Supporting Information

An efficient strategy for cancer therapy using a tumor- and lysosome-targeted organic photothermal agent

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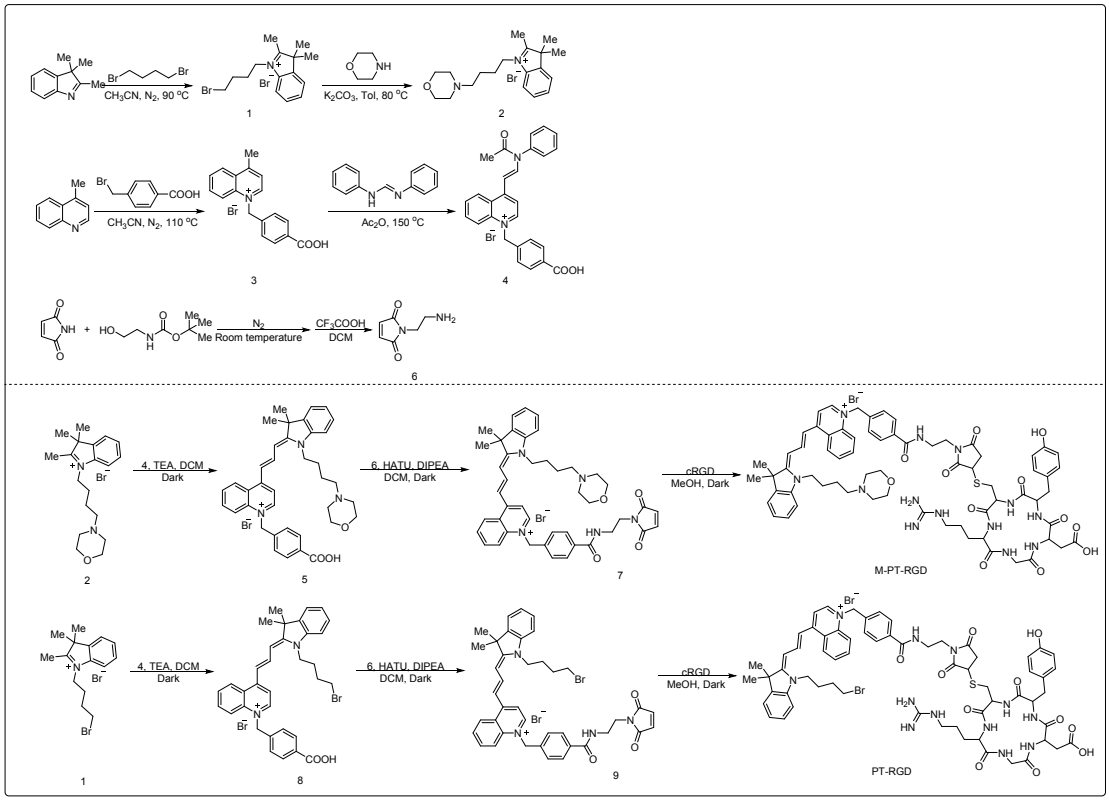
Materials and Instruments.

Materials.

1,4-Dibromo butane, morpholine, 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU), triphenylphosphine and N,N-diisopropylethylamine (DIPEA) were purchased from Adamas Reagent, Ltd. 4-Methylquinoline was purchased from Shanghai Macklin Biochemical Co., Ltd. 2,3,3-Trimethyl-3H-indole, 4-(bromomethyl)benzoic acid, maleimide, trifluoroacetic acid, diethylazodicarboxylate and 2-(boc-amino)-1-ethanol were obtained from Tianjin Heowns Biochemical Technology Co., Ltd. N,N'-diphenylformamidine was purchased from Alfa Aesar (Tianjin, China). Cyclic Arg-Gly-Asp (cRGD) was purchased from Shanghai Dechi Biosciences Co., Ltd. Dialysis Membranes and acridine orange were obtained from Beijing Solarbio Science & Technology Co., Ltd. Methanol, acetonitrile, dichloromethane (DCM), acetic anhydride, dimethyl sulfoxide (DMSO), toluene and triethylamine (TEA) were purchased from China National Pharmaceutical (Shanghai, China). Lyso-Tracker Red was obtained from Beyotime (Shanghai, China). 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI) and calcein acetoxymethyl ester (Calcein AM) were purchased from Sigma-Aldrich (USA). The mouse breast cancer cell line (4T1) was purchased from KeyGEN biotechnology (Nanjing, China). Analytical grade reagents were used with no further purification. All aqueous solutions were prepared using distilled-deionized water of 18.2 $M\Omega \cdot cm^{-1}$.

Instruments.

Transmission electron microscopy (TEM) was carried out on a HT7700 electron microscope (Hitachi, Japan). Fluorescence spectra were acquired with fluorescence spectrometer (FLS-980, Edinburgh, UK). UV-Vis absorption spectra were measured on a pharmaspec UV-1700 UV-Visible spectrophotometer (Shimadzu, Japan). Confocal fluorescence imaging studies were performed with a TCS SP8 confocal laser scanning microscopy (Leica, Germany). Absorbance was measured in a microplate reader (Synergy 2, Biotek, USA) in the MTT assay. Size distribution was confirmed by monitoring with a Malvern Zeta Sizer Nano (Malvern Instruments). NMR spectra was recorded on a Bruker NMR spectrometers. Chemical shifts (δ) for ^{1}H NMR (400 Hz) were given in ppm. Data were reported as follows: chemical shift, intergration, multiplicity (s = single, d = doublet, t = triplet, br = broad, m = multiplet) and coupling constants (Hz).



Synthesis of M-PT-RGD and PT-RGD

Scheme S1. Synthesis of M-PT-RGD and PT-RGD.

The product 1 was prepared according to the previous report.[1] The products 3 and 4 were prepared according to the previous report.[2]

Synthesis of 2.

A mixture of 1 (2.00 mmol), morpholine (1.00 mmol) and K_2CO_3 (1.2 mmol) in toluene (5.00 mmol) was stirred at 80 °C for 8 h. After completion of the reaction, the mixture was transferred to centrifuge tube with methanol and the supernatant was collected by centrifugation into a round-bottom flask. After removal of the methanol solvent under vacuum, the crude product was purified by column chromatography over silica gel.

Synthesis of 5 or 8.

Compound 4 (1.00 mmol) and TEA (10.00 mmol) were dissolved in dichloromethane (10.00 mL). After reacting at 25 ºC for 0.5 h in the dark, compound 2 or 1 (1.00 mmol) was added to the solution. After 24 h, the solvent was removed and the crude product was purified by column chromatography over silica gel using CH_2Cl_2/MeOH (v/v, 10:1) as the eluent.

Synthesis of 6.

Maleimide (6.44 mmol), triphenylphosphine (6.32 mmol), THF (30.00 mL), 2-(boc-amino)-1-ethanol (5.86 mmol) and diethylazodicarboxylate (7.02 mmol) were successively added into 100 mL round-bottom flask. After reacting at 25 ºC under a nitrogen atmosphere overnight, the solvent was removed and the crude product was purified by column chromatography over silica gel using petroleum ether/ethyl acetate (v/v, 2:1) as the eluent.

The product was dissolved in a mixture of trifluoroacetic acid (10.00 mL) and DCM (6.00 mL). After reacting at 25 ºC for 2 h, the solvent was removed. The crude product was then dissolved in methanol (3.00 mL) and added anhydrous diethyl ether to precipitate the desired product. The produce was collected through filtration and dried under high vacuum.

Synthesis of 7 or 9.

Compound 5 or 8 (0.25 mmol), DIPEA (1.72 mmol) and HATU (0.50 mmol) were dissolved in dichloromethane (10.00 mL). After reacting at 25 ºC for 1 h in the dark, compound 6 (0.54 mmol) was added to the solution. After 12 h, the solvent was removed and the crude product was purified by column chromatography over silica gel using CH_2Cl_2/MeOH (v/v, 20:1) as the eluent.

Synthesis of M-PT-RGD or PT-RGD.

Compound 7 or 9 (0.05 mmol) and cRGD (0.05 mmol) were dissolved in methanol (5 mL). After reacting at 25 ºC for 12 h in the dark, the solvent was removed and the crude product was further purified by dialysis and high performance liquid chromatography (HPLC) (The yield of M-PT-RGD was 17.2%). **^{1}H-NMR (400 Hz, $CDCl_3$)** δ 8.65 – 8.49 (m, 3H), 7.95 – 7.92 (m, 2H), 7.89 – 7.85 (m, 1H), 7.81 – 7.77 (m, 2H), 7.74 – 7.70 (m, 1H), 7.47 – 7.45 (d, J = 8.0 Hz, 1H), 7.39 – 7.35 (m, 3H), 7.28 – 7.25 (d, J = 12.0 Hz, 1H), 7.20 – 7.16 (m, 2H), 6.95 – 6.93 (d, J = 8.0 Hz, 1H), 6.87 – 6.85 (d, J = 8.0 Hz, 1H), 6.62 – 6.59 (m, 2H), 6.36 – 6.33 (d, J = 12 Hz, 1H), 5.97 – 5.96 (br, 2H), 4.80 – 4.74 (m, 3H), 4.52 – 4.42 (m, 1H), 4.30 – 3.68 (m, 14H), 3.59 – 3.37 (m, 7H), 3.27 – 3.05 (m, 9H), 2.91 – 2.70 (m, 5H), 2.63 – 2.56 (m, 1H), 2.40 – 2.35 (m, 1H), 1.92 (br, 6H), 1.77 (br, 7H), 1.68 – 1.52 (m, 4H).

Synthesis of M-PT-RGD nanoparticles.

M-PT-RGD (1.00 μmol) were dissolved in anhydrous methanol (1.00 mL). The solution was then added dropwise into deionized water (25.00 mL) and stirred in dark at 50 °C for 0.5 h. M-PT-RGD nanoparticles was produced by vacuum freeze drying.

Experimental Section

UV-Vis absorbance Curves of M-PT-RGD.

M-PT-RGD solutions of different concentrations were prepared in water and the absorption spectra of 400-800 nm were recorded.

Fluorescence Curves of M-PT-RGD.

M-PT-RGD solution was prepared in water and the fluorescence properties were further recorded by fluorescence spectrophotometer under 610 nm excitation.

Photothermal effect.

The photothermal effect of different concentrations of M-PT-RGD solutions under different power density of 635 nm laser irradiation were studied. A digital thermometer was used for temperature detection and recorded data every 20 s. The M-PT-RGD solution (0.50 mM, 0.50 W/cm^2) was imaged by Infrared Thermal Camera.

The photothermal conversion efficiency (η) was calculated according to the following formula.[3]

$$\eta = \frac{hS(\Delta T_1 - \Delta T_2)}{I(1-10^{-A})} \qquad hS = \frac{mc}{\tau_s} \qquad t = -\tau_s \ln(\theta) \qquad \theta = \frac{T - T_{surr}}{T_{max} - T_{surr}}$$

T is the temperature at moment t in the cooling process. T_{max} is the maximum temperature of sample. T_{surr} is the surrounding temperature. m and c are the mass and heat capacity of solvent, respectively. ΔT_1 and ΔT_2 are the maximum temperature change of sample and H_2O, respectively. I is the laser power. A is the absorbance of sample at 635 nm.

Cell Culture.

4T1 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (Gibco) and 100 units/mL of 1% antibiotics penicillin/streptomycin (Gibco). All the cells were cultured in a humidified incubator at 37 ºC with 5% CO_2.

Co-localization analysis of M-PT-RGD in 4T1 cells by CLSM.

4T1 cells seeded in confocal dishes were incubated in M-PT-RGD (50.00 μM) for 4 h, 8 h, and 12 h at 37 °C under 5% CO_2. After washing three times with PBS (10.00 mM, pH = 7.4), 4T1 cells were incubated with Lyso-Tracker Red at 37 °C for 10 min in darkness. Then, each dish was washed with PBS (10.00 mM, pH = 7.4) for another three times, and the fluorescence images of the cells were obtained using TCS SP8 confocal laser scanning microscopy (Leica, Germany).

4T1 cells seeded in confocal dishes were incubated in M-PT-RGD (50.00 μM) or PT-RGD (50.00 μM) for 12 h at 37 °C under 5% CO_2. After washing three times with PBS (10.00 mM, pH = 7.4), 4T1 cells were incubated with Lyso-Tracker Red at 37 °C for 10 min in darkness. Then, each dish was washed with PBS (10.00 mM, pH = 7.4) for another three times, and the fluorescence images of the cells were obtained using TCS SP8 confocal laser scanning microscopy (Leica, Germany).

MTT Assays.

4T1 cells seeded in 96-well plates were incubated in different concentrations of M-PT-RGD solutions for 12 h. Subsequently, the cells were cultured with fresh complete medium and with or without 635 nm laser irradiation (0.50 W/cm^2, 5 min). After incubation for 2.5 h, 200.00 μL MTT solution (0.50 mg/mL) was added to each well. After 4 h, the remaining MTT solution was removed, and 150.00 μL DMSO was added to each well to dissolve the formazan crystals. The absorbance was measured at 490 nm with a RT 6000 microplate reader.

Live/Dead Cell Staining Assays.

4T1 cells seeded in confocal dishes were divided into five groups (PBS, Laser, M-PT-RGD, PT-RGD+Laser, M-PT-RGD+Laser) and incubated with different materials (50.00 μM) for 12 h. After washing three times with PBS (10.00 mM, pH = 7.4), 4T1 cells were cultured with fresh complete medium and with or without 635 nm laser irradiation at 0.50 W/cm^2 for 5 min. After 2 h, the calcein-AM and PI solutions were stained in PBS buffer for 20 min. Finally, the cells were washed three times with PBS and imaged by confocal fluorescence imaging.

Lysosomal Membrane Permeability (LMP) Assays.

4T1 cells seeded in confocal dishes were divided into five groups (PBS, Laser, M-PT-RGD, PT-RGD+Laser, M-PT-RGD+Laser) and incubated with different materials (50.00 μM) for 12 h. After washing three times with PBS (10.00 mM, pH = 7.4), 4T1 cells were cultured with fresh complete medium and with or without 635 nm laser

irradiation at 0.50 W/cm^2 for 5 min. After 20 min, 4T1 cells were incubated with acridine orange (5.00 mg/mL) at 37 °C for 10 min in darkness. Finally, the cells were washed three times with PBS and imaged by confocal fluorescence imaging.

Tumor Model Establishment.

All animal experiments performed were performed in accordance with the Laboratory Animal Care Principles (ROC). Balb/c mice (4-6 weeks old, female, ~18g) were fed for 12 h under normal conditions of light and dark circulation, and mice were provided plenty of food and water. 4T1 cells were injected subcutaneously into mice. After the tumor size reached approximately 85-115 mm^3, the mice were randomly divided into 5 groups (PBS, Laser, M-PT-RGD, PT-RGD+Laser, M-PT-RGD+Laser) for subsequent experiments.

***In vivo* Fluorescence, Photoacoustic and Photothermal Imaging.**

M-PT-RGD in saline (150.00 μL, 0.50 mM) were injected into 4T1 tumor-bearing mice via the intravenous injection. We used IVS animal imaging system, Endra Nexus 128, and Infrared Thermal Camera to perform fluorescence, photoacoustic and photothermal imaging of mice injected with M-PT-RGD at different time points, respectively.

***In vivo* Biosafety Experiment and Therapeutic Effect.**

4T1 tumor-bearing mice were divided into five groups (PBS, Laser, M-PT-RGD, PT-RGD+Laser, M-PT-RGD+Laser). Different materials in saline (150.00 μL, 0.50 mM) were injected into each mouse by intravenous injection. After 12 h, the tumor site was irradiated with or without a 0.50 W/cm^2 635 nm laser for 10 min.

The tumors were dissected 12 h later and the main organs (heart, liver, spleen, lung and kidney) were dissected 7 days later for H&E staining.

On the day 16, blood was taken from the eyes of the mice. After 20 min at room temperature, the samples were centrifuged at 4 ºC and the supernatants were stored at -80 ºC for biochemical analysis.

Tumor size and body weight were measured every other day after treatment (tumor volume = $L \times W^2/2$, W = width, L = length). The relative tumor volume of each mouse was calculated as V/V_0 (V_0 is the tumor volume at the start of treatment).

Supporting Figures

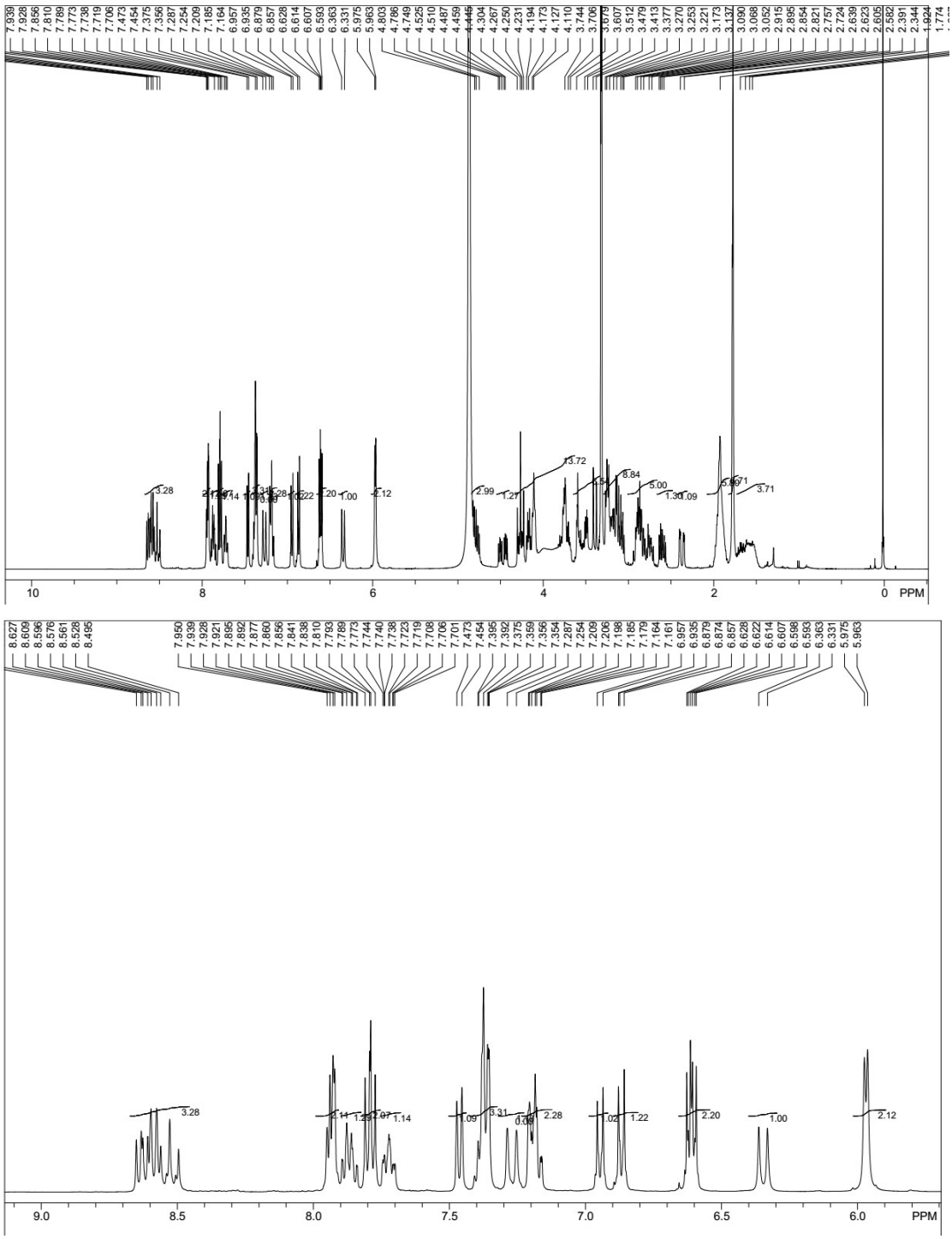


Figure S1. ^{1}H-NMR spectrum of M-PT-RGD.

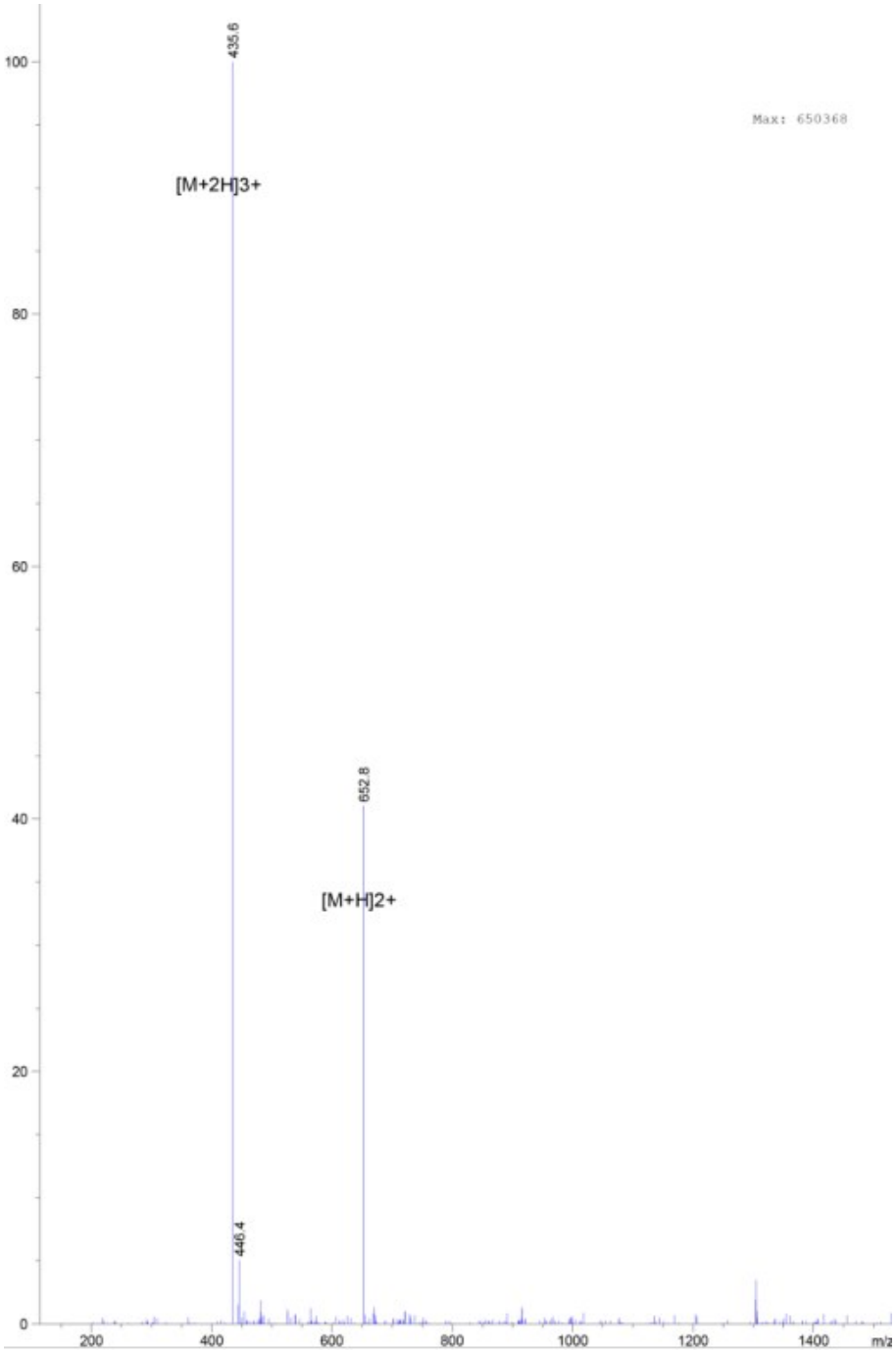
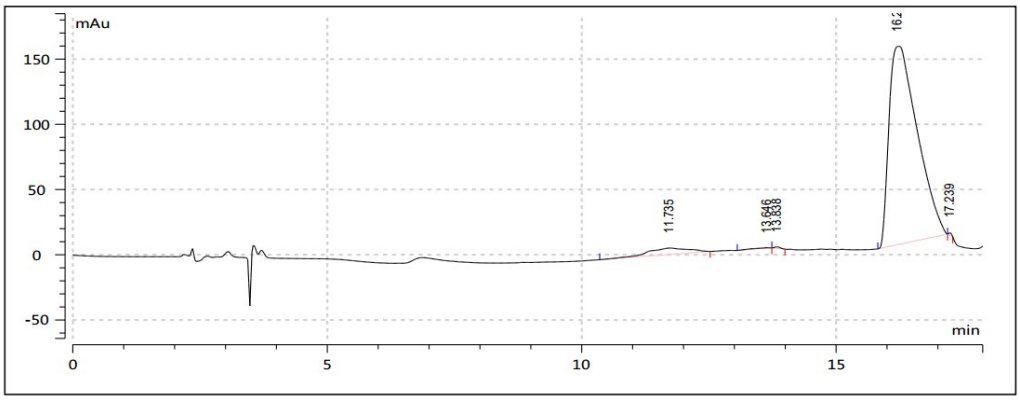


Figure S2. MS spectrum of M-PT-RGD.

Solvent A: 0.1% Trifluoroacetic in 100% Acetonirile
Solvent B: 0.1% Trifluoroacetic in 100% Water

Gradient:	A	B
0.01 min	5%	95%
25.0 min	70%	30%

Flow rate: 1.0 mL/min
Wavelength: 214 nm



Rank	Name	Time	Area	% Area
1		11.735	269.06689	4.364
2		13.646	12.07363	0.196
3		13.838	8.67085	0.141
4		16.226	5867.78074	95.170
5		17.239	8.01152	0.130

Figure S3. HPLC spectrum of M-PT-RGD.

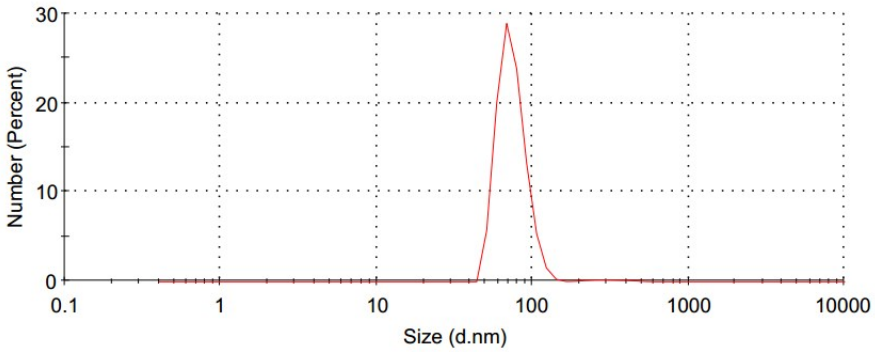


Figure S4. Size distribution of M-PT-RGD in water *via* dynamic light scattering analysis.

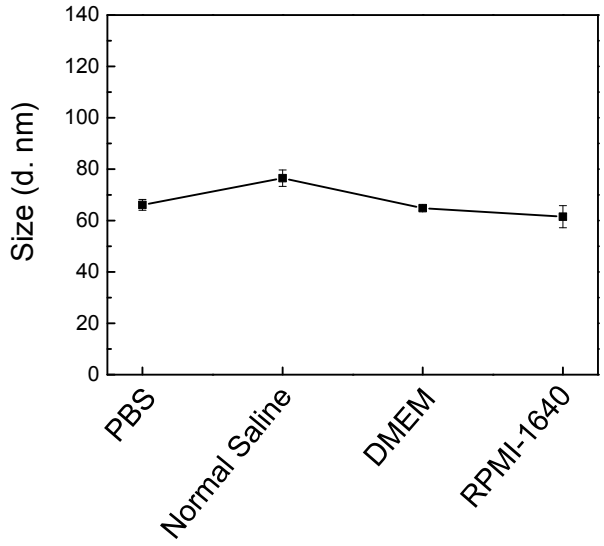


Figure S5. DLS size of M-PT-RGD with different treatments for 48 h.

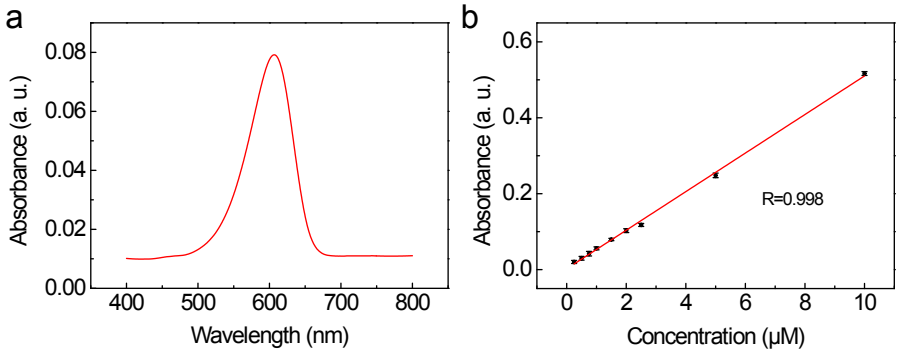


Figure S6. (a) The UV-Vis-NIR spectra of M-PT-RGD in water. (b) The linear curve of concentration and absorbance of M-PT-RGD at 610 nm.

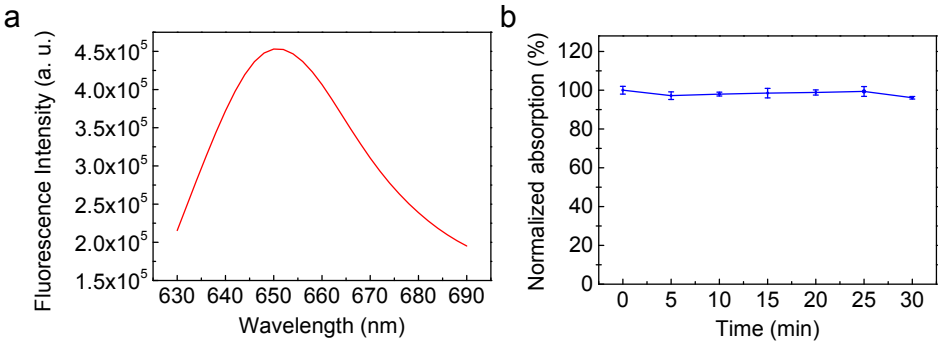


Figure S7. (a) The fluorescence emission spectrum and (b) photostability of M-PT-RGD in water.

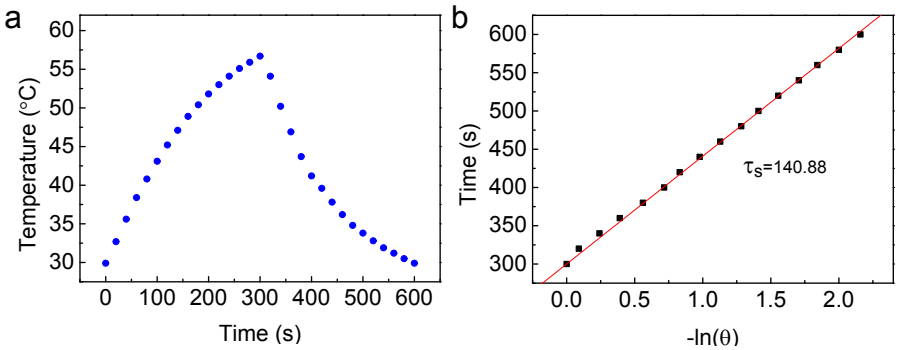


Figure S8. (a) Heating and cooling curves of M-PT-RGD (0.5 mM) under 635 nm laser irradiation (0.5 W/cm^2, 0-300 s). (b) Linear relationship in plots of the time versus the –ln(θ).

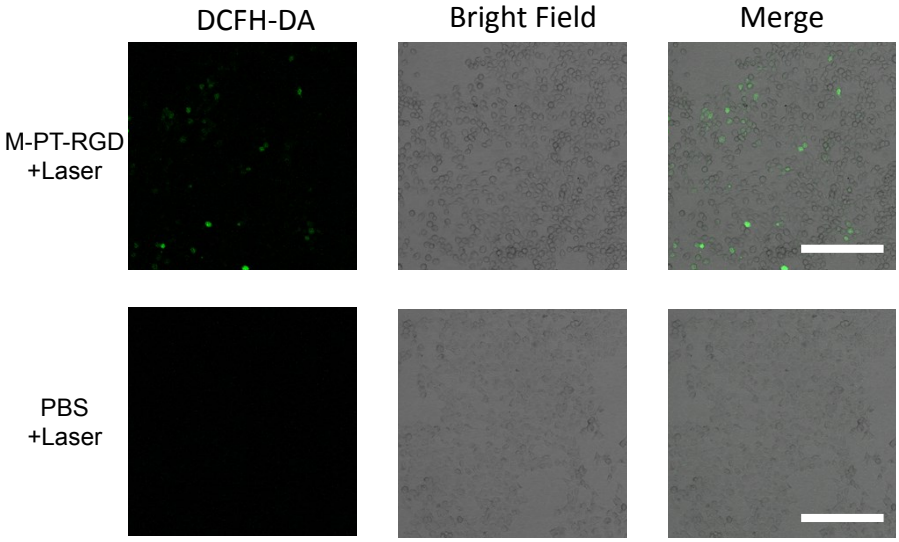


Figure S9. CLSM images of 4T1 cells with different treatments stained with DCFH-DA (green). Scale bars are 250 μm.

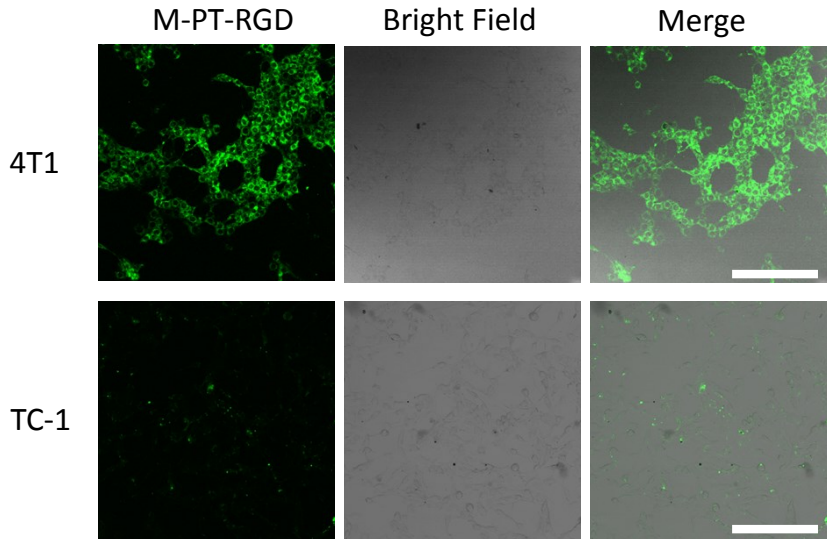


Figure S10. CLSM images of 4T1 and TC-1 cells treated with M-PT-RGD. Scale bars are 250 μm.

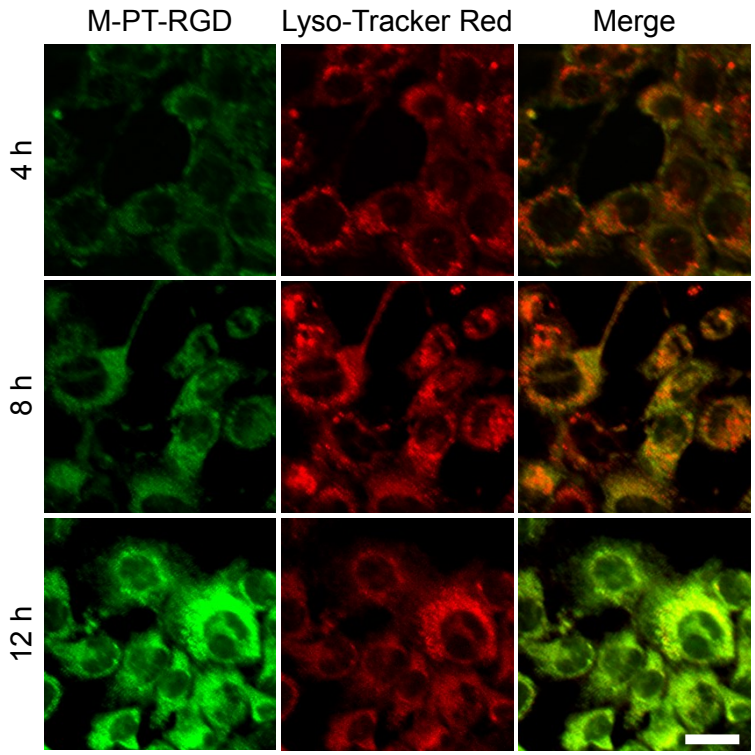


Figure S11. Co-location fluorescence imaging of M-PT-RGD and Lyso-Track Red at different times (4, 8 and 12 h). Scale bar is 20 μm.

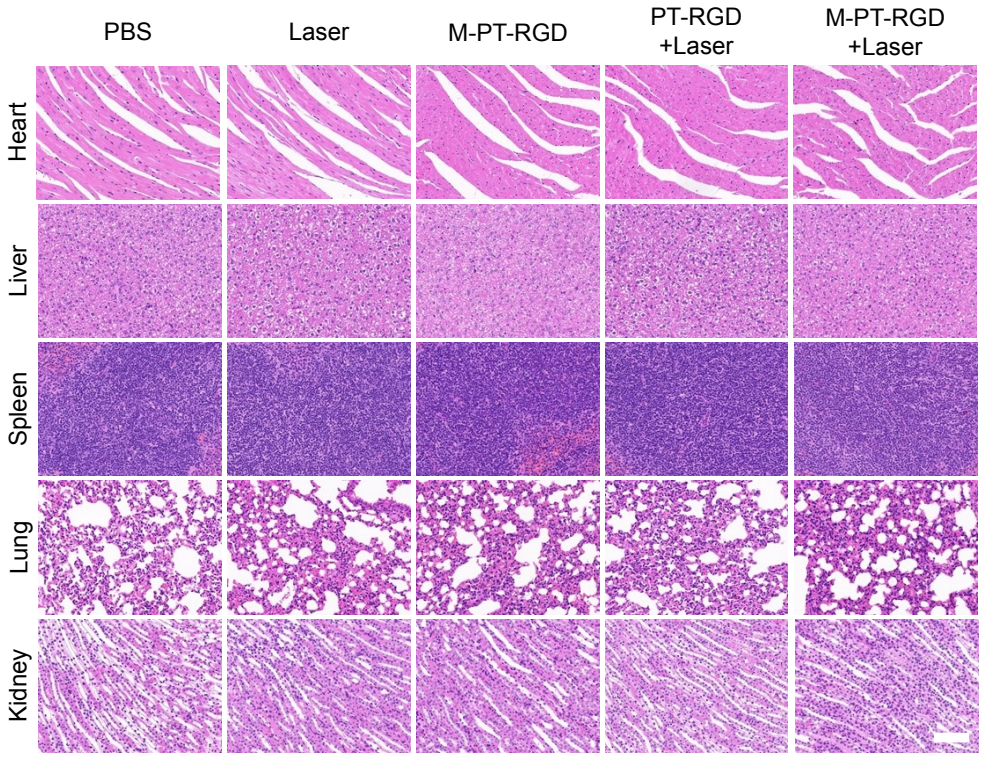


Figure S12. H&E stained images of the heart, liver, spleen, lung and kidney in 4T1 tumor-bearing mice after different treatments. Scale bar is 100 μm.

References

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