Supporting Information

Zwitterionic Polypeptide Nanocomposite with Unique NIR-I/II Photoacoustic Imaging for NIR-I/II Cancer Photothermal Therapy

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Submitted to ***J Mater Chem B***

Materials

Acetonitrile (99%), dimethyl sulphoxide (DMSO, 99%), aqueous hydrogen peroxide (30%) and tetrachloroauric acid hydrate ($HAuCl_4$-$4H_2O$) were purchased from Shanghai Sinopharm Corp. and used as received. Dimethylformamide (DMF, 99.5%) was distilled from calcium hydride under reduced pressure and stored over 4 Å molecular sieves. D/L-Cysteine (97%, Aldrich), o-nitrobenzyl bromide (98%, Aldrich), triphosgene (99%, Aladdin Chemistry Ltd), Dimethylphenylphosphine (DMPP, 99%, Aldrich) and 2-methacryloyloxyethyl phosphorylcholine (MPC, 97%, Aladdin) were used as received. Dulbecco's Modified Eagle's Medium (DMEM, PAA Laboratories), fetal bovine serum (FBS, PAA Laboratories), methylthiazolyldiphenyl-tetrazolium bromide (MTT, ultrapure, Aldrich), calcein-AM (ultrapure, Yeasen), and propidium iodide (PI, ultrapure, Yeasen) were used as received. Alexa fluorR 488 annexin V/dead cell apoptosis assay kit was purchased from Invitrogen and used as received. Both MCF-7 (a human breast carcinoma cell line) and L929 (a mouse fibroblastic cell line) cell lines were received from Shanghai Institute of Biochemistry and Cell Biology.

Methods. Fourier transform infrared (FT-IR) spectroscopy was recorded on a Perkin Elmer Spectrum 100 spectrometer. ^1H nuclear magnetic resonance (NMR) (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Varian Mercury- 400 spectrometer at room temperature using tetramethylsilane as an internal standard. X-ray photoelectron spectroscopy (XPS) was conducted on a VG ESCALAB MKII spectrometer and analyzed by the XPS PEAK software (Version 4.1). Dynamic light

scattering (DLS) was used to determine the average hydrodynamic diameter and polydispersity index (PDI) of nanoparticles on a Malvern ZS90 instrument. Transmission electron microscopy (TEM, JEM-2010, JEOL) and field emission transmission electron microscopy (FE-TEM, TALOS F200X, FEI) were performed without negative staining at 200 kV accelerating voltage, during which a little nanoparticle solution was dropped on the Formvar-carbon film-coated copper grids and allowed to dry in air; the energy-dispersive X-ray (EDX) spectroscopy was obtained simultaneously to analyze the elemental composition. The UV-Vis-NIR spectroscopy was recorded on a Perkin-Elmer Lambda 750S spectrometer. Molecular weights and polydispersities (Mw/Mn) of polymers were determined on a gel permeation chromatograph (GPC, HLC-8320, Tosoh Corporation, Japan) equipped with two HLC-8320 columns (TSKgel Super AWM-H, pore size: 9 μm; 6 × 150 mm, Tosoh Corporation) and a double-path, double-flow refractive index (RI) detector (Bryce) at 30 °C. Field emission scanning electron microscopy (SEM) was performed with a FEI Nova NanoSEM 450 instrument with an accelerating voltage of 2 kV. Atomic force microscopy (AFM) measurements were carried out on an environment-controlled scanning probe microscope (Nanonavi E-Sweep, NSK Ltd, Japan) in tapping mode (TM) at room temperature. The samples were prepared by depositing several drops of the nanoparticle solution on the surface of fresh mica and they were freeze-dried under vacuum after excess solution was removed with a spin coater. Thermogravimetric analysis (TGA) was performed on a PerkinElmer TGA 7 under a nitrogen flow (10 mL min^{-1}) from room temperature to 900 ^{o}C at 20 ^{o}C min^{-1}.

Inductively coupled plasma mass spectrometry (ICP-MS) was performed on a Thermofisher ICAPQ machine for determining the Au content. The fluorescent spectroscopy was recorded on a Perkin-Elmer LS-50B spectrometer. Super-resolution multiphoton confocal laser scanning microscopy (CLSM) was performed on a Leica TCS SP8 STED 3X instrument. The NIR-I/II irradiations were carried out by using a continuous wave diode laser (808 nm or 1064 nm; Shanghai SFOLT Corp., FC-960-6000-MM) with tunable power (0–1650 mW), during which process the spot size was tuned by a fiber collimator connected by a fiber optic patch cable (FC/PC/200UM/1M).

Preparation of the zwitterionic polypeptide PMC. By utilizing ring-opening polymerization of α-amino acid N-carboxyanhydride, UV photolysis, thiol-ene click reaction, the zwitterionic polypeptide PMC (yield 82%) was prepared from poly(*S*-(o-nitrobenzyl)-$_{D,L}$-cysteine)$_{40}$ and 2-methacryloyloxyethyl phosphorylcholine according to our previous publications (**Fig. S1**).[1,2] ^{1}H NMR of PMC (DMSO-d_6/CF_3COOD (v:v =9:1): δ(ppm) = 4.42-4.85 (s, COC*H*NH, 40H), 3.88-4.39 (m, $(CH_3)_3N^+CH_2CH_2OPO_3CH_2CH_2$, 60H), 3.45-3.71 (s,$COOCH_2CH_2OPO_3$, 20H), 2.94-3.24 (s, $(CH_3)_3N^+CH_2$, 90H), 2.38-2.90 (d, $COCH(CH_2SCH_2)NH$, 100H), 1.91-2.03 (q, CH_2CH_2NH, 2H), 1.01-1.32 (m, $CH_3CH_2CH_2CH_2CH_2CH_2$, $COCH(CH_3)CH_2SCH_2$, 38H), 0.72-0.86 (t, CH_3CH_2, 3H). FT-IR (KBr, cm^{-1}): 3365 (ν_{N-H}), 3050, 2965, 2933 (ν_{C-H}), 1729 ($\nu_{O=C-O}$), 1668 (amide I), 1525 (amide II), 1236 ($\nu_{-P=0}$) , 860 ($\nu_{-N+-(CH3)3}$), 713 (ν_{C-S}). $M_{n,GPC}$ = 4820, M_w/M_n = 1.52.

Preparation of Zwitterionic Polypeptide Nanocomposite of PMC@AuNP.

Zwitterionic polypeptide nanocomposites were fabricated in two steps. Firstly, PMC (5.0 mg) was dissolved in 5 mL of DMSO, followed by gradual addition of distilled water (50 μL/min) under vigorous stirring and then dialyzed against distilled water (8 × 1 L) for 48 h to produce PMC micelles. In the second step, 50 μL of $HAuCl_4$ (10 mg/mL, 1.21 μmol) and 1 μL of H_2O_2 (30%, 9.79 mol/L) was added into 1 mL of the above solution (0.25 mg/mL), vortexed for 10 min and then reacted for 12 h in the dark and at room temperature. The resulting nanocomposite solution of PMC@AuNP was further dialyzed against distilled water (3 × 1 L) or PBS (10 mM, pH 7.4) for 24 h, and stored at 4 °C for further use.

NIR-Mediated Photothermal Properties of PMC@AuNP. To study and compare the NIR-I or NIR-I mediated photothermal property, 200 μL of PMC@AuNP solution (0.5 mg/mL) was added into a 96-well plate and then vertically irradiated by a continuous-wave diode laser (808 nm or 1064 nm, 1 W/cm^2, 10 min), respectively, during which the solution temperature and the repeated heating–cooling cycles were recorded were recorded by a digital thermometer every 30 s. The photothermal conversion efficiency (η) was calculated according to the previous method.[8]

In vitro NIR-I/II PTT and Cell Internalization of PMC@AuNP. MCF-7 cell lines were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in DMEM (10% FBS, 100 IU mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin). Briefly, MCF-7 cell suspension (200 μL, 1 × 10^4 cells per well) in a 96-well plate were incubated for 24 h. The PMC@AuNP solutions with different concentrations were added into wells with/without NIR-I/II irradiation (808 nm or 1064 nm, 1 W cm^{-2}, 10 min) and

incubated 48 h at 37 ^{o}C. The cell viability was assessed by MTT assay. Half maximal inhibitory concentration (IC_{50}) was calculated by the GraphPad Prism 6 software and all cell viabilities were tested using eight samples. The IC_{50} value of PTT was calculated on the PMC@AuNP concentration.

Cellular uptake was studied by means of dark-field microscopy and ICP-MS. MCF-7 cells were incubated with PMC@AuNP at 37 °C for 4 h with/without NIR-I/II irradiation (808 nm or 1064 nm, 1 W/cm^2, 10 min), washed twice with PBS, and then observed under a dark-field microscope. For quantitative cell entry of nanocompositite, the MCF-7 cells were seeded into 6-well plates at 5 × 10^5 cells per well and cultured overnight. After further incubation with PMC@AuNP for 0.5, 1, 2 and 4 h at 37 ^{o}C with/without NIR-I/II irradiation, respectively, ICP-MS was performed on a Thermofisher ICAPQ machine for determining the Au content of the cellular internalized nanocomposites.

PTT-mediated Live−Dead Cell Observation and Cellular Death Pathway. MCF-7 cells were seeded in 6-well plates at a density of 5 × 10^5 cells per well. After 24 h incubation, the cells were treated with PMC@AuNP at 37 °C for 4 h with/without NIR-I/II irradiation, and then incubated for another 20 h. The treated cells were harvested by centrifugation and washed with PBS for three times, stained in propidium iodide (PI) and annexin V-FITC for 30 min, and finally analyzed by flow cytometry (BD FACS Calibur, USA). Data were obtained from 10 000 cells for each sample. After similar treatment, the cells were co-stained with calcein AM and PI at room temperature for 30 min and then observed by confocal laser scanning

microscopy (CLSM).

In vivo X-ray CT and NIR-I/II PAI. Five week-old male Balb/c nude mice (~ 20 g) were purchased from the Chinese Academy of Sciences (Shanghai, China). The animal experiments (approval # A2019017) were performed in accordance with the guidelines for the care and use of laboratory animals and approved by the Animal Ethics Committee of Shanghai Jiao Tong University. In order to check the CT signals as a function of concentration, PMC@AuNP with gradient concentrations (0.32, 0.63, 1.25, 2.5, and 5 mg/mL) in PBS (pH 7.4, 10 mM) were used for in vitro CT. For in vivo CT, the mice bearing MCF-7 tumors (~200 mm^3, n = 5) were injected intravenously with 200 μL PMC@AuNP (3 mg/mL), and CT was then performed at 0, 2, 4, 6, 8, 10 and 12 h post-injection on a clinical Brilliance 64-slice CT imaging system (80 kV, 450 mA, Philips Healthcare, Andover, MA) with a slice thickness of 45 mm.

For high-resolution NIR-I/II PAI (808/1200 nm), the mice bearing MCF-7 tumors were intravenously injected with PMC@AuNP (200 μL, 3 mg/mL, n = 4). PAI was obtained at 0, 2, 4, 6, 8, 10 and 12 h post-injection on a multi-mode Ultrasound/PA Imaging System (Fujifilm VisualSonics/VEVO LAZR-X, USA).

In vivo Biodistribution. The MCF-7 tumor-bearing nude mice (n=3) were intravenously injected with 200 μL PMC@AuNP (3 mg/mL). At the pre-determined time points after administration, the mice were sacrificed, and the major organs and tumors were collected at 4, 10, and 12 h postinjection. Finally, ICP-MS was used to quantify the accumulation contents of PMC@AuNP in different organs and tumors.

Data are presented as the percentage injected dose per gram tissue (% ID/g).

In Vivo Infrared Thermography, NIR-I/II PTT-Mediated Anticancer Efficacy, and Histological Analyses. MCF-7 tumor-bearing mice (~100 mm^3) were randomly divided into seven groups (n = 4). PMC@AuNP (200 μL, 3 mg/mL), MPC and PBS were intravenously injected into mice on day 0, respectively. After 10 h post-injection, the tumors in those groups (PBS+1064, PBS+808, PMC@AuNP+1064, PMC@AuNP+808) were irradiated for 10 min (808 nm or 1064 nm, 1 W/cm^2), and then photothermal imaging was performed on an infrared camera (AXT100, Ann Arbor Sensor Systems).

For in vivo NIR-I-II PTT, the volume of the tumors and the weight of each mouse were measured every two days. The tumor volume (V) was calculated according to the equation of $V = L \times W^2/2$, where W and L are the width and length of tumors, respectively. The tumor inhibitory rates (TIR) of various treatments were calculated using the equation: TIR (%) = 100× (mean tumor volume of the PBS group – mean tumor volume of others)/(mean tumor volume of the PBS group). On day 24, one mouse from each group was sacrificed and their tumors and the major organs (heart, liver, spleens, lung and kidneys) were harvested, photographed and fixed with 4% formaldehyde for histology and TUNEL assays. Data are represented as average ± standard error.

Statistical Analysis. The statistical significance of the results was estimated by a one-tailed Student's t test. ***$P < 0.001$ and **$P < 0.005$ was considered to be highly significant and significant, respectively.

Table S1. The NIR-II absorbance (A_{1064nm}) and photothermal data of PMC@AuNP synthesized at different Au/S ratios when irradiated by NIR-II laser (1064 nm, 1W/cm^2, 10 min).

Entry	Au/S	A_{1064nm}	ΔT_{max}(℃)	η(%)
PMC@30μL $HAuCl_4$	0.8	1.92	21.2	43.3
PMC@40μL $HAuCl_4$	1.0	2.62	22.8	48.9
PMC@50μL $HAuCl_4$	1.2	2.65	23.0	49.5
PMC@60μL $HAuCl_4$	1.4	2.14	20.3	40.1
PMC@70μL $HAuCl_4$	1.6	1.92	17.7	37.7

Table S2. The absorbance at 1064 nm (A_{1064nm}) and NIR-II photothermal temperature-elevating data of PMC@AuNP synthesized at different H_2O_2 amounts (NIR-II laser: 1064 nm, 1W/cm^2, 10 min).

Entry	H_2O_2 (μL)	Diameter (nm)	A_{1064nm}	ΔT_{max} (℃)
PMC@50μL $HAuCl_4$	0.5	101 ± 42	0.43	9.2
PMC@50μL $HAuCl_4$	1.0	48 ± 2	2.65	23.0
PMC@50μL $HAuCl_4$	2.0	24 ± 3	2.63	21.7

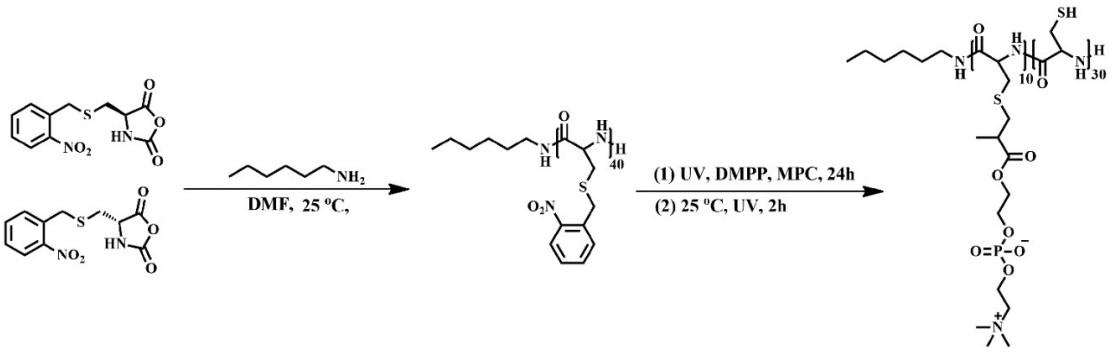

Fig. S1 Synthesis of zwitterionic polypeptide PMC.

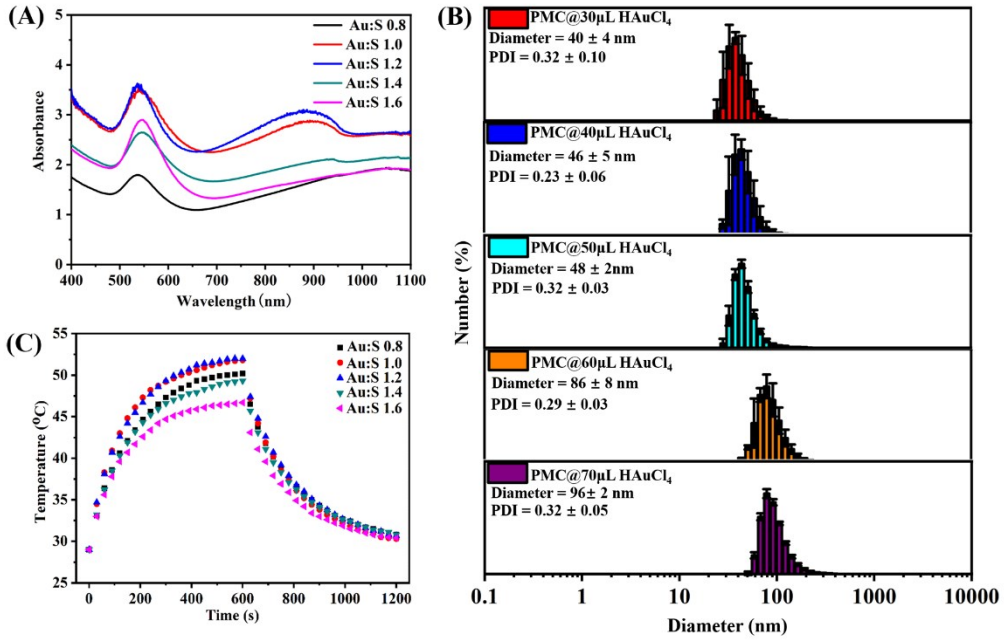

Fig. S2 (A) Vis-NIR curves; (B) the DLS-determined diameters; and (C) NIR-II-mediated heating-cooling curves of PMC@AuNP synthesized at different Au/S ratios.

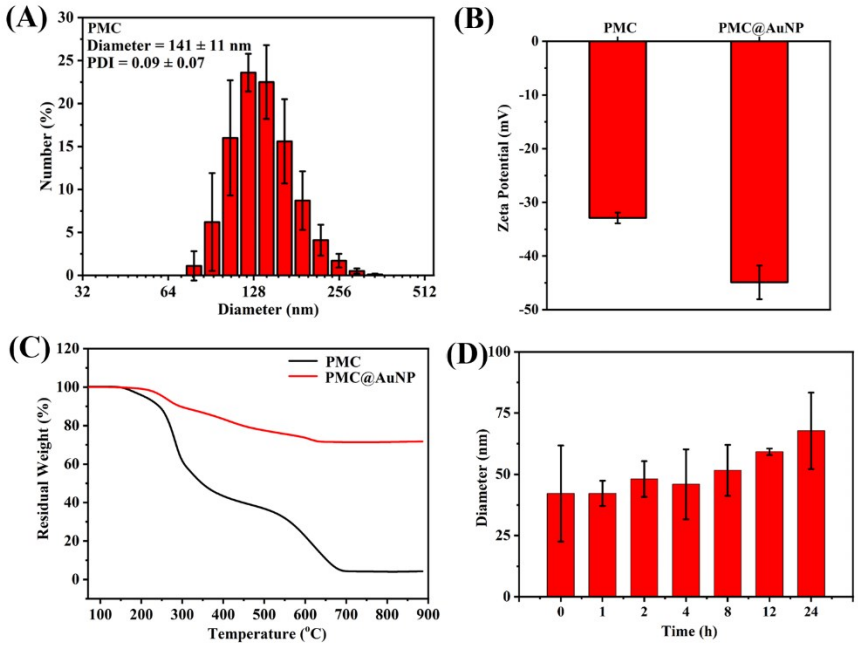

Fig. S3 (A) The DLS-determined diameter of PMC micelle; (B) Zeta potentials and (C) TGA curves of PMC micelle and PMC@AuNP; (D) Hydrodynamic diameter of PMC@AuNP over incubation time when incubated in 10% FBS at 37 °C (n = 3).

Note: As shown in Fig S3C, the remaining weight percentage of the PMC@AuNP nanocomposites and PMC is 72.2 wt% and 4.3 wt% by TGA. The content of gold in

nanocomposites PMC@AuNP = X, and (1–X) × 4.3% + X = 72.2%. Based on the above equation, X = 71 wt%, i.e. the percentage of Au within PMC@AuNP is calculated to 71 wt%. These data are basically consistent with each other.

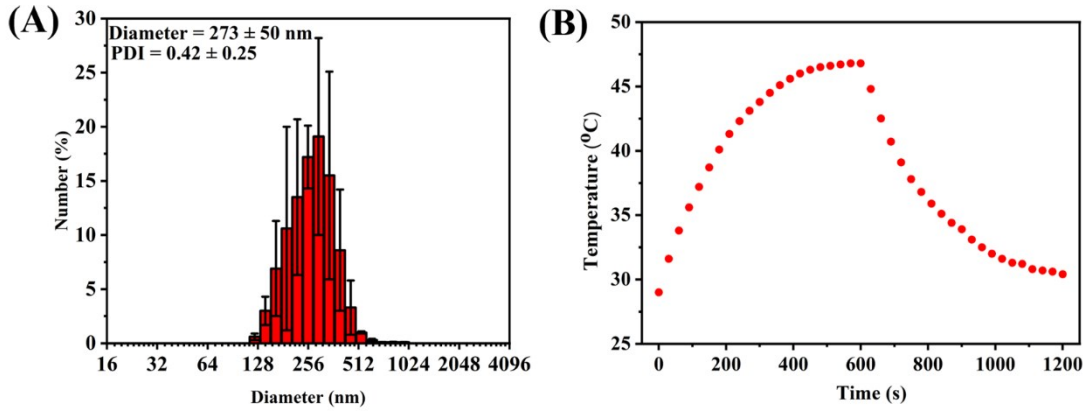

Fig. S4 (A) the DLS-determined diameter and (B) NIR-II-mediated heating-cooling curve of the bigger aggregates formed from the unimers of amphiphilic copolymers with $HAuCl_4$.

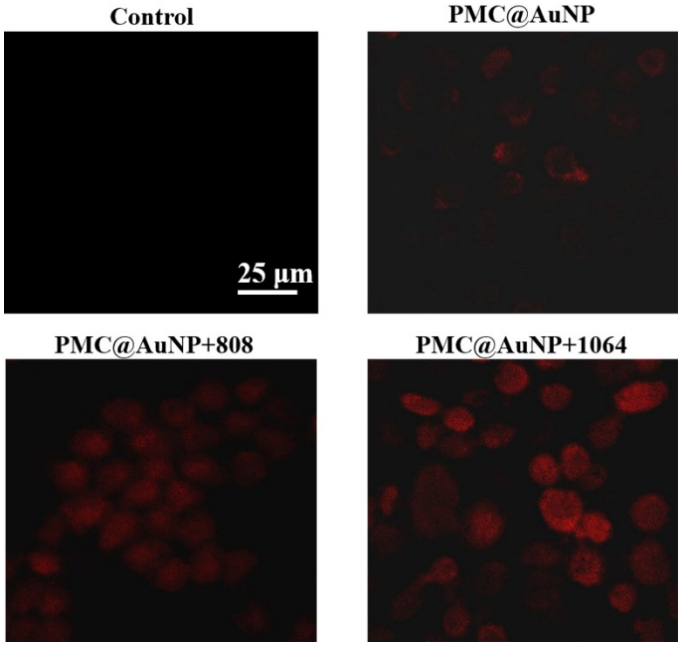

Fig. S5 Dark-field microscopy images of MCF-7 cells and those incubated with PMC@AuNP or PMC@AuNP with NIR-I/II irradiation (808 or 1064 nm, 1 W/cm^2, 10 min). The scale bar represents 25 μm.

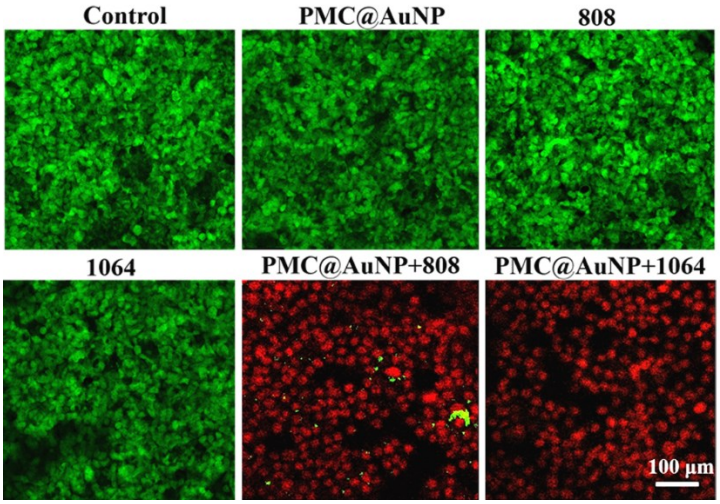

Fig. S6 CLSM images of calcein-AM (green, live cells) and PI (red, dead cells) co-staining MCF-7 cells treated with NIR-I/II irradiation or PMC@AuNP treated with NIR-I/II irradiation (808 or 1064 nm, 1 W/cm^2, 10 min). The scale bar = 100 μm.

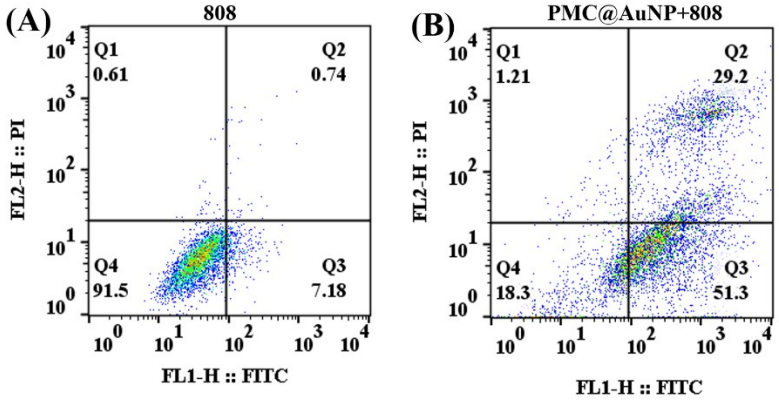

Fig. S7 Flow cytometry analyses of MCF-7 cells treated with NIR-I irradiation (A) or those incubated with PMC@AuNP plus NIR-I irradiation after co-staining with propidium iodide (PI) and annexin V-FITC (B).

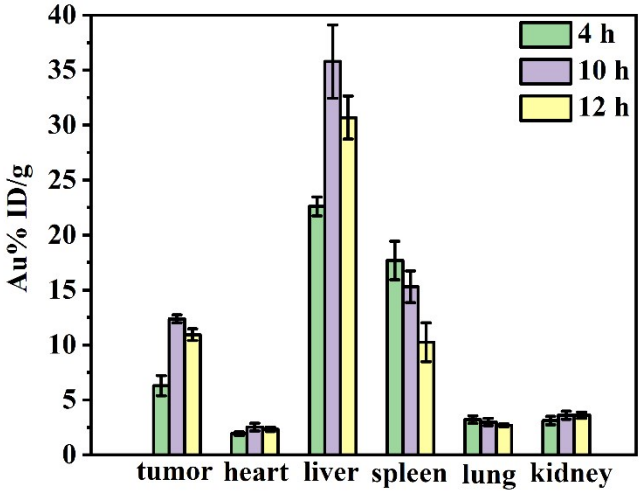

Fig. S8 In vivo biodistribution of PMC@AuNP in MCF-7 tumor-bearing mice.

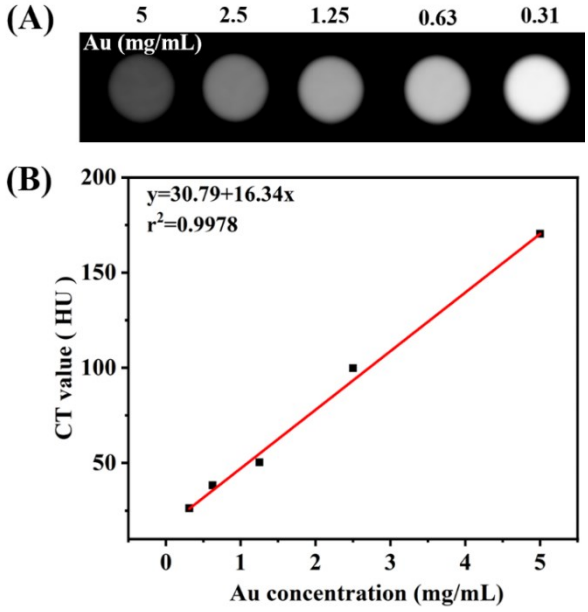

Fig. S9 In vitro CT images (A) and CT value (HU) (B) of PMC@AuNP as a function of the Au concentration.

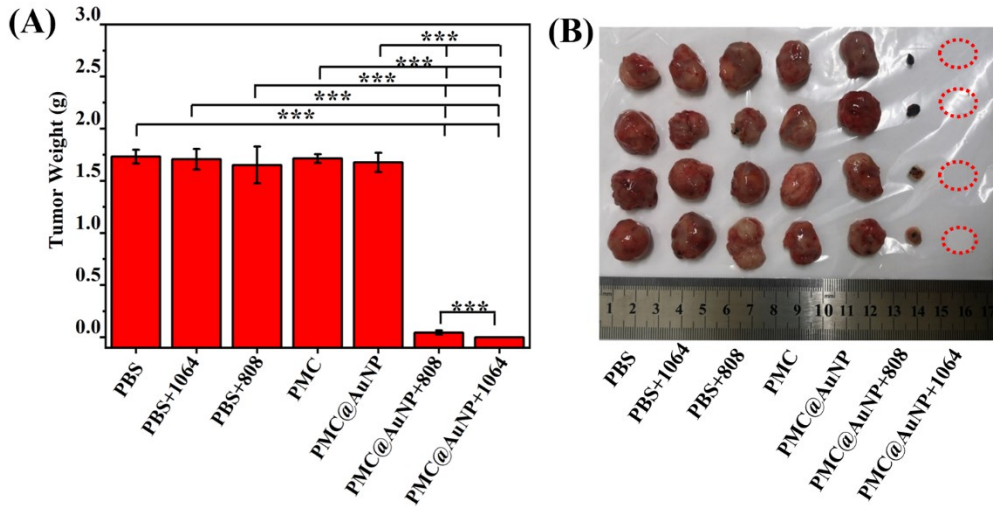

Fig. S10 The dissected tumors' weights (A) and photographs (B) after various treatments on day 24.

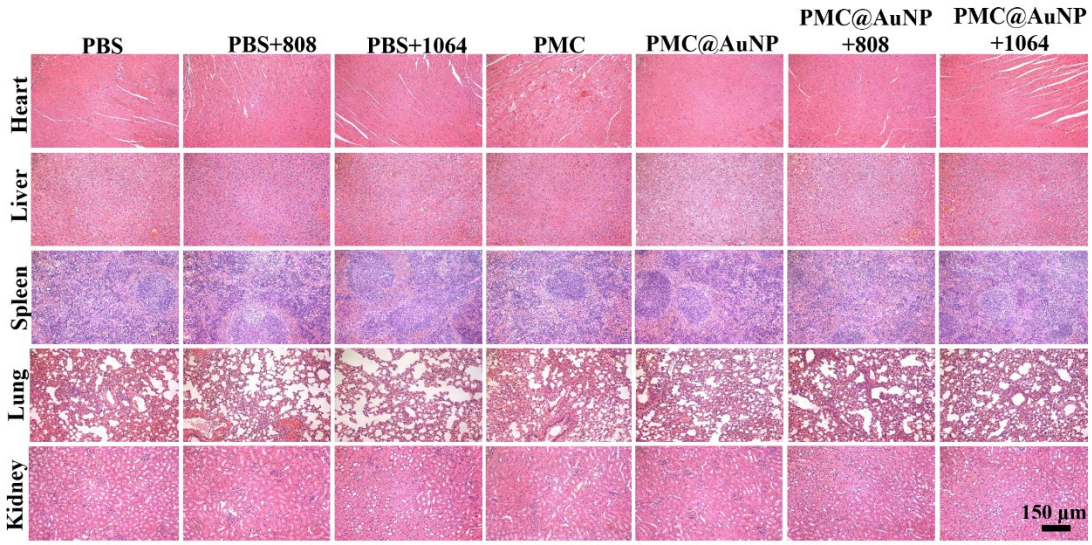

Fig. S11 HE staining images of the tissue sections from the main organs including liver, kidneys, spleen, lung, and heart from the mice after various treatments on day 24, and the scale bar represents 150 μm.

References

(1) X. J. Wu, L. Zhou, Y. Su, C.-M. Dong, *Polym. Chem.* 2015, **6**, 6857–6869.

(2) Y. Ding, C. Du, J. Qian, C. -M. Dong, *Polym. Chem.* 2019, **10**, 4825–4836.