of Ce6-doped NPs and ICG-Ce6-doped NPs (C_{Ce6} = 2 μg/mL) were detected by a spectrometer (Shimadzu UV-2600) for comparison of the absorption spectra of different doping ratios and spectra changes after the degradation of ICG. Fluorescent spectra were recorded by a fluorescence spectrometer (Hitachi F-4500) with excitation at 400 nm.

Evaluation of the Singlet Oxygen Generation Ability of NPs

Singlet oxygen sensor green (SOSG) was used to evaluate the singlet oxygen generation of ICG-Ce6-doped NPs and Ce6 upon light irradiation. In these NPs, ICG acted as the singlet oxygen quencher of the PDT process. SOSG was dissolved in methanol (5 mM) and diluted to 100 μM with MilliQ water. The NP solutions with different doping ratios containing the same concentration of Ce6 (2 μg/mL) were mixed with SOSG so that the final concentration of SOSG in the mixed solution was 10 μM. The mixed solution was irradiated by a 660-nm light emitting diode (LED, 50 mW/cm^2) for different lengths of time, and the fluorescence spectra were recorded. Then the fluorescence intensity of SOSG (Ex = 460 nm, Em = 525 nm) in different nanoparticle solutions was used to evaluate the generation of singlet oxygen. The

florescence of SOSG was regarded as the control to be removed from each part, so changes in the fluorescence were used to show the generation of singlet oxygen within 10 min. In addition, singlet oxygen generation of different NPs after being activated by near-infrared light was also evaluated by SOSG. Before the evaluation of singlet oxygen generation, NPs with the same Ce6 concentration (2 μg/mL) were irradiated by NIR light at 808 nm (1 W/cm^2) for 5 min. When the ICG was removed completely, SOSG was added to repeat the above procedures to test the singlet oxygen generation ability.

***In Vivo* NIR Fluorescence Imaging of NPs**

All experiments involving animals followed regulations and guidelines stipulated by the Animal Care and Use Committee (IACUC) of Southern University of Science and Technology. BALB/c mice were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The female BALB/c ageing from 5-6 weeks were used for experiments. To build the 4T1 tumor model, each mouse was injected with 2×10^6 4T1 cells in 100 μL PBS to the back. After that, the mice bearing 4T1 tumors were bred at an animal care center until the tumor size grew to 100 mm^3.

To investigate the changes in NIR fluorescence, the tumor-bearing mice were intratumorally injected with 50 μg/mL of ICG-Ce6-doped NPs (mass-ratio$_{Ce6:\ ICG}$ = 1:4). After 24 h, the ICG fluorescence signal at the tumor sites was recorded by a Near-Infrared II small living animal fluorescence imaging system (NIR-OPTICS Series III 900/1700) with an optical filter (LP 1000). Then, the mouse was moved to a small animal imaging system (IVIS Lumina Series III PerkinElmer) to detect the

fluorescence changes of Ce6 with excitation at 525 nm and emission at 660 nm. When ICG was totally removed by directly irradiating the tumor sites for 10 min with NIR light (808 nm, 1 W/cm^2), the disappearance of the ICG fluorescent signal was observed by a Near-Infrared II imaging system. Meanwhile, the changes in the Ce6 fluorescence signal before and after ICG degradation were visually observed under the same setting conditions.

In Vivo Synergistic Therapy with NPs

For thermal imaging of the PTT process, the tumor-bearing mice were randomly divided into two groups after the tumor sizes reached ~100 mm^3. The mice injected with PBS (60 μL) were marked as the control group, and the mice injected with NPs (50 μg/mL, 60 μL) were marked as the experimental group. When the tumor region of the mice was irradiated using NIR light at 808 nm (1 W/cm^2), the temperature increases in the tumor region were recorded by the FLIR E6 thermal imaging camera (FLIR System, Inc., USA).

For the evaluation of synergistic therapy effects of NPs, BALB/c mice bearing 4T1 were randomly divided into five groups of four. When the tumor sizes reached ~100 mm^3, the experiments were carried out under different light conditions: (i) injection of PBS (60 μL) without irradiation; (ii) injection of NPs (50 μg/mL, 60 μL) without irradiation; (iii) injection of NPs (50 μg/mL, 60 μL), irradiated with red light (660 nm, 50 mW/cm^2, 10 min); (iv) injection of NPs (50 μg/mL, 60 μL), irradiated with NIR light (808 nm, 1 mW/cm^2, 10 min); and (v) injection of NPs (50 μg/mL, 60 μL), irradiated with NIR light (808 nm, 1 mW/cm^2, 10 min) then irradiated with a red

light (660 nm, 50 mW/cm^2, 10 min). After that, the mice were continuously kept in the sterile environment of the animal experimental center for 10 days, and the weight and tumor volume of each mouse were recorded every two days from day 0. The tumor volume was measured using a digital caliper, and weight was recorded by a digital balance. An equation was used to calculate the tumor volume: volume = 1/2×(tumor length)×(tumor width)^2. For histology analysis, the organs of mice were fixed in 4% neutral buffered paraformaldehyde. The main organs including heart, liver, spleen, lung, and kidney were collected for H&E staining.

Evaluation of the Stability of NPs

ICG-Ce6-doped NPs were placed under dark conditions for 14 days, and the changes in the emission and absorption spectra were compared before and after the 14 days. Meanwhile, fetal bovine serum (FBS) (10%) was added to ICG-Ce6-doped NPs to simulate the environment in vivo, as FBS can cause swelling conditions to cause leakage of small dyes. Under dark conditions for 24 h, the solution mixture was centrifuged 5 times by ultrafiltration centrifugation (MWCO, 100 kDa), and then the absorbance of the solution at the bottom of centrifuge tube was measured.

Cell Culture and Cytotoxicity Assay

4T1 cells (mouse breast cancer cells) were cultured in an incubator containing 5% CO_2 with a constant temperature at 37^{o}C. The cells were cultivated by RPMI 1640 containing 1% penicillin/streptomycin and 10% FBS. The activities of various types of NPs were detected by MTT assay, and 4T1 cells were seeded in the 96-well cell

culture plates with a density of 2×10^4 cells per well and incubated at 37°C for 24 h. After that, the fresh medium containing free Ce6 and ICG-Ce6-doped NPs with various concentrations of Ce6 (0-8 μg/mL) were added to specific wells, and then the cells with NPs were incubated for 24 h until the NPs were internalized by cells. Next, the medium containing NPs was washed with PBS three times, and then the cells were cultured in fresh medium for 8 h. After that, a 660-nm LED light at 50 mW/cm^2 was used to irradiate cells for 10 min. Subsequently, MTT (20 μL, 5 mg/mL) was added to each well, and the materials were incubated for an extra 4 h before the addition of dimethyl sulfoxide (150 μL well^{-1}). Finally, the cell viability was recorded by a microplate reader (BioTek Cytation 3) evaluating the absorbance value (OD_{490}) of each well. Cell viability of ICG-Ce6-doped NPs was also detected under different light conditions, including no light, irradiation at 808 nm (1 W/cm^2, 10 min), and irradiation at 808 nm (1 W/cm^2, 10 min) followed by irradiation at 660 nm (50 mW/cm^2, 10 min). Other procedures were the same as the aforementioned. After incubation for 24 h, the conventional MTT assay was carried out.

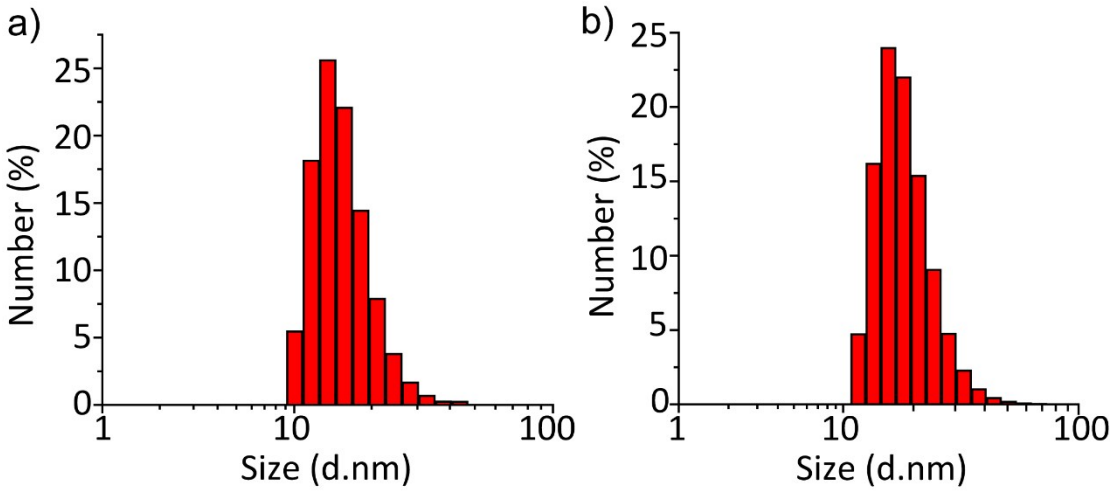

Figure S1. (a) DLS size distribution of Ce6：ICG (1:2). (b) DLS size distribution of Ce6：ICG (1:1).

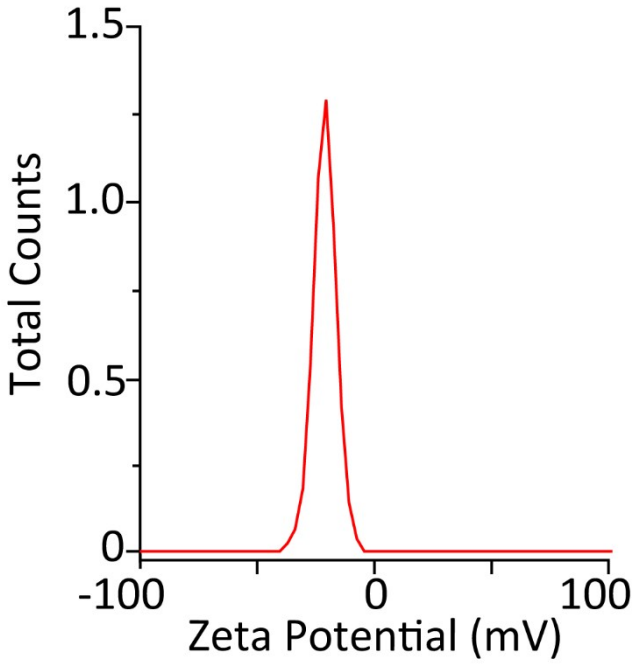

Figure S2. The Zeta potential of Ce6-ICG-doped PLGA nanoparticles.

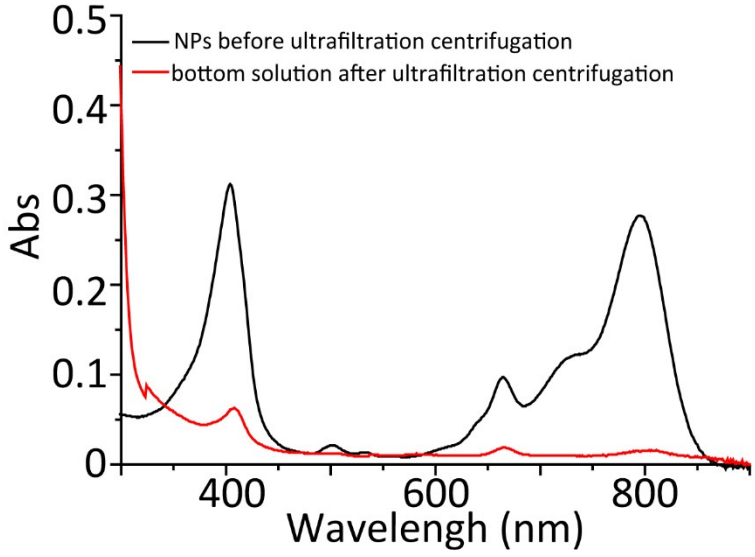

Figure S3. The simulation of micro-environment in vivo with 10% FBS, the US-vis-NIR spectra of NPs and bottom solution after ultrafiltration centrifugation.

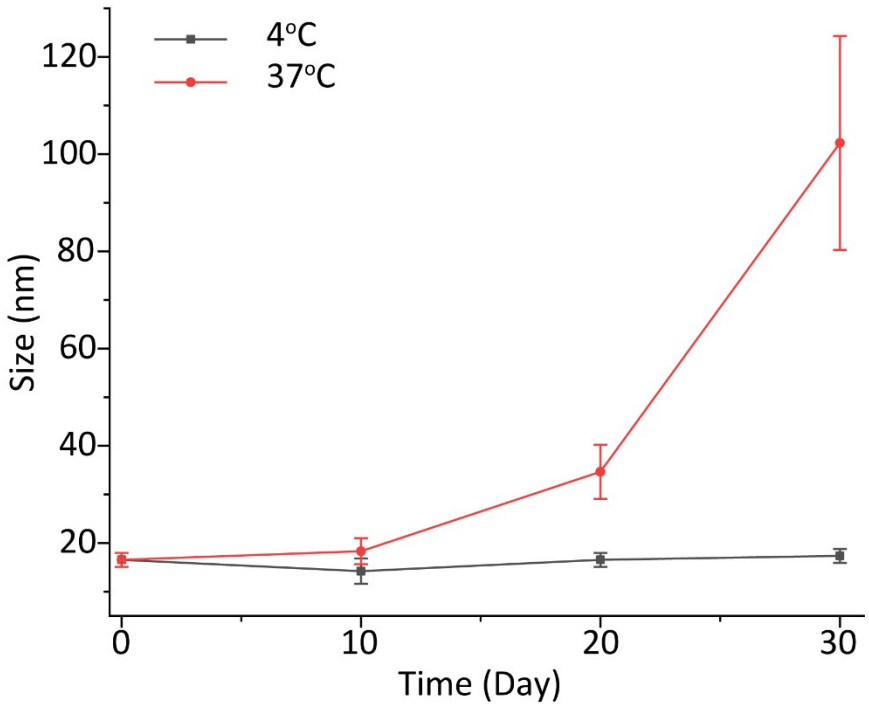

Figure S4. The size distributions of nanoparticles stored at 37°C and 4°C.

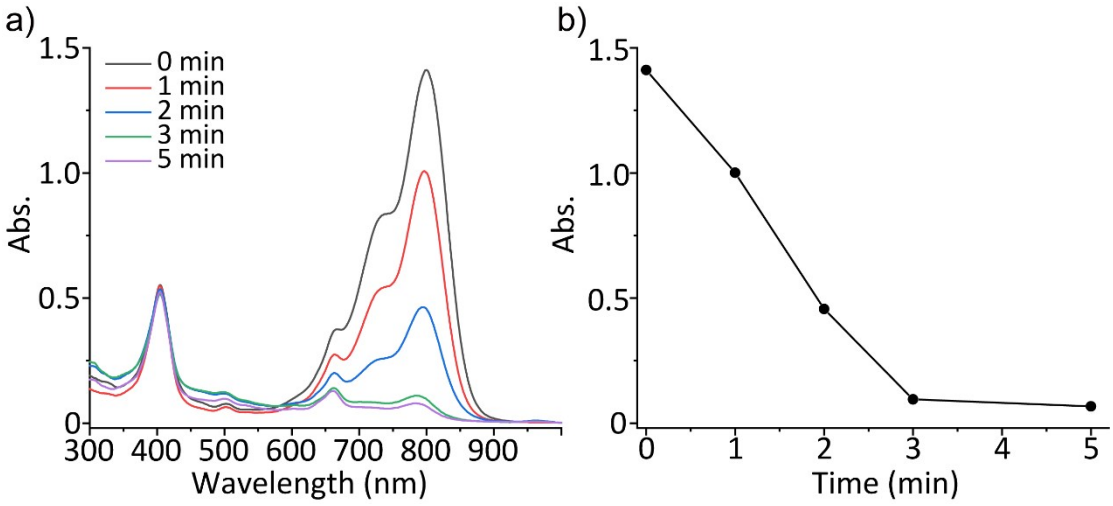

Figure S5. The absorbance spectra of Ce6-ICG-doped nanoparticles irradiated at 808 nm for different time (a) and the corresponding absorbance at 800 nm for different time.

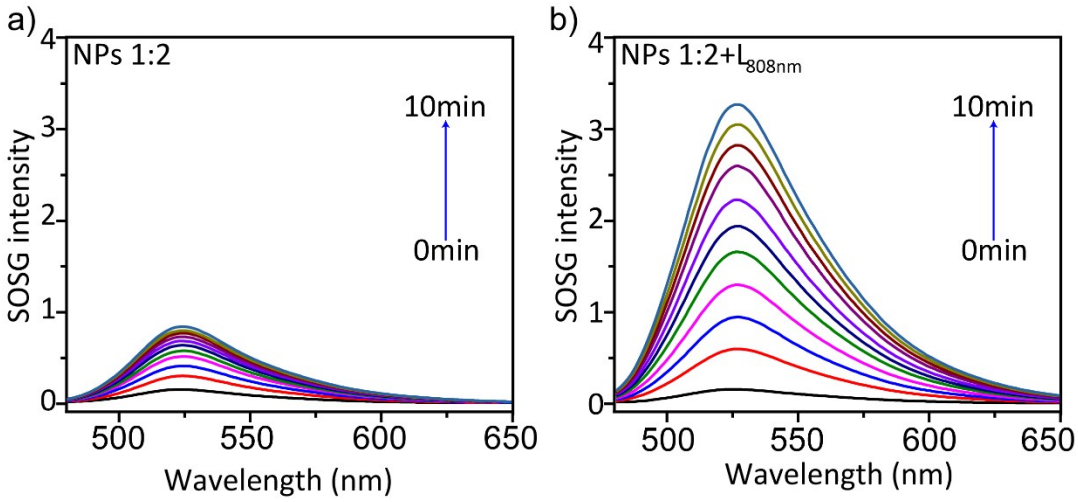

Figure S6. Spectral changes of SOSG in the presence of Ce6：ICG (1:2) after light irradiation at 660 nm for 10 min.

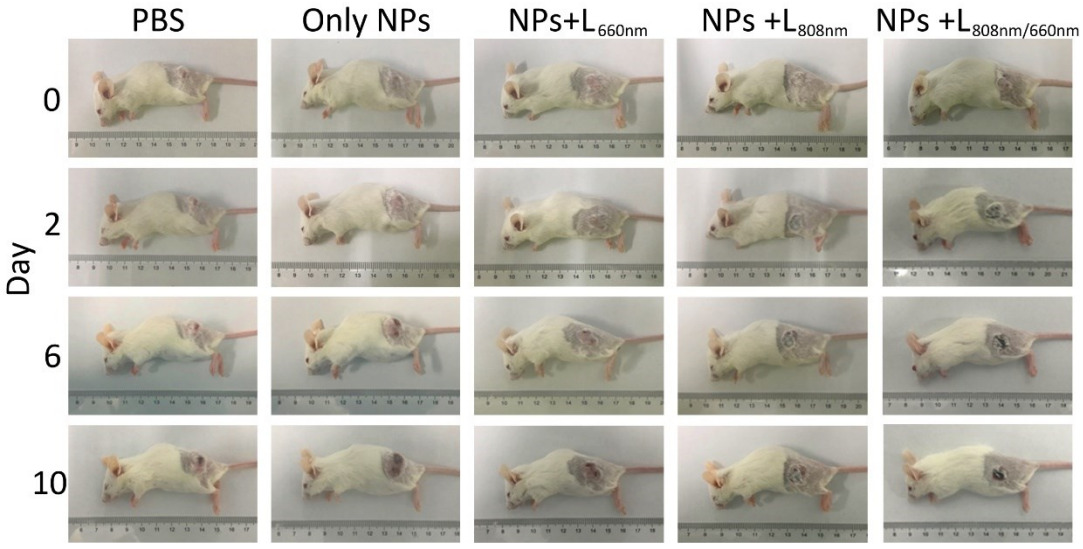

Figure S7. Picture of representative tumor-bearing mice with different conditions, including PBS and NPs without light irradiation; NPs with 660 nm LED light irradiation; NPs with 808 nm laser irradiation and NPs with 660 nm LED light irradiation after 808 nm laser irradiation.

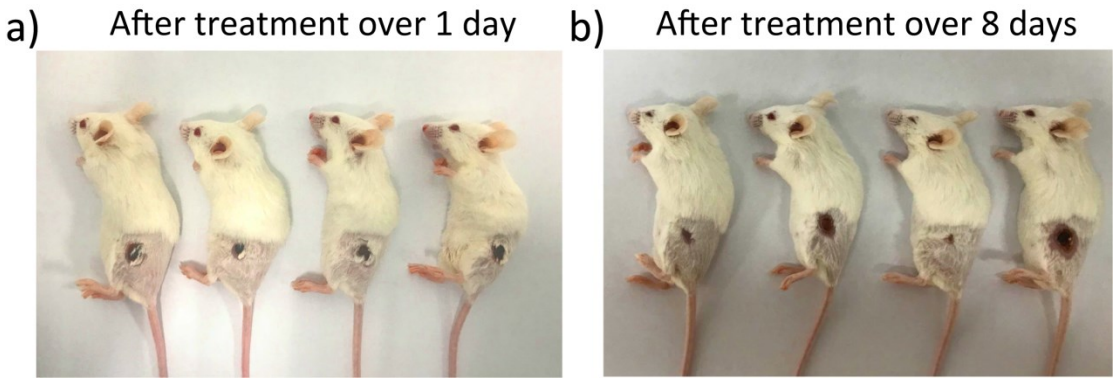

Figure S8. Comparison of survival of mice after PTT combined activatable PDT treatment over 1 day and 8 days.

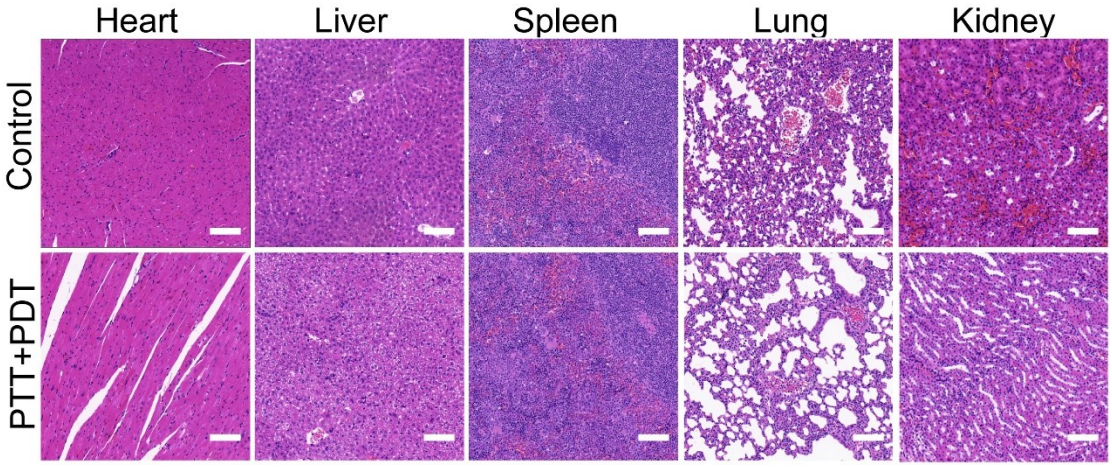

Figure S9. H&E staining of organs collected from tumor-bearing mice after treatment. Scale bar: 200 μm