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Supplementary Information

Tumor-targeted nanoflowers to regulate glutamine metabolism and amplify oxidative stress for synergistic therapy

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Chemicals.

Meso-Tetra(4-carboxyphenyl)porphine and Zirconyl chloride octahydrate were purchased from Shanghai Macklin Biochemical Co., Ltd. Bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulfide (BPTES) was purchased from APExBIO Technology LLC. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Rhodamine 123 were purchased from Sigma. Annexin V-FITC Apoptosis Detection Kit and Calcein-AM/PI Double Stain Kit were purchased from Beyotime (Nantong, China). ATP content determination kit was purchased from Shanghai Enzyme-linked Biotechnology Co., LTD. MDA-MB-231 cells were obtained from Aoluo Biotechnology Co. Ltd. (Shanghai, China). The normal immortalized human mammary epithelial cell line (MCF-10A) was purchased from Procell. (Wuhan, China). All the other reagents and solvents were analytical grade and used directly without further purification. Sartorius ultrapure water (18.2 $M\Omega \cdot cm^{-1}$) was used for experiments.

Measurement and characterization.

Transmission electron microscopy (TEM, HT7700, Japan) was employed to characterize the morphologies of the nanomaterials. Powder X-ray diffraction (XRD) pattern was obtained on a Rigaku Smart Lab SE X-Ray Powder Diffractometer with Cu Kα line focused radiation (λ = 1.5405 Å). Absorption spectra were measured on the UV-1700 UV-visible spectrophotometer (Shimadzu, Japan). Fourier infrared spectrometer (Nicolet iS50 FT-IR) was used to characterize the infrared spectrum. In the MTT assay, the absorbance was measured using a microplate reader (Synergy 2, Biotek, USA). Cell disruption was performed using a homogenizer (IKA, Germany, T10 basic ultra-turrrax). All pH measurements were performed with a digital pH-meter (pH-3e, LeiCi, China). Confocal fluorescence images were captured using TCS-SP8 confocal laser scanning microscope (Leica, Germany). In vivo fluorescence images were captured using live animal imaging system (IVIS Lumina III, US).

Synthesis of PCN-224.

H_2TCPP (100 mg, 0.13 mmol), $ZrOCl_2 \cdot 8H_2O$ (300 mg, 0.93 mmol) and benzoic acid (2.8 g, 23 mmol) were dissolved in DMF (250mL) and the mixture was heated at 90 °C for 5 h under stirring. PCN-224 were gathered by centrifugation at 12000 revolutions per minute (rpm) for 15 min and washed with fresh DMF for three times. The obtained nanoparticles were dispersed in DMF for further analysis and characterization.

Synthesis of PCN-224@MnO_2（PM）.

3 mg as-prepared PCN-224 nanoparticles were dispersed in 10mL of water. Subsequently, 1 mL (9 mg) $KMnO_4$ were added in the solution and the mixture was sonicated for 3 hours. PCN-224@MnO_2 were separated by centrifugation at 12000 revolutions per minute (rpm) for 15 min and washed with water for three times. The PCN-224@MnO_2 were dispersed in water for further analysis and characterization.

Synthesis of BPTES@PCN-224@MnO_2 (BPM).

The inhibitor loading experiment was performed before coating PEG-FA, through the mixing of BPTES (10 mg) and PCN-224@MnO_2 (10 mg) into 20 mL deionized at room temperature for 24 h. The prepared BPTES@PCN-224@MnO_2 were collected by centrifugation and washed three times.

Synthesis of BPTES@PCN-224@MnO_2-PEG-FA (BPMF) and the release of BPTES in vitro.

20 mg NH_2-PEG-FA was fully dissolved in 10 mL DMSO and water (v/v = 1:1), then the BPTES@PCN-224@MnO_2 were added into the mixed solution. Subsequently the mixed solution was stirred overnight, and the BPTES@PCN-224@MnO_2-PEG-FA were collected by centrifugation. The prepared BPTES@PCN-224@MnO_2-PEG-FA were dispersed in aqueous solution for further use.

The synthesized BPMF was dispersed in PBS buffer solution with pH=5.5 and 7.4, GSH with different concentrations was added, and stirred at 600 rpm at room temperature for different times (0, 1, 2, 3, 4, 8, 10, 12, 24, 48 h). The solution was collected and centrifuged at 15000 rpm to obtain the supernatant. Its absorbance was measured by UV-vis spectrophotometer.

Detection of GSH.

The experiment of GSH consumption was implemented with DTNB. The BPM with different concentrations were dissolved in the GSH solution (5 mM). The solution reacted in Tris-HCl (pH=8.0) at 37 ℃ for 60 min and the supernatant was mixed with DTNB (5 mM) for 10 min. The absorption at 412 nm was detected by a microplate reader.

Cell culture.

All the cells were maintained in a humidified atmosphere with 5 % CO_2 at 37 ℃. Dulbecco' s modified Eagle 's medium (DMEM) medium containing 10 % fetal bovine serum and 1 % penicillin reptomycin were used to culture MDA-MB-231 cells. MCF-10A cells were cultured with the special culture medium.

Cytotoxicity test.

MDA-MB-231 cells were dispersed in 96-well plates and incubated for 24 h, PF, PMF and BPMF with different concentrations (0,25,50,75,100 μg/mL) in DMEM medium were incubated for 6 h. Then fresh culture medium was added and incubate for another 12 hours. Then the culture medium was removed from the 96-well, and 150 μL of MTT solution (0.5 mg/mL) was added. After 4 h, the MTT solution was removed, and 150 μL of DMSO was added. The absorbance at 490 nm was monitored by a microplate reader.

Detection of ROS in cells.

MDA-MB-231 cells were cultured in confocal dishes for 24 h and random divided into 5 groups (PBS, Laser, PF+Laser, PMF+Laser, BPMF+Laser). The cells were treated with PF, PFM, BPMF at a concentration of 100 μg/mL and incubated for 4 h. For all the laser irradiation groups, 635 nm laser (0.52 W/cm^2) was utilized to irradiate the cells for 10 min. All the groups were further cultured for overnight. Before laser irradiation, cells from all groups were incubated with DMEM containing of

DCFH-DA (10 μM) for 30 min before laser irradiation. Finally, the cells were washed three times with PBS and imaged with CLSM

Detection of mitochondrial transmembrane potential (ΔΨm).

MDA-MB-231 cells were cultured in confocal dishes for 24 h and random divided into 5 groups (PBS, Laser, PF+Laser, PMF+Laser, BPMF+Laser). The cells were treated with PF, PFM, BPMF at a concentration of 100 μg/mL and incubated for 4 h. For all the laser irradiation groups, 635 nm laser (0.52 W/cm^2) was utilized to irradiate the cells for 10 min. All the cells were further cultured for 12 hours. Finally, the cells were stained with Rhodamine 123 at 37 ℃ for 30 min and washed with PBS and analyzed with CLSM.

Detection of GSH in cells.

MDA-MB-231 cells were inoculated in a 60 mm petri dish for 24 hours and were randomly divided into 5 groups (PBS, Laser, PF+Laser, PMF+Laser and BPMF+Laser). Cells were treated with 100 μg/mL PF, PFM and BPMF, respectively, and incubated for 4 h. All groups were irradiated with 635 nm (0.52 W/cm^2) laser for 10 min and then cultured for 12 h. wash with PBS for one time. The three times volume of cell precipitate protein removal reagent S solution were added. Then the solution was froze-thawed twice using liquid nitrogen and 37℃ water. The solution was placed in ice bath for 5 minutes. The supernatant was acquired by ultracentrifuge and was used for the determination of total glutathione.

Live/dead cell staining assay.

MDA-MB-231 cells were cultured in confocal dishes for 24 h. The cells were divided into 5 groups (PBS, Laser, PF+Laser, PMF+Laser, BPMF+Laser). The cells were treated with PF, PFM, BPMF at a concentration of 100 μg/mL and incubated for 4 h. For all the laser irradiation groups, 635 nm laser (0.52 W/cm^2) was utilized to irradiate the cells for 10 min. All the groups were further cultured for 12h. Subsequently, calcein-AM/PI probe was added to the confocal dishes and incubated for another 15 min. Finally, the cells were washed three times with PBS and imaged with CLSM (Ex: 488 nm, Em: 500-560 nm).

Flow cytometry analysis of apoptosis.

MDA-MB-231 cells were cultured in cell dishes with DMEM for 24 h and randomly divided into five groups (PBS, Laser, PF+Laser, PMF+Laser, BPMF+Laser). Each group of cells incubated with PF, PFM, BPMF at a concentration of 100 μg/mL and incubated for 4 h. For all the laser irradiation groups, 635 nm laser (0.52 W/cm^2) was utilized to irradiate the cells for 10 min. All the groups were further cultured for 12h. After that, the cells were incubated with Annexin V-FITC/PI for 15 min and washed with PBS for three times. Finally, MDA-MB-231 cells were resuspended in 50 μL PBS for flow analysis.

ATP content determination:

MDA-MB-231 cells were inoculated in 100 mm petri dish for 24 h, and then cultured with 5, 15, 25, 50 and 100 μg/mL BPMF for 24 h. Then, the cells were washed three times with PBS and collected into

the centrifugal tube, and the supernatant was centrifuged. Extract (0.4 mL) was added and ultrasound was used to break cells (ice bath, 200W, ultrasound 3S, 10S interval, repeat 30 times). The mixture was centrifuged at 12000 rpm for 10min. The supernatant was taken and placed on ice for testing.

Establishment of tumor model.

Animal experiments were reviewed and approved by the Ethics Committee of Shandong Normal University, Jinan, P. R. China (approval number AEECSDNU2021072). All animal experiments were conducted and obeyed the Principles of Laboratory Animal Care (People's Republic of China) and the Guidelines of the Animal Investigation Committee, Biology Institute of Shandong Academy of Science, China. Female nude mice (6 weeks old, ~16 g) were raised under normal circumstances of free access to water and goods. 1×10^7 MDA-MB-231 cells in 100 μL of serum-free RPMI DMEM medium were injected subcutaneously into the right axillary region of the nude mice. The mice were used into subsequent therapy experiments until the tumor volume had reached approximately 75-100 mm^3.

In vivo targeting and biodistribution analysis.

The synthesis of IR808@PCN-224@MnO_2 and IR808@PCN-224@MnO_2-PEG-FA were according to the methods previously reported. The prepared equal amount (50 μL, 4 mg/mL) of IR808@PCN-224@MnO_2 or IR808@PCN-224@MnO_2-PEG-FA were injected into each group of nude mice through the caudal vein. the IR808 fluorescence of nude mice were recorded with a live body imaging system at different time points. Afterward, the nude mice bearing MDA-MB-231 tumors were sacrificed and the fluorescence of the tumor, heart, liver, spleen, lung, and kidney were acquired using a live body imaging system.

In vivo antitumor experiment.

The MDA-MB-231 tumor bearing nude mice were randomly divided into six groups (PBS, Laser, PF+Laser, PMF+Laser, BPMF, BPMF+Laser). The nanoparticle (10mg/kg) was administered to each mouse by intravenous injection. For all the laser irradiation groups, 635 nm laser (0.52 W/cm^2) was utilized to irradiate the tumor site for 10 min. After that, the tumor growth and body weight change situations of the nude mice were observed and recorded within 14 days. For H&E staining assay: 14 days after different treatments, all the nude mice were sacrificed, the major argan were collected and staining with hematoxylin and eosin.

Statistical Analysis

All data were reported as the mean ± s.d.; n = 3 for all in vitro. n = 5 for in vivo anticancer studies. The difference between two groups was analyzed by independent sample t-test. The statistical significance was indicated as $p < 0.05$, and ex-pressed as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

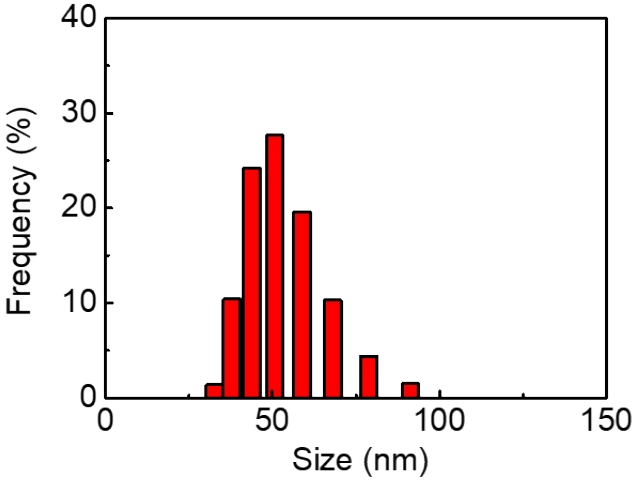

Fig. S1. The size of PCN-224 measured by DLS in water.

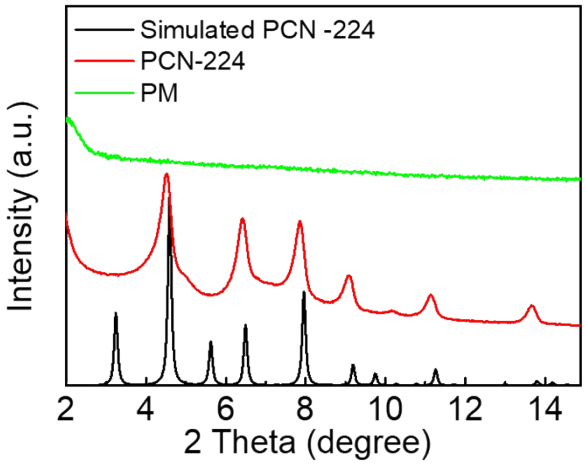

Fig. S2. PXRD patterns for simulated PCN-224, experimental PCN-224 and PM.

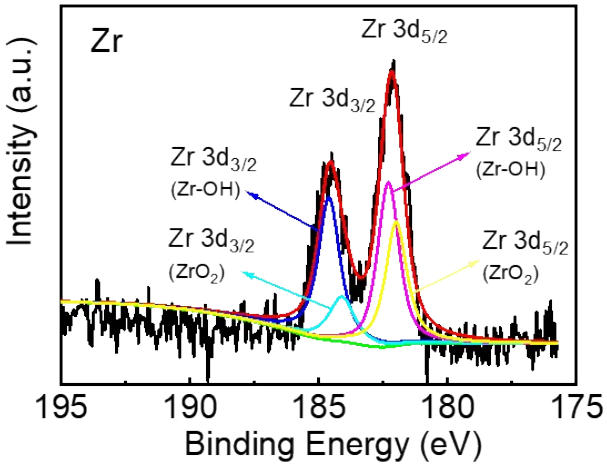

Fig. S3. The high-resolution XPS spectra of Zr in PCN-224.

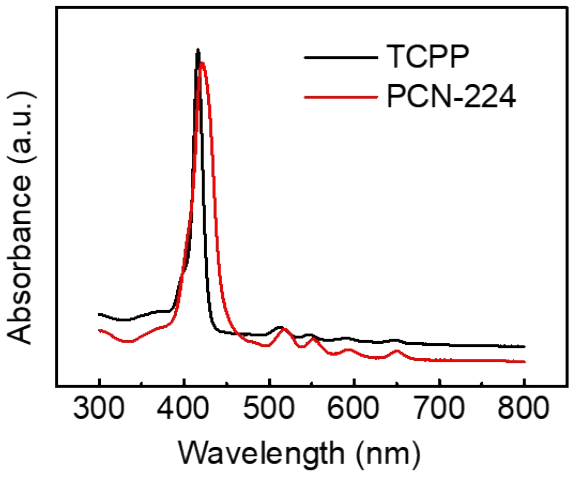

Fig. S4. UV-vis spectrum of TCPP and PCN-224.

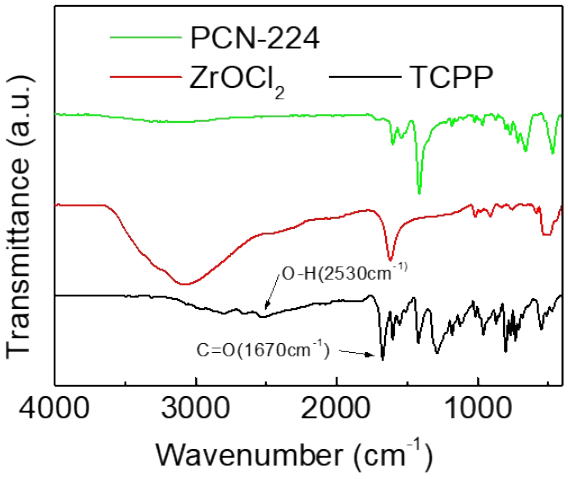

Fig. S5. FT-IR spectra of TCPP, $ZrOCl_2$ and PCN-224.

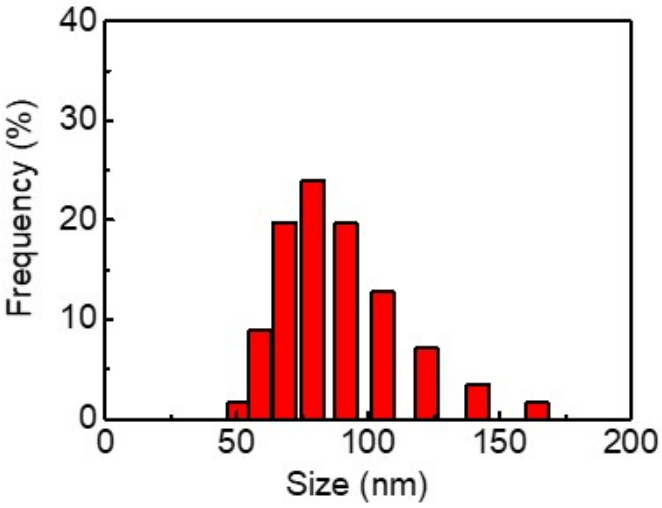

Fig. S6. Size distribution of PM NFs measured by DLS in water.

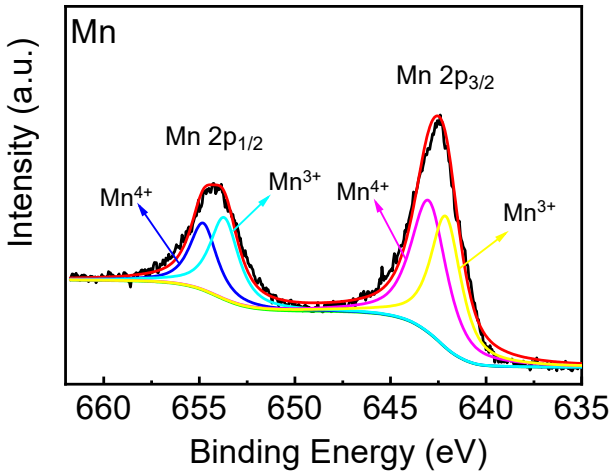

Fig. S7. The Mn $2p_{1/2}$ peaks in the high-resolution XPS spectra of PM NFs.

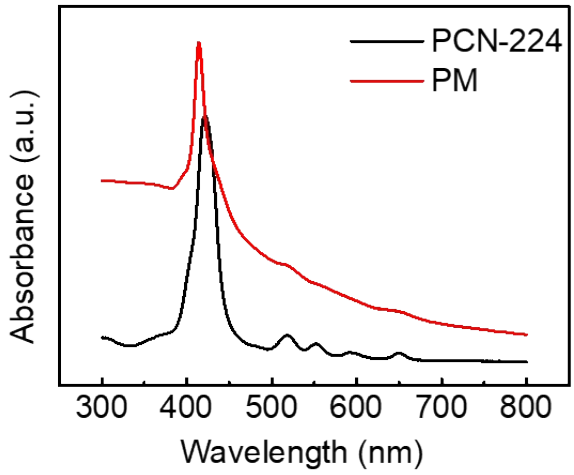

Fig. S8 The UV-vis spectra of PCN-224 and PM.

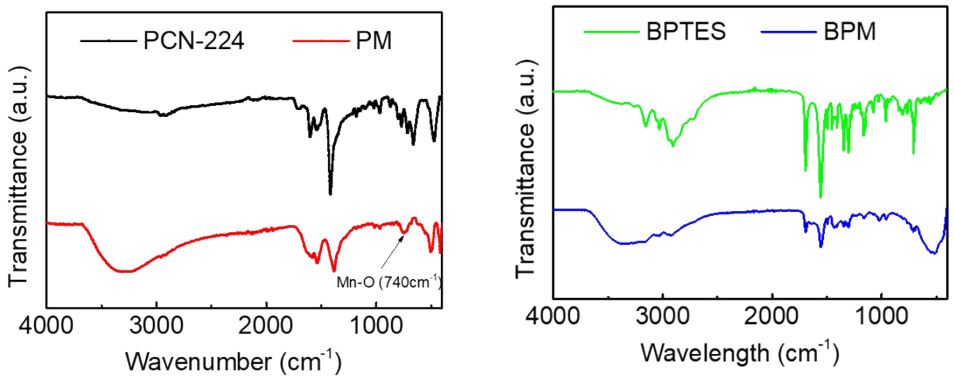

Fig. S9. The FT-IR spectra of PCN-224, PM, BPTES and BPM.

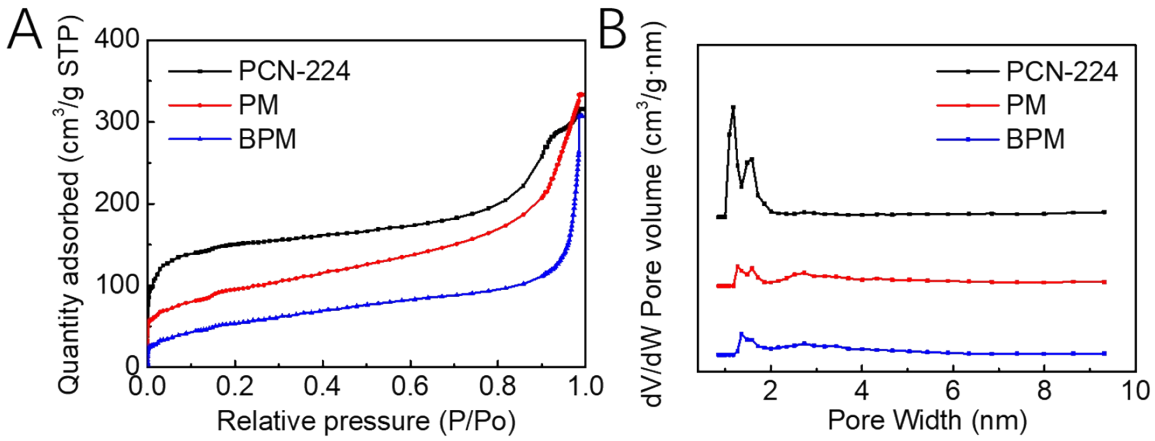

Fig. S10. N_2 adsorption-desorption isotherms (A) and pore size distributions (B) of PCN and PM.

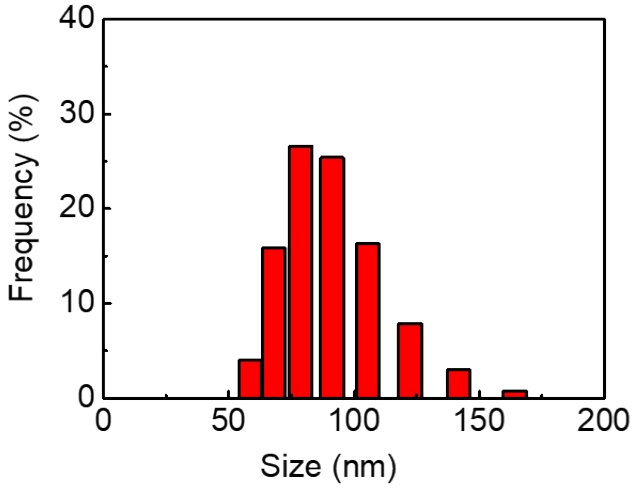

Fig. S11. Size distribution of BPM measured by DLS in water.

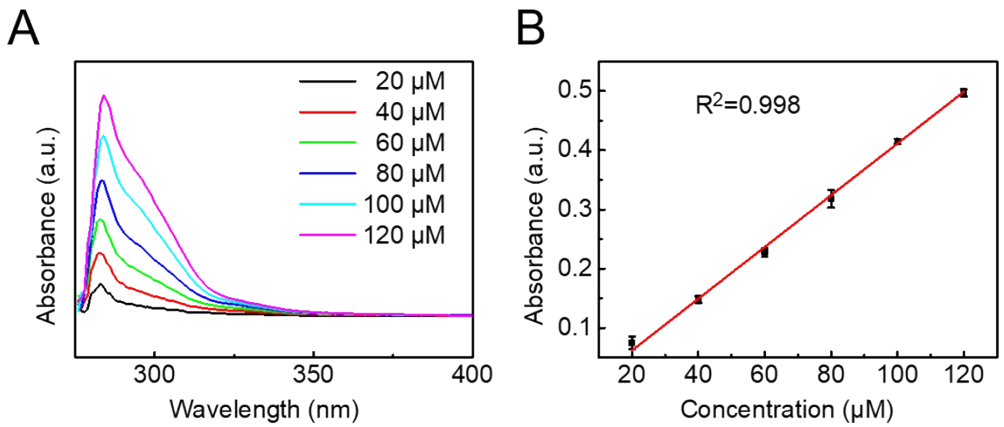

Fig. S12. UV-vis spectra (A) and linear standard curve (B) of BPTES from 5-40 μM.

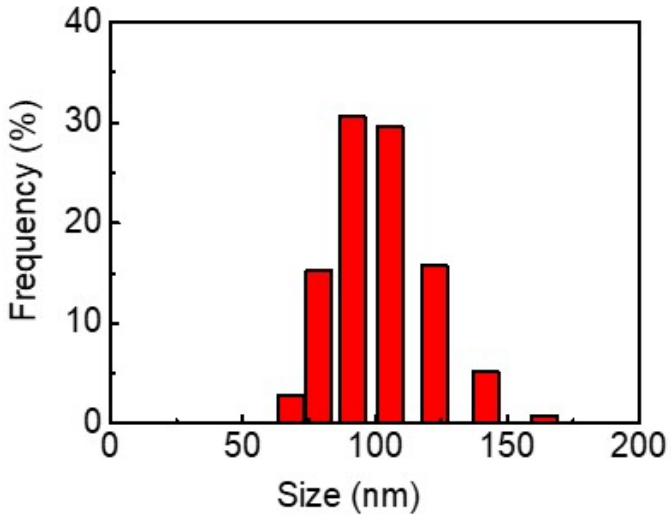

Fig. S13. Size distribution of BPMF measured by DLS in water.

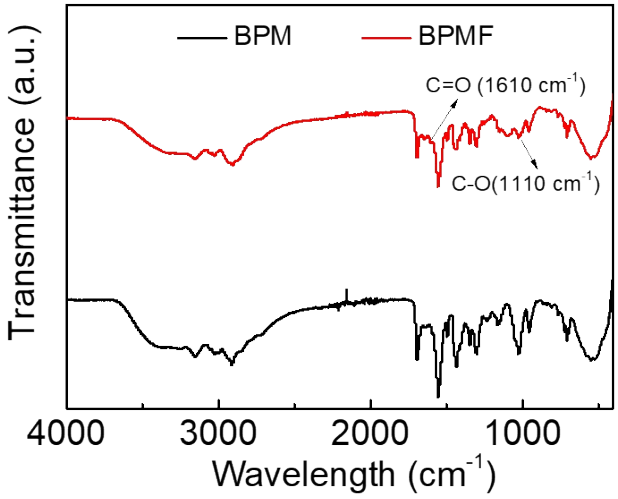

Fig. S14. The FT-IR spectra of BPM and BPMF.

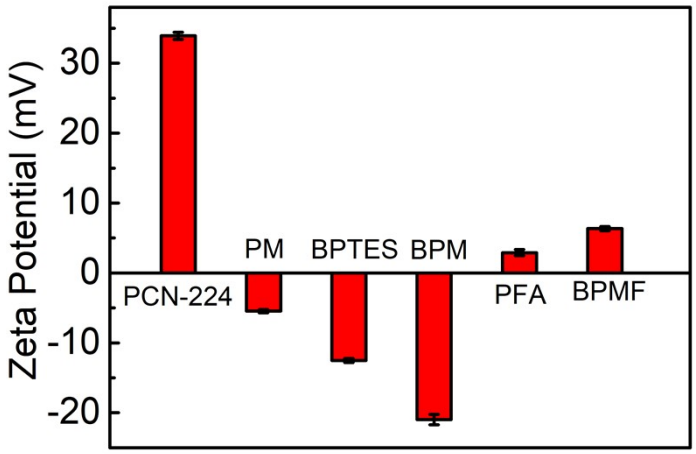

Fig. S15. Zeta potential of PCN-224, PM, BPTES, BPM, PFA and BPMF.

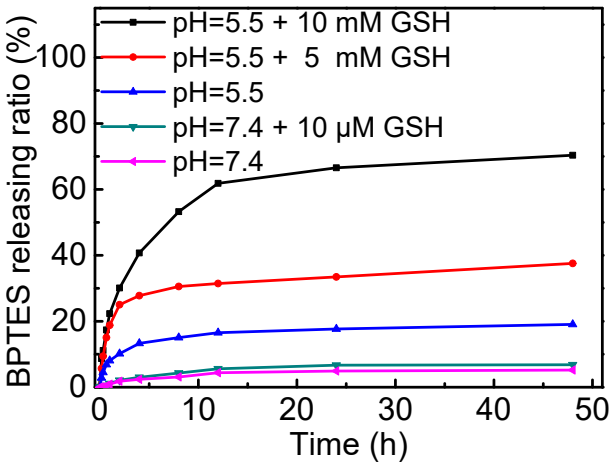

Fig. S16. BPTES releasing curves from PM NFs under different conditions.

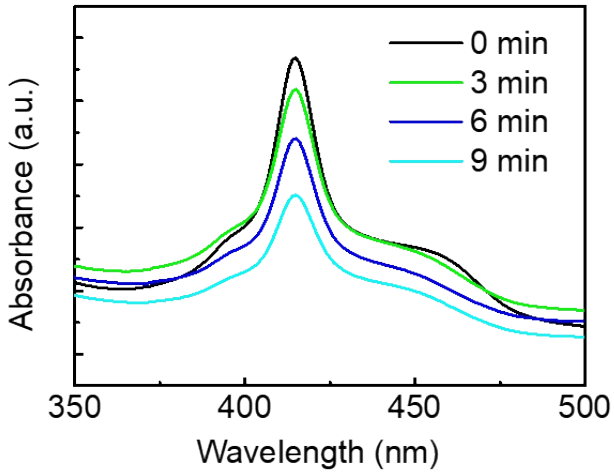

Fig. S17. UV-vis absorption spectra of DPBF with PM NFs under different incubating time.

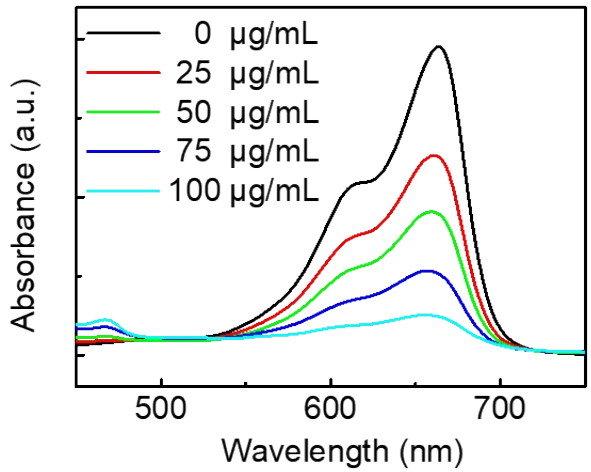

Fig. S18. UV-vis absorption spectra of MB under different concentration of PM NFs.

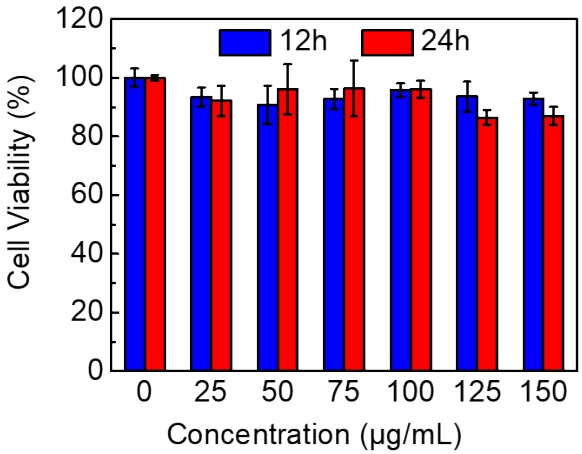

Fig. S19. Viability of MDA-MB-231 cells after incubated with different concentrations of PMF NFs for 12 h and 24 h.

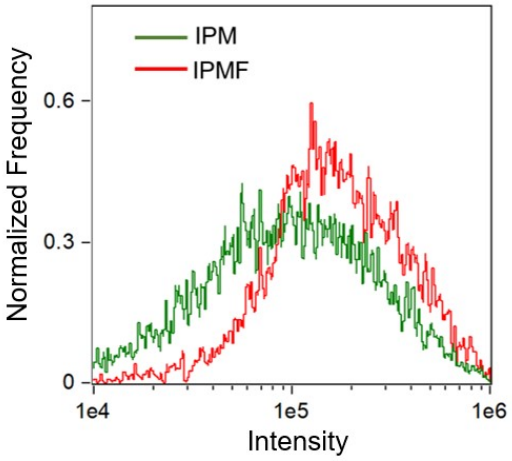

Fig. S20. Flow cytometry analysis of the cells treated with IPM and IPMF.

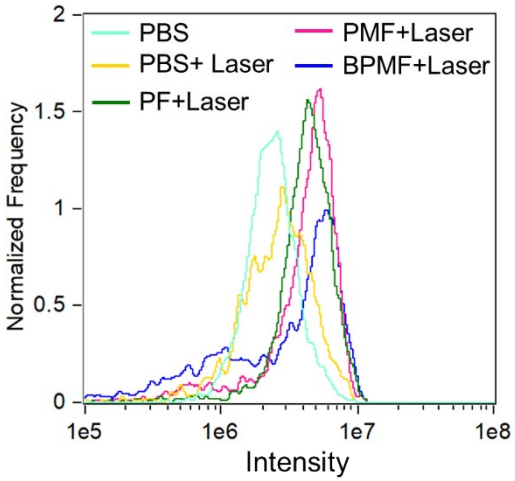

Fig. S21. Flow cytometry analysis of ROS accumulation in MDA-MB-231 cells with different treatments.

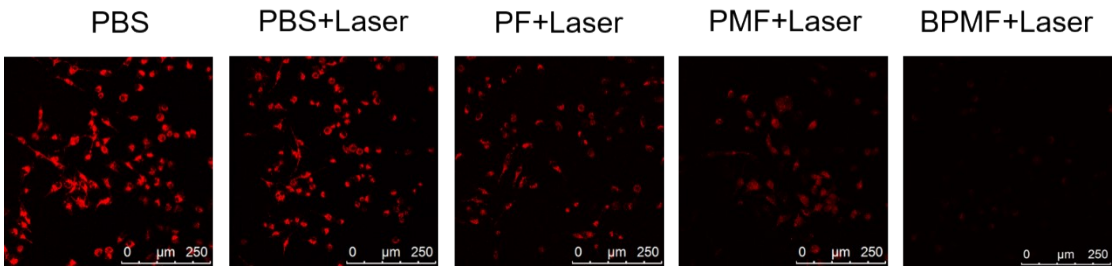

Fig. S22 Rhodamine 123 stained MDA-MB-231 cells with different treatments.

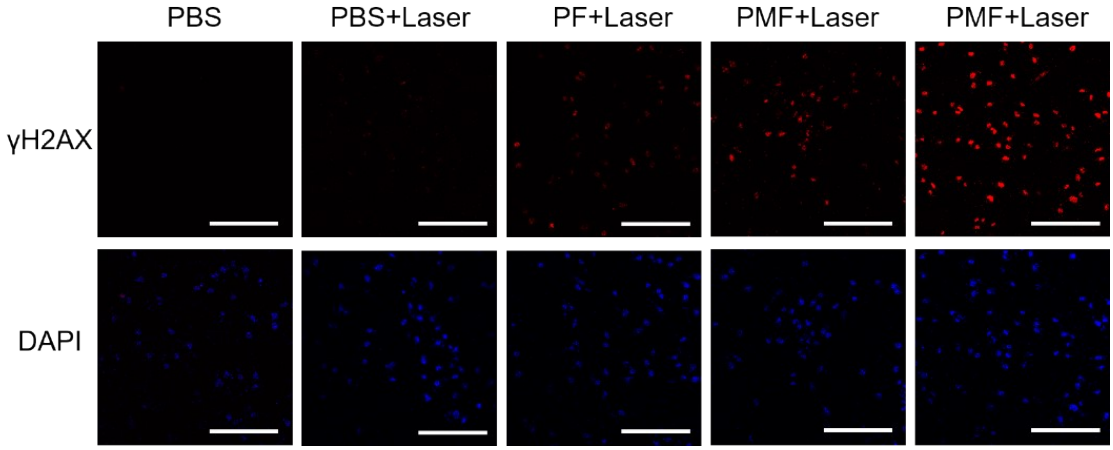

Fig. S23 DAPI and γ H2AX stained MDA-MB-231 cells with different treatments. All scale bars are 250 μm.

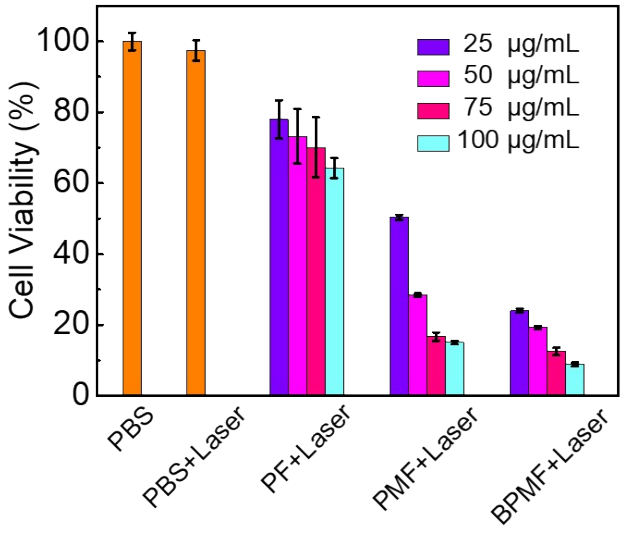

Fig. S24. MTT assay of MDA-MB-231 cells with different treatments.

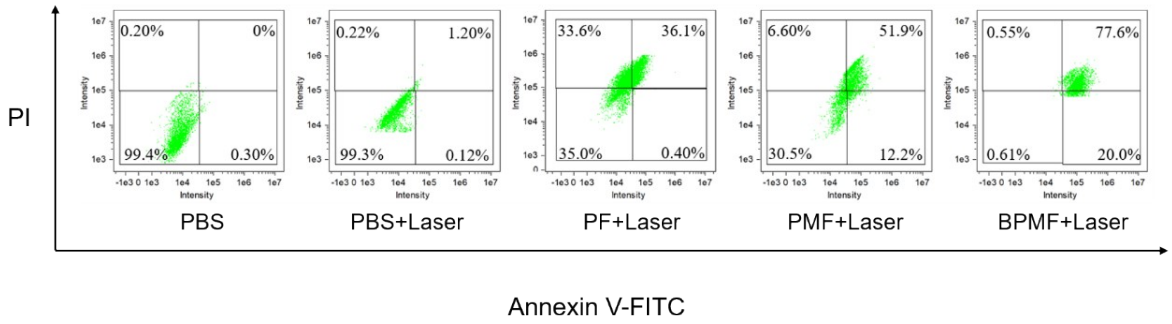

Fig. S25. Flow cytometry was used to verify cell viability.

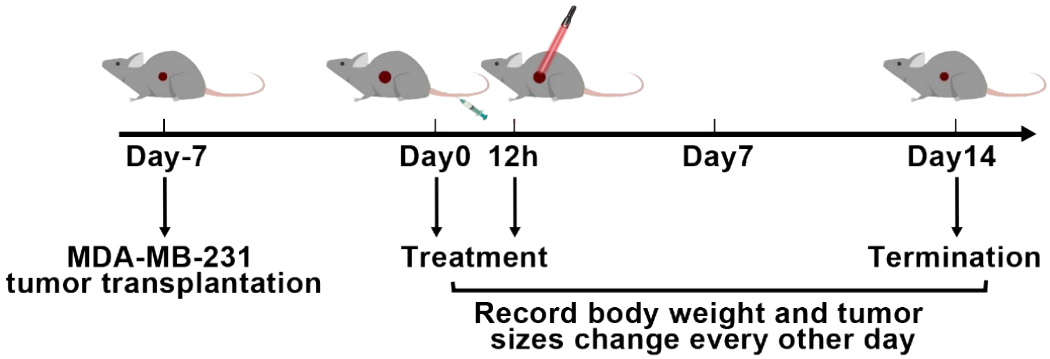

Fig. S26. Diagrammatic instruction of the therapeutic process.

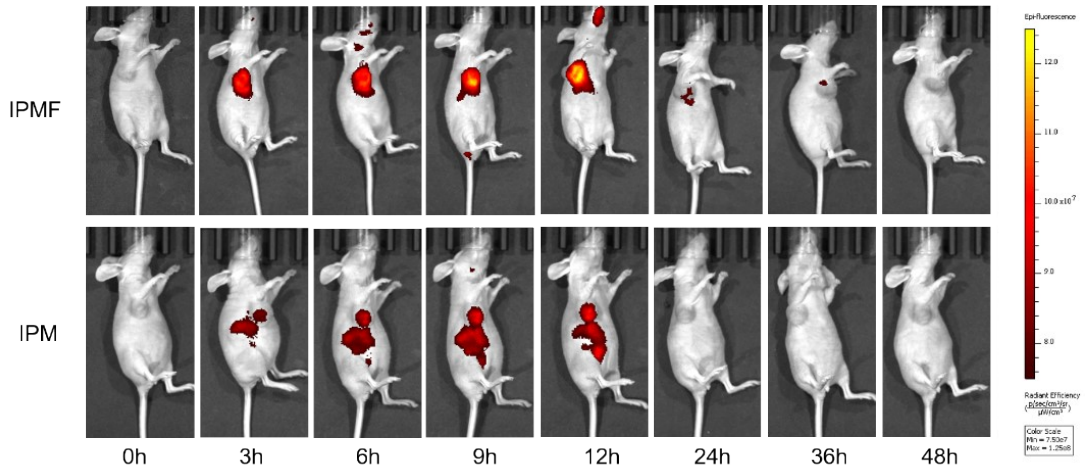

Fig. S27. In vivo fluorescence imaging of MDA-MB-231 tumor-bearing nude mice with intravenous injection of IPM or IPMF after different time.

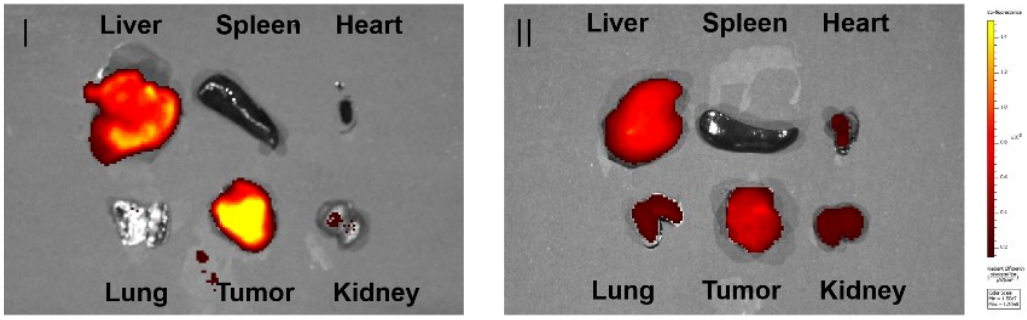

Fig. S28. Fluorescent imaging of organs and tumors 12 h after intravenous injection of IPMF (I) and IPM (II).

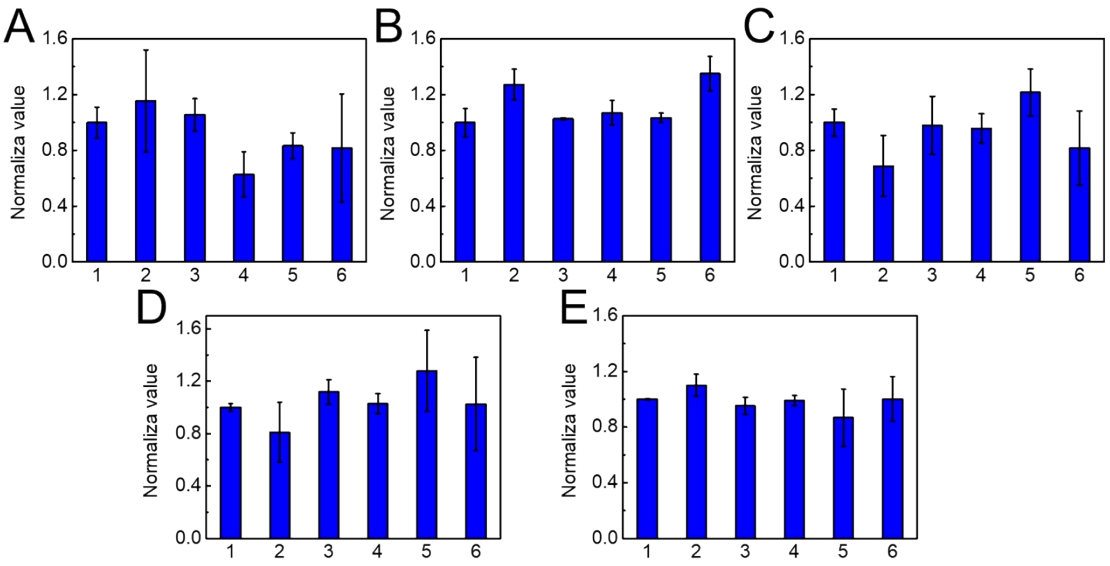

Fig. S29. Haematological data of the mice in different group. (A) white blood cells (WBC), (B) platelets (PLT), (C) monocyte (Mon), (D) neutrophile granulocyte (Gran), and (E) lymphocyte (Lymph). (1) PBS, (2) PBS+Laser (3) PF+Laser (4) PMF+Laser (5) BPMF and (6) BPMF+Laser.

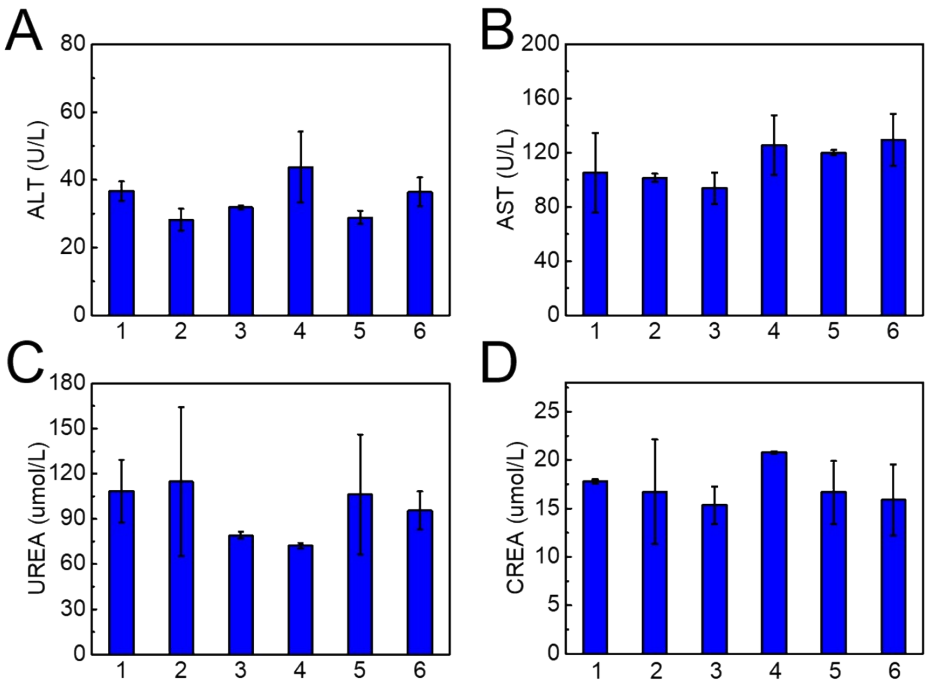

Fig. S30. Blood routine examination of the mice with different treatments. (A) alanine aminotransferase (ALT), (B) aspartate aminotransferase (AST), (C) UREA and (D) creatinine. (1) PBS, (2) PBS+Laser (3) PF+Laser (4) PMF+Laser (5) BPMF and (6) BPMF+Laser.

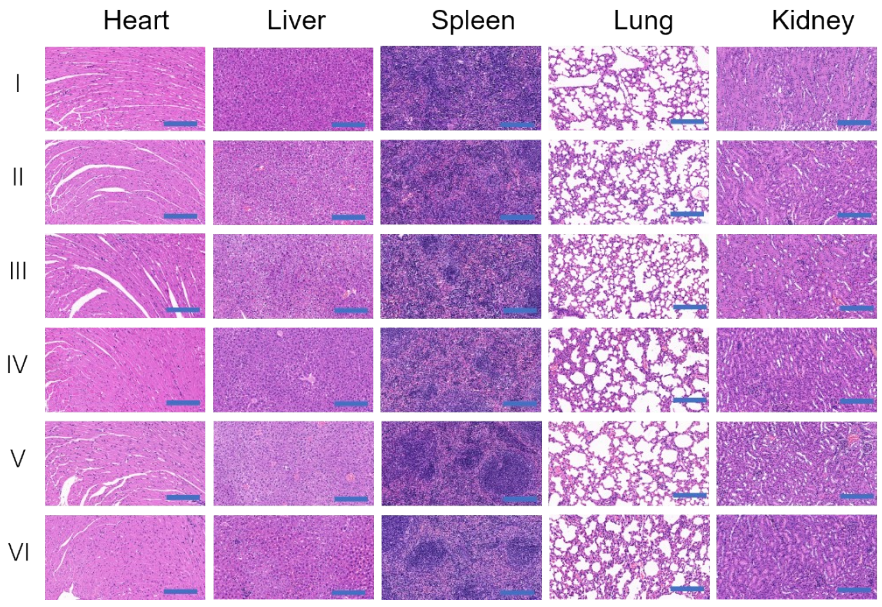

Fig. S31. H&E staining of major organs (heart, liver, spleen, lung and kidney) with different treatments. Scale bars are 200 μm. (I) PBS, (II) PBS+Laser (III) PF+Laser (IV) PMF+Laser (V) BPMF and (VI) BPMF+Laser.