Electronic Supplementary Material (ESI) for Nanoscale.

Supporting Information

A Self-Assembly of Tumor-Targeted Photothermal Agent for Enhanced Anti-Inflammatory Cancer Therapy

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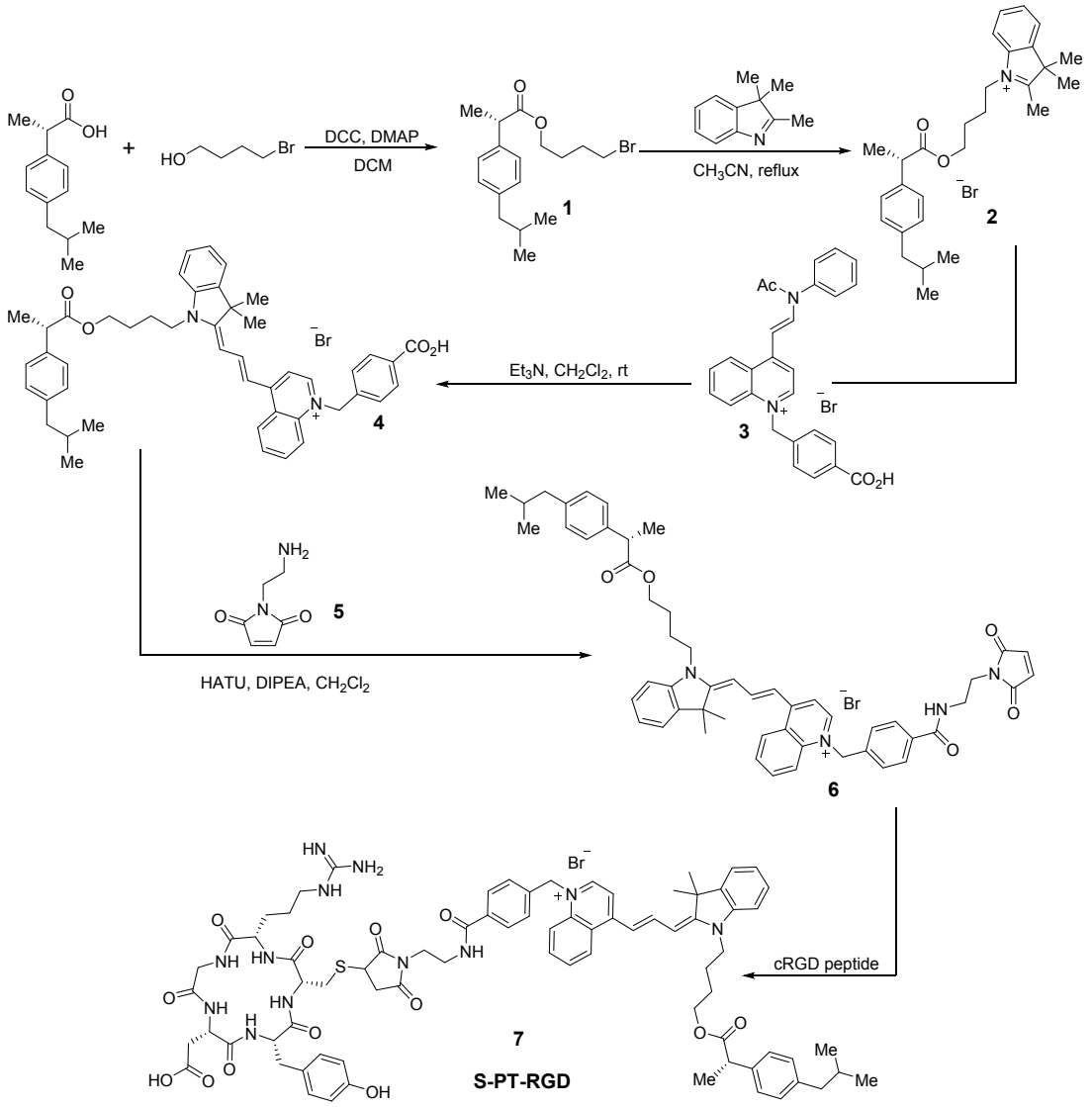
1. General information

Chemicals： 4-Methylquinoline was purchased from Shanghai Macklin Biochemical Co., Ltd. (S)-(+)-ibuprofen, naproxen, maleimide, 4-bromo-1-butanol were obtained from Shanghai Energy Chemical Co., Ltd. 4-(bromomethyl)benzoic acid, Acetylsalicylic acid, 2,3,3-trimethyl-3H-indole, Trifluoroacetic acid (TFA), 2-(Boc-amino)-1-ethanol 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) diisopropyl azodicarboxylate（DIAD）were obtained from Tianjin Heowns Biochemical Technology Co., Ltd. N-ethyldiisopropylamine (DIPEA) was purchased from Adamas Reagent, Ltd. Methanol, acetonitrile, dichloromethane (DCM), toluene, acetic anhydride dimethyl sulfoxide （DMSO） dicyclohexylcarbodiimide (DCC) and triethylamine (TEA) were purchased from China National Pharmaceutical (Shanghai, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2h-tetrazoliubromide (MTT) and dialysis Membranes were obtained from Beijing Solarbio Science & Technology Co., Ltd. Cyclo (RGDyC) was purchased from Shanghai Dechi Biosciences Co., Ltd. N,N'-diphenylformamidine was purchased from Alfa Aesar (Tianjin, China). Annexin V-Alexa Fluor 488/PI Apoptosis Detection Kit was purchased from Yeasen (Shanghai, China). Tracker Green (MTG) was obtained from Beyotime (Shanghai, China). Mito-Tracker Green (MTG) was purchased from Molecular Probes (Invitrogen, U.S.). The mouse breast cancer cell line (4T1), A549 and Hela cell line were 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: High resolution transmission electron microscopy (HRTEM) was carried out on a JEM-2100 electron microscope. 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. Success of each reaction step was confirmed by monitoring the changes in zeta potential with a Malvern Zeta Sizer Nano (Malvern Instruments). Photoacoustic imaging was accomplished using an Endra Nexus 128 (Ann Arbor, Michigan). All the NMR spectra were recorded on a Bruker NMR spectrometers. High resolution mass spectra (HRMS) were recorded on a Bruker Daltonics maXis UHR-TOF MS.

2. Synthesis of Organic Photothermal Agent (S-PT-RGD).



Compound 1: A mixture of (*S*)-(+)-ibuprofen (618.84 mg, 3.0 mmol), 4-dimethylaminopyridine (36.6 mg, 0.3 mmol), dicyclohexylcarbodiimideand (865.2 mg, 4.2 mmol) and 4-bromo-1-butanol (1093 μL, 12 mmol) in dichloromethane was stirred at 25 °C for 12 h. The solvent was evaporated off. The crude product was purified by column chromatography over silica gel using petroleum ether/ethyl acetate (v/v, 5:1) as the eluent (90% yield).

Compound 2: A mixture of 2,3,3-Trimethyl-3H-indole (642 μL, 4mmol) and compound 1 (1364 mg, 4 mmol) in anhydrous acetonitrile was stirred at 25 °C for 12 h. After removal of the acetonitrile solvent under vacuum, the crude product was purified by column chromatography over silica gel using CH_2Cl_2/MeOH (v/v, 20:1) as the eluent (86% yield).

Compound 4: A mixture of compound **2** (0.420 g, 1.0 mmol), compound **4** (0.423 g, 1.00 mmol) and triethylamine (1.391 mL, 10.0 mmol) in dichloromethane (10 mL) was stirred at 25 °C for 24 h. After removal of the dichloromethane solvent under vacuum, the crude product was purified by column chromatography over silica gel using CH_2Cl_2/MeOH (v/v, 10:1) as the eluent (60% yield).

Compound 5: Maleimide (624 mg, 6.44 mmol) triphenylphosphine (1658 mg, 6.32 mmol) and THF (30 mL) were added into 100 mL round-bottom flask, and then N-(tert-butoxycarbonyl) ethanolamine (5.00 mL, 29.3 mmol) and diisopropylazidodicarboxylate (6.80 mL, 35.1 mmol) were mixed in succession. The flask was stirred under a nitrogen atmosphere overnight, the reaction mixture was concentrated on a rotary evaporator, and the crude product was filtered through a plug of silica gel using CH_2Cl_2/MeOH (v/v, 40:1) as the eluent.

The product was added in 10 mL of trifluoroacetic acid and 20 mL of DCM. The reaction mixture was stirred for 12 h and then dried under vacuum. The crude product was then dissolved in 2 mL of methanol and anhydrous diethyl ether was added to precipitate the desired product. The produce was collected through filtration and dried under high vacuum.

Compound 6: A mixture of compound **5** (0.354 g, 0.5 mmol), N,N′-diisopropylethylamine (0.3 μL) and HATU (0.38 g, 1mmol) in DCM (10 mL) was stirred at 25 °C for 30 min. Then, Compound 6 (0.165 g, 1 mmol) and triethylamine (417 μL, 3.0 mmol) were added to the mixture, and stirred at 25 °C for 24 h. After removal of the dichloromethane solvent under vacuum, the crude product was purified by column chromatography over silica gel using CH_2Cl_2/MeOH (v/v, 5:1) as the eluent (50% yield).

Compound 8: 41.5 mg (0.05 mmol) of compound 7 and 30 mg (0.05 mmol) of c(RGDyC) was dissolved in 5 mL methanol. The mixture was stirred at room temperature for 12 h and then precipitated into ethyl acetate to obtain compound 8. Compound 8 was further purified by dialysis in water and dried as a blue powder (40% yield). ^{1}H-NMR (400 MHz, D^6-DMSO) δ 8.59-8.44 (m, 3H), 8.31-8.29 (d, *J* = 8 Hz, 1H), 7.91-7.81 (m, 3H), 7.79-7.77 (d, *J* = 8 Hz, 1H), 7.77-7.75 (d, *J* = 8Hz, 1H), 7.71-7.67 (m, 1H), 7.45-7.43 (d, *J* = 8Hz, 1H), 7.37-7.33 (m, 3H), 7.25-7.22 (d, *J* = 12 Hz, 1H), 7.19-7.15 (dt, *J* = 8Hz, *J* = 1.6 Hz, 1H), 7.14-7.12 (d, *J* = 8Hz, 2H), 7.09-7.07 (dd, *J* = 8Hz, *J* = 1.6Hz, 1H), 6.96-6.93 (m, 3H), 6.88-6.86 (d, *J* = 8Hz, 1H), 6.63-6.58 (dd, *J* = 8Hz, 2H), 6.25-6.22 (d, *J* = 12Hz, 1H), 5.94-5.93 (d, *J* = 4Hz, 1H), 4.80-4.74 (m, 1H), 4.53-4.40 (br, 1H), 4.30-4.23 (m, 3H), 4.20-4.11 (m, 2H), 3.92 (s, 2H), 3.79-3.66 (m, 4H), 3.59-3.43 (m, 2H), 3.39-3.35 (d, *J* = 16Hz, 1H), 3.24-3.04 (m, 4H), 2.94-2.69 (m, 4H), 2.63-2.53 (m, 1H), 2.38-2.33 (dd, *J* = 16Hz, *J* = 4Hz, 1H), 2.28-2.26 (d, *J* = 8Hz, 2H), 1.93-1.83 (br, 1H), 1.74 (s, 9H), 1.71-1.64 (m, 3H), 1.60-1.48 (br, 2H), 1.42-1.40 (d, *J* = 8Hz, 3H), 1.28-1.26 (m, 1H), 0.80 (s, 3H), 0.75 (s, 3H); HRMS (ESI) m/z calcd for $C77H92N12O13S^{2+}$ [$(M+H-Br)^{2+}$]: 1425.7129; found: 1425.9 and 712.6.

3. Experimental Section.

Cell culture. 4T1 cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium, A549 cells were cultured in dulbecco's modified eagle medium, and Hela cells were cultured in minimum Eagle's medium. All cells were supplemented with 10% fetal bovine serum (BI) and 100 units/mL of 1% antibiotics penicillin/streptomycin (Gibco) to maintain at 37 in a 100% humidified atmosphere containing 5% CO_2.

Co-localization analysis of S-PT-RGD in 4T1 cells by CLSM. 4T1 cells were cultured in RPMI-1640 containing 10% fetal bovine serum and antibiotics penicillin/streptomycin at 37 °C in a 5% CO_2/95% air incubator. For fluorescence imaging, cells (4×10^3/well) were passed on confocal dishes and incubated for 24h. Immediately before the staining experiment, cells were washed three times with PBS (10 mM, pH = 7.4), and then incubated with 25 μM S-PT-RGD for 4 h at 37 °C. Then the petri dish was washing with PBS (10 mM, pH = 7.4) for another three times, and incubating with Mito-Tracker Green (20 nM) for 15 min at 37 °C. Finally, wash each dish with PBS (10 mM, pH = 7.4) for three times, and analyzed with a TCS SP8 confocal laser scanning microscopy.

Cytotoxicity Assays. 4T1, A549, Hela cells were seeded in 96-well plates at an amount of 5×10^3 for 24 h and incubated with S-PT-RGD (0 μg/mL, 5 μg/mL, 10 μg/mL, 25 μg/mL, 50 μg/mL and 100 μg/mL) for another 24 h. Subsequently, the cells were cultured with fresh complete medium and irradiated or not with 635nm laser at a power density of 0.5W/cm^2 for 5 min. Next, 150 μL MTT solution (0.5 mg/mL) was added to each well. After 4 h, the remaining MTT solution was removed, and 150 μL of DMSO was added to each well to dissolve the formazan crystals. The absorbance was measured at 490 nm with a RT 6000 microplate reader.

4T1 cells (2.0×10^5 per dish) were seeded on 35 mm confocal dishes and allowed to stabilize for 24 h.

For Calcein-AM and PI co-staining Assay, 4T1 cells incubated with S-PT-RGD were or not exposed to 635 nm light source at 0.5W/cm^2 for 5 min. After another 4 h of incubation, the cells were stained with calcein-AM and PI for 30 min to evaluate the PTT efficacy using a fluorescence spectrometer.

Animal tumor xenograft models. All animal experiments were prepared and agreed with the Principles of Laboratory Animal Care (People's Republic of China). Balb/c mice (6-8 weeks old, female, ~20 g) were fed with normal conditions. For the purpose of establishing the tumor xenograft models, 4T1 cells (1×10^6) suspended in 50 μL RPMI 1640 were subcutaneously injected into the back of the mice. The treatments were carried out only once when the tumor volume reaches to about 50 mm^3.

Cytokine Detection. Tumor-bearing mice were divided into for 3 groups to detect serum cytokines after the following treatments: (1) only PBS; (2) PT-RGD + 635 nm NIR laser for 10 min; (3) S-T-RGD + 635 nm NIR laser for10 min. Serum samples were separated from mice after various treatments and diluted for analysis. TNF-α and IL-6 (all from Sangon Biotech (Shanghai) Co., Ltd.) were analyzed with ELISA kits according to the vendor’s instructions.

In vivo fluorescence imaging and Photoacoustic imaging (PA). Tumor targeting characteristics of PT and S-PT-RGD were studied in 4T1 tumor-bearing mice. It was randomly

divided into two groups: first group, intravenous injection of PT; second group, intravenous injection of S-PT-RGD. S-PT-RGD (150 μL, 0.5 Mm) or PT in PBS were intravenously injected via tail vein. We used IVS animal imaging system to perform fluorescence imaging of mice injected with different materials at different time points (12, 24, 48 hours). The excitation wavelength was 620−635 nm and the emission wavelength was 650−700 nm. For in vivo PA imaging, 4T1 tumor-bearing mice were injected intravenously with 150 μL S-PT-RGD (0.5 mM) and detected by in Endra Nexus 128 at various time points (12, 24 and 48 hours).

In vivo biodistribution of S-PT-RGD. In vivo biodistribution of S-PT-RGD in 4T1 tumor bearing mice was performed with an IVS animal imaging system. Mice treated with S-PT-RGD were sacrificed and their major organs including heart, liver, spleen, lung, and kidney were removed for visualization under the imaging system at different time points (12, 24 and 48,hours). The fluorescence intensity of each tissue organ was analyzed by the system software.

In vivo antitumor efficacy via injection. When the tumor size reached about 50 mm^3, the tumor-bearing mice (n=5) were divided into five groups: Group 1: only PBS injection; Group 2: only PT injection; Group 3: only laser; Group 4: PT injection and laser; Group 5: S-PT-RGD injection and laser. Various materials in PBS was injected via tail vein. After 24 hours, 635 nm laser treatment was performed on group 3, 4, and 5 by irradiating the tumor region at power of 0.5W/cm^2 for 10 min. The effect of different treatment groups were monitored by measuring the tumor size using a Vernier caliper after the treatment. The tumor volume (V) was computed as $V=L\times W^2/2$ by measuring length (L) and width (W).

In vivo biosafety experiment. Biosafety of materials in 4T1 tumor bearing mice was tested by using H&E slice analysis major organs and measurement mice body weight. The mice with the same age in each treatment group were sacrificed, and their major organs including heart, liver, spleen, lung, and kidney and tumor were carefully removed. The major organs were dissected for H&E staining.

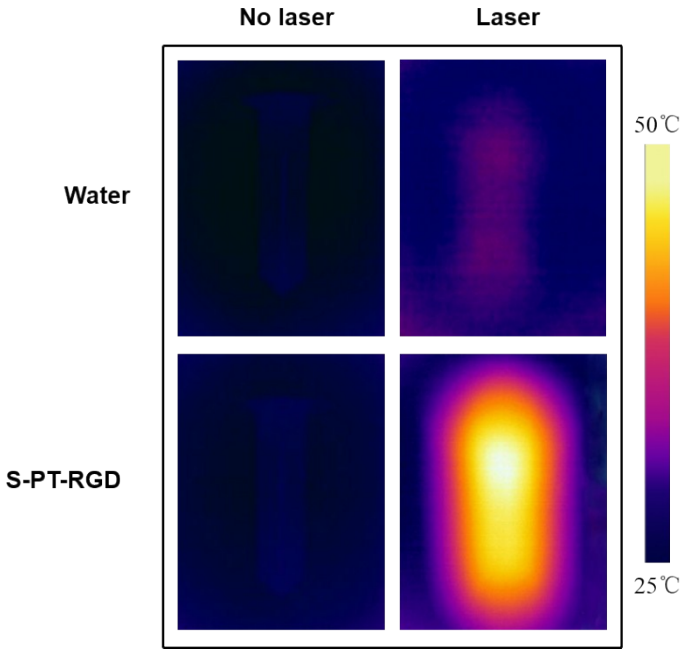


Figure S1. IR thermal images of S-PT-RGD dispersed in water under irradiation by the 635-nm light (0.5 W/cm^2) for 5 min.

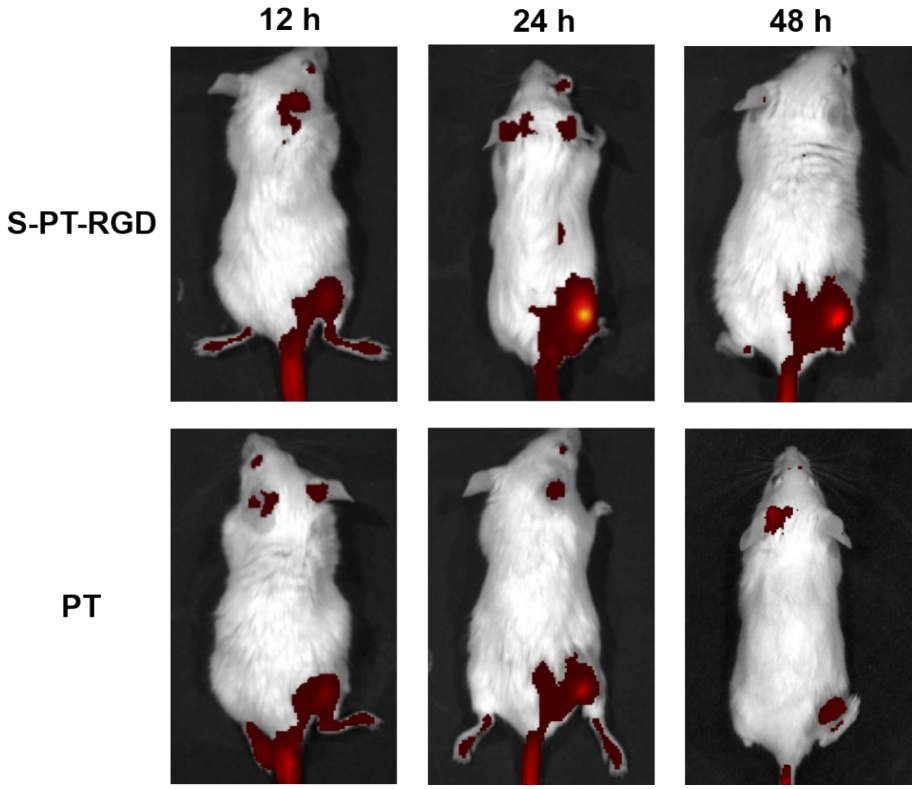


Figure S2. In vivo fluorescence imaging of 4T1 tumor-bearing mice after intravenous injection of PT or S-PT-RGD at 12, 24 and 48 hours.

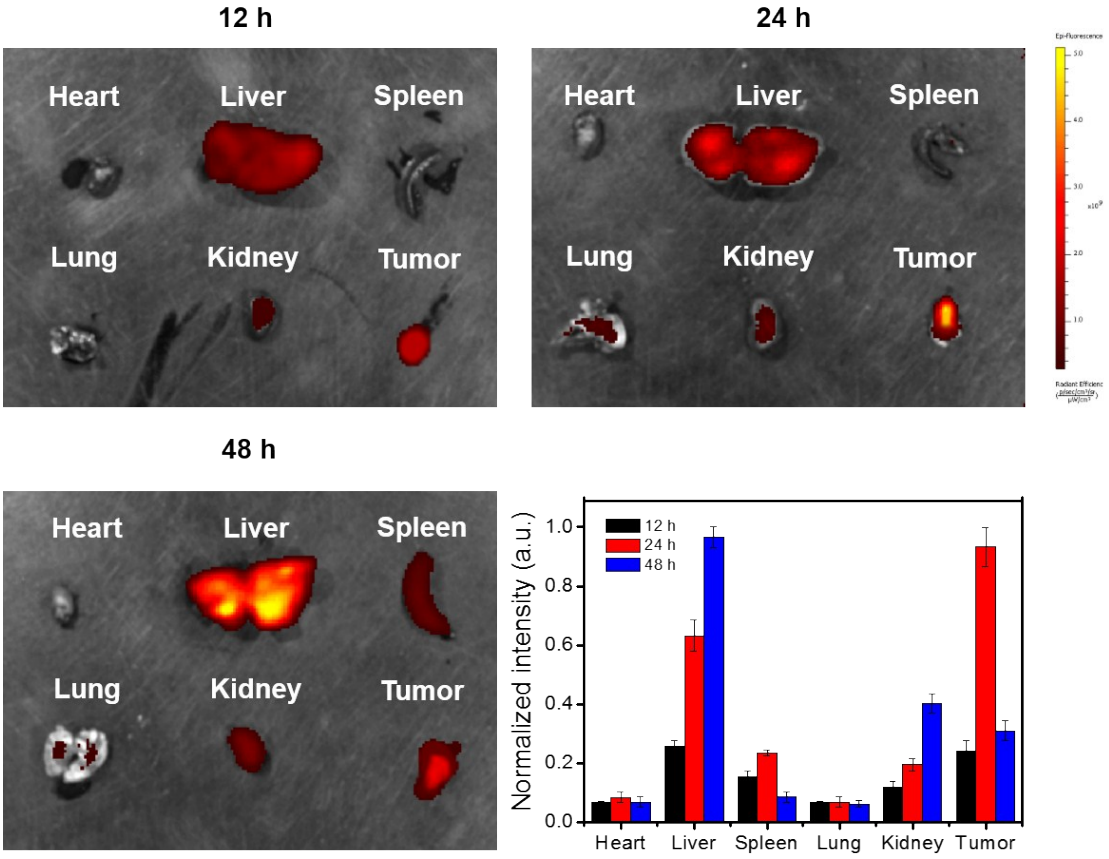


Figure S3. Bio-distribution of S-PT-RGD in tumor-bearing mice. Exe vivo fluorescence imaging of major organs (heart, liver, spleen, lung, and kidney) and tumor dissected from 4T1 tumor-bearing mice after intravenous injection of S-PT-RGD at 12, 24 and 48 hours. And quantitative fluorescence intensity of major organs and tumors.

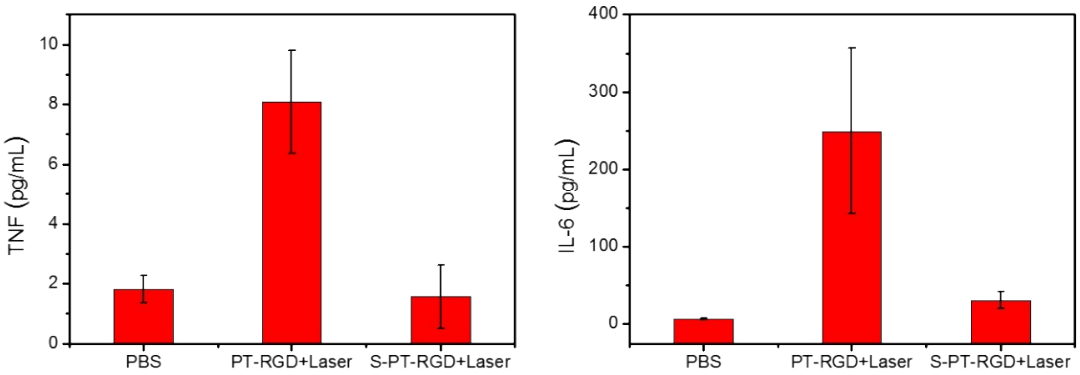


Figure S4 Cytokine levels in sera of mice after various treatments.

4. NMR and HRMS Spectra of S-PT-RGD.

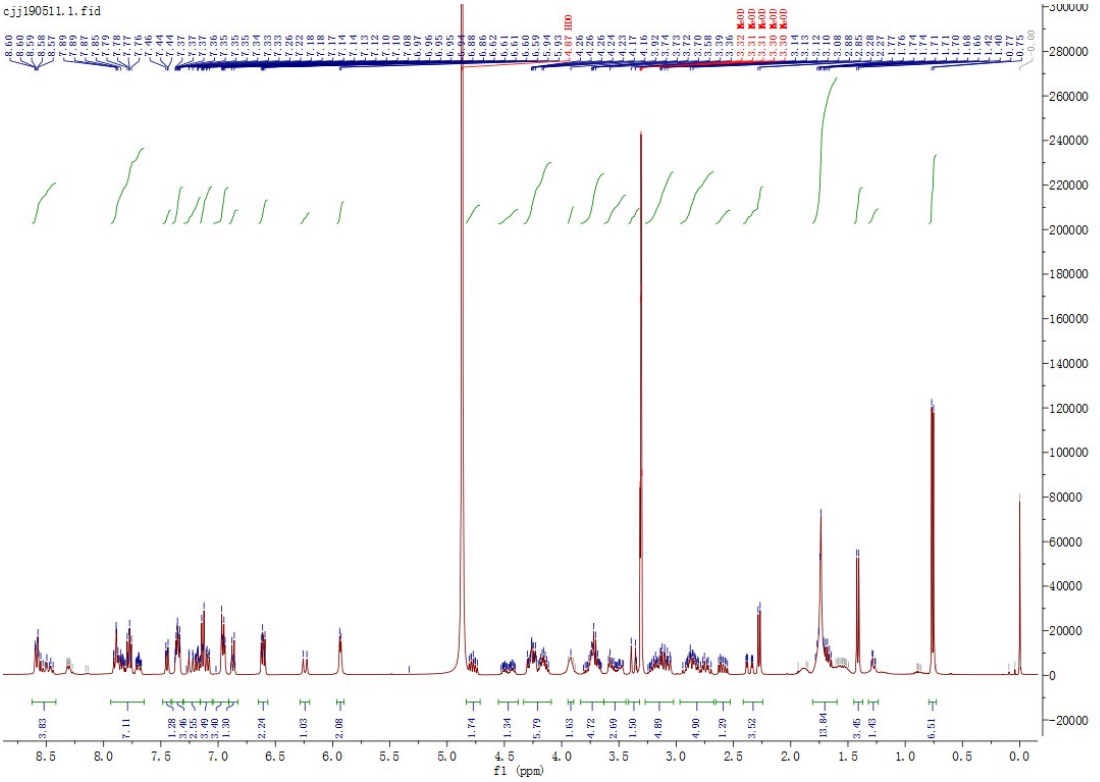


Figure S6. ^{1}H-NMR spectrum of S-PT-RGD.

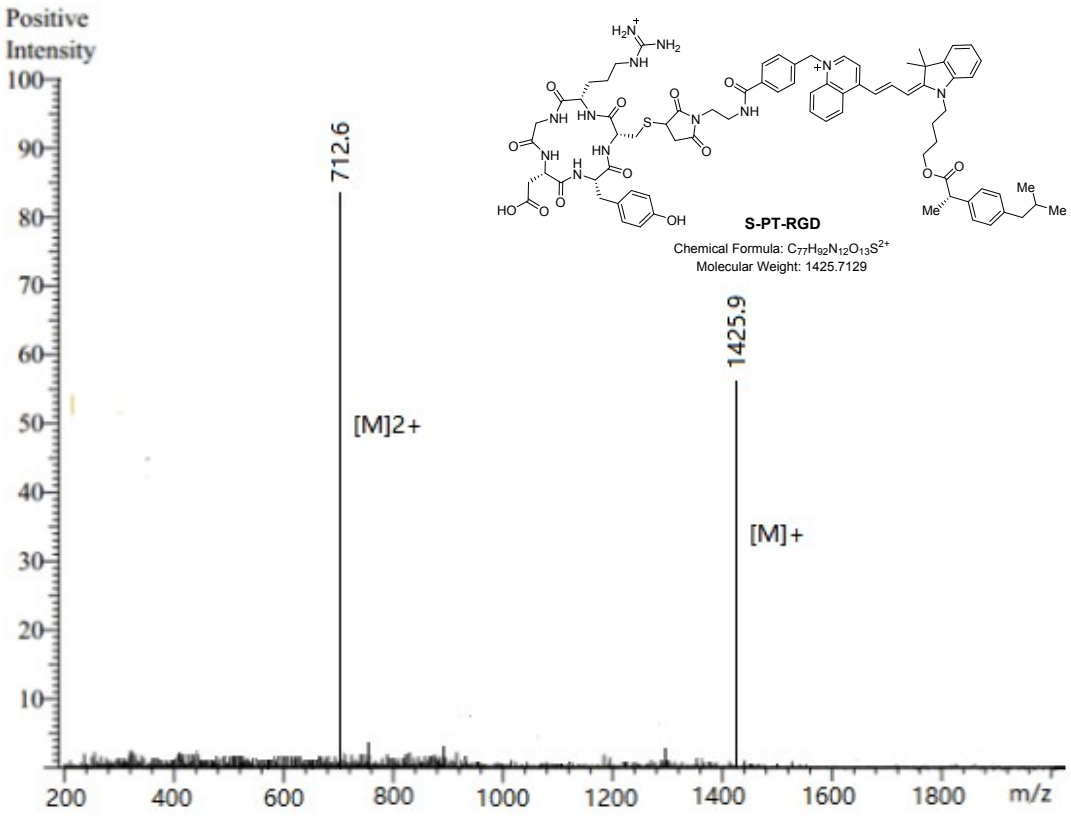


Figure S7. HRMS (ESI) spectrum of S-PT-RGD.

5. HPLC Analysis Report.

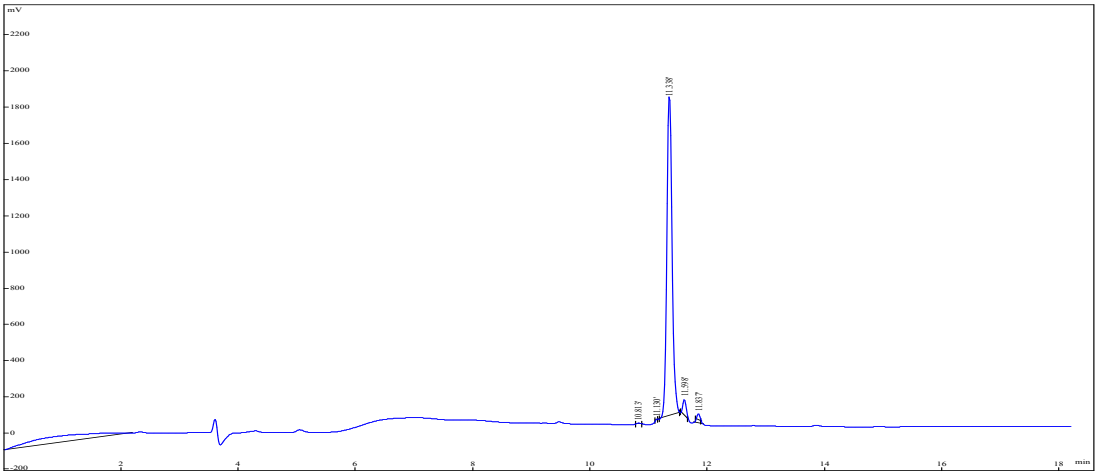
Solvent A: 0.1%Trifluoroacetic in 100% Acetonitrile.

Solvent B: 0.1%Trifluoroacetic in 100% Water.

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

Flow rate :1.0ml/min

Wavelength :214nm



Rank	Time	Name	Conc.	Area
1	10.813		0.1559	17989
2	11.130		0.07794	8991
3	11.338		95.96	11069624
4	11.598		2.917	336524
5	11.837		0.8941	103141
Total			100	11536269

Figure S8. HPLC spectrum of S-PT-RGD.

6. References:

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[3] Ji, C.; Gao, Q.; Dong, X.; Yin, W.; Gu, Z.; Gan, Z.; Zhao, Y.; Yin, M. *Angew. Chem. Int. Ed*. **2018**, *57*, 11384.