Electronic Supplementary Information

Targeted NIR-II Emissive Nanoprobes for Tumor Detection in Mice and Rabbits

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Experimental section

Materials and characterization

All reagents were obtained commercially and used without further purification. Tetrahydrofuran (THF) and toluene were distilled from sodium benzophenone ketyl under dry argon immediately before use.[1] ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded on Bruker AV400 spectrometers (Bruker, Billerica, MA, USA). ^{1}H NMR and ^{13}C NMR spectra used tetramethylsilane (TMS) as an internal standard in $CDCl_3$. High-resolution mass spectra were measured using Q-Exactive with Dionex Ultimate 3000 (Thermo Fisher Scientific, Waltham, MA, USA).

Synthesis of TTB

The TTB molecule was synthesized according to our previously reported procedures.[11] To the 1 M solution of compound **1** (1.1 mL, 2.2 equiv.) in toluene was added 4,7-dibromobenzo[1,2-*c*:4,5-*c*']bis([1,2,5]thiadiazole) (BBTD) (0.16 g, 0.5 mmol) and tetrakis(triphenylphosphine)palladium(0) [$Pd(PPh_3)_4$] (0.06 g, 0.05 mmol, 0.1 equiv.) and dissolved in toluene (3 mL) under argon protection. After refluxing for 24 h and then cooling to room temperature, the mixture was quenched with 1 M KF aqueous solution and washed with ethyl acetate. The combined organic phase was dried over anhydrous Na_2SO_4 and evaporated in vacuum. The crude product was subjected to column chromatography on silica gel with petroleum ether: ethyl acetate (10:1) to obtain TTB as a black green solid (0.27 g, 50%). ^{1}H NMR (400 MHz, $CDCl_3$), δ (ppm): 7.49 (d, *J* = 8.4 Hz, 4H), 7.29 (s, 2H), 7.23-7.18 (m, 9H), 7.08-6.95 (m, 15H), 2.51 (t, *J* = 7.6 Hz, 4H), 1.57-1.54 (m, 8H), 1.23-1.02 (m, 8H), 0.82-0.80 (m, 8H), 0.74 (t, *J* = 7.2 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$), δ (ppm): 152.18, 146.54, 146.40, 145.93, 144.62, 128.31, 127.12, 125.72, 123.75, 123.61, 122.33, 122.16, 115.02, 30.74, 29.45, 29.29, 28.67, 28.40, 28.26, 28.10, 21.58, 13.06. HRMS (ESI): m/z [$M+H^+$] calcd for $C_{66}H_{65}N_6S_4$ 1069.41481; found: 1069.41479.

Synthesis and characterization of the nanoprobes

The nanoprobes were prepared using hydrophobic TTB precursor (1 mg) encapsulated in a mixture of DSPE-PEG_{2000}-Maleimide (1 mg) and DSPE-PEG_{2000} (1 mg), following the same procedures from our previous reports.[40] The obtained TTB NPs were conjugated with thiol-functionalized cRGD to afford cRGD-TTB NPs, through the reaction between the maleimide groups on nanoprobes and thiol groups in cRGD peptides. The individual solution was concentrated using Amicon Ultra-16 Centrifugal Filters (Merck KGaA, Darmstadt, Germany). The average particle sizes of AIE nanoprobes at room temperature were determined using a Zetasizer Nano ZS equipment (Malvern, Worcestershire, UK). The morphology of nanoprobes was observed under the JEOL JEM-2100F Transmission Electron Microscope (TEM) (JEOL, Ltd., Tokyo, Japan). The absorption and NIR-II fluorescence spectra of nanoprobes were measured separately using UV-2600 UV-Vis-NIR spectroscopy (Shimadzu, Kyoto, Japan) and iHR320 spectrometer (Horiba, Kyoto, Japan). To determine the quantum yield (ϕ) values of nanoprobes, the samples were dispersed in water and IR-26 was dissolved in dichloroethane in a series of concentrations. The fluorescence intensities of these samples were recorded under 808 nm laser excitation. The integrated area of fluorescent profile was plotted against concentration to obtain the slope. The ϕ was calculated in the following manner:

$$\phi_{sample} = \phi_{ref} \frac{slope_{sample}}{slope_{ref}} \left[\frac{n_{sample}}{n_{ref}}\right]^2$$

Note: ϕ_{ref} of IR-26 in dichloroethane is 0.5%; the refractive index (n_{sample} and n_{ref}) is 1.33 and 1.414, respectively.

Cell culture

The cells were purchased from Xiehe Cell Bank of the Chinese Academy of Medical Sciences. The 3T3 mouse embryonic fibroblasts and VX2 rabbit hepatoma cells were cultured in DMEM medium at 37 °C with 5% CO_2. The rat C6-Luc glioma cells were cultured in high-glucose DMEM medium at 37 °C with 5% CO_2.

Cytotoxicity study of nanoprobes

The metabolic viability of mouse 3T3, rat C6 cells and rabbit VX2 cells was evaluated individually *via* Cell Counting Kit-8 (CCK-8) assay. The cells were seeded in 96-well plates at a density of 5×10^3 cells per well. The cells were cultured in an incubator for 24 h, followed by medium replacement using 200 μL of cRGD-TTB NPs solutions at concentrations of 100, 50, 20, 10, 5 μg/mL. Upon further incubation for 24 h, 10 μL of CCK-8 solution was added into each sample well. After another 2 h incubation, the absorbance from each sample well was measured using a microplate reader at 450 nm (n = 6 in each loading concentration). Cell viability was expressed as the absorbance ratio of the cells treated with nanoprobes over the cells incubated with culture medium only.

Cellular uptake of the non-functionalized and cRGD-functionalized nanoprobes

The cellular uptake of TTB NPs and cRGD-TTB NPs by cancer cells was first evaluated, individually. Upon individual incubation with C6 and VX2 cells for 4 h at 40 μg/mL of NPs, the cells were detached and free NPs were discarded by centrifugation. After washing with 1× PBS buffer (7.2-7.4) twice, the cells were collected in 1.5 mL centrifuge tubes for imaging (Series III 900/1700-D NIR-II imaging system). The excitation wavelength is 808 nm and the NIR-II fluorescence was collected using a 1319 nm long-pass filter.

The targeting ability of cRGD-functionalized NPs was further investigated using flow cytometry and confocal microscopy. To meet the requirement of commercial flow cytometry analyzer and confocal microscopy, red-emissive NPs were synthesized by encapsulating a red emissive AIEgen (TPETPAFN)[42] instead of TTB to prepare FN NPs and cRGD-FN NPs, following the same synthetic procedures of TTB NPs and cRGD-TTB NPs. TPETPAFN (1 mg) was mixed with DSPE-PEG_{2000}-Maleimide (1 mg) and DSPE-PEG_{2000} (1 mg), instead of using TTB. To investigate the targeting efficiency to C6 or VX2 cells, the cells were first cultured in confocal chambers or six-well plates to achieve 80% confluence, respectively. The cells were then co-cultured with 40 μg/mL of cRGD-FN or FN NPs for 4 h, respectively. In the blocking group, the cells were first treated with 1 mM of cRGD for 2 h to block the $\alpha_v\beta_3$ integrin receptors, followed by addition of 40 μg/mL of cRGD-FN NPs for another 4 h. After washing with 1× PBS buffer (pH = 7.2-7.4) to eliminate the free NPs, the cells in confocal chambers were fixed by 4% paraformaldehyde for confocal imaging (Excitation: 488 nm; Filter: 620-700 nm). The cells in six-well plates were detached by 1× trypsin and resuspended for flow cytometry analysis (Excitation: 561 nm; Filter: 640-700 nm). The cells without NP treatment were used as control.

Animal handling

All animal protocols were approved by the Institutional Animal Care and Use Committee at the Southern University of Science and Technology and the Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences. Female BALB/c nude mice and SPR New Zealand white rabbits were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. and Qingdao Kangda Biotechnology Co., Ltd., respectively. A glioma orthotopic tumor model was established by implanting C6-Luc glioma cells (5×10^5 cells, 5 μL) into the mouse brain striatum (bright lateral: 1.6 mm, bregma: 4.6 mm, depth: 3.5 mm) using a mouse adaptor (RWD Life Science, Shenzhen, China). The rabbit tumor model was developed through a two-step approach. First, rabbit VX2 tumor cells (5×10^7 cells) dispersed in 500 μL 1× PBS (pH = 7.2 -7.4) were subcutaneously injected into the leg of SPF New Zealand white rabbit to establish a VX2 tumor-bearing rabbit model. Second, the obtained VX2 tumors were cut into 1 mm pieces, mixed with the medium, and transplanted into the leg muscle of SPF New Zealand white rabbit. After three weeks, the rabbit tumor model was successfully established.

NIR-II imaging in glioma bearing mice

The NIR-II fluorescence imaging was conducted once the C6-Luc tumors were successfully formed through bioluminescence imaging confirmation. Upon intravenous injection of TTB or cRGD-TTB NPs (10 mg/kg), the NIR-II fluorescence imaging of tumors was performed at different time points (0, 3, 6, 9, 12, 24 h post-injection), using an 808 nm laser for excitation and a 1319 nm long-pass filter to collect the fluorescent signal with a uniform exposure time (500 ms). After in vivo imaging, the major organs of mice with different treatments including heart, liver, kidney, lung, spleen and brain were collected for ex vivo fluorescence imaging.

Toxicology evaluation

Healthy BALB/c mice (4 mice per group) were intravenously injected with 1× PBS (pH = 7.2-7.4) or cRGD-TTB NPs at 10 mg/kg. After one-week of treatments, mouse blood (~0.6 mL) was collected for a complete blood panel test. The major organs of mice, including heart, liver, kidney, lung and spleen, treated with nanoprobes were harvested for H&E staining analysis.

Targeted NIR-II imaging in VX2-tumor bearing rabbits

The NIR-II fluorescence imaging was conducted once the VX2 tumor size reached to 600 mm^3. The legs bearing tumors were shaved before NP injection and imaging. Upon injection of TTB or cRGD-TTB NPs (2 mg/kg) through the retention needle, the real-time NIR-II fluorescence change in its hindlimb vasculature was recorded as a video, using 808 nm diode laser as an excitation and a 1250 nm long-pass filter to collect the fluorescent signal. The camera was focused on the tumor vasculature during imaging. The NIR-II fluorescence imaging was performed under the same parameters 10 min, 3, 6, 9, 12, 24, and 48 h post-injection.

Notes and references

1 J.-S. Ni, T. Min, Y. Li, M. Zha, P. Zhang, C. L. Ho, and K. Li, *Angew. Chem., Int. Ed.*, 2020, **59**, 10179-10185.

2 Y. Li, D. Hu, Z. Sheng, T. Min, M. Zha, J.-S. Ni, H. Zheng and K. Li, *Biomaterials*, 2021, **264**, 120365.

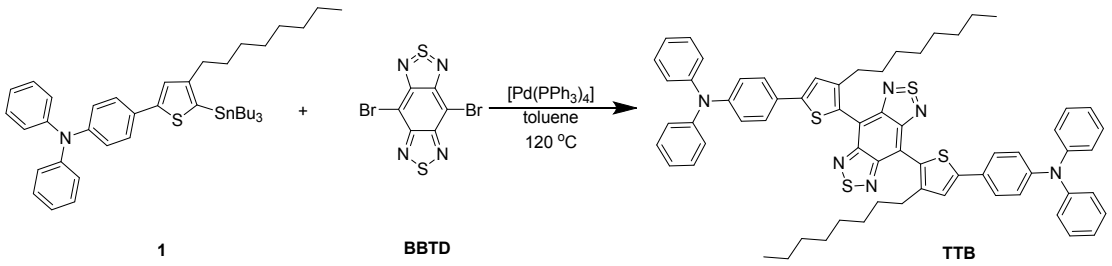

Figure S1. Synthetic route of TTB.

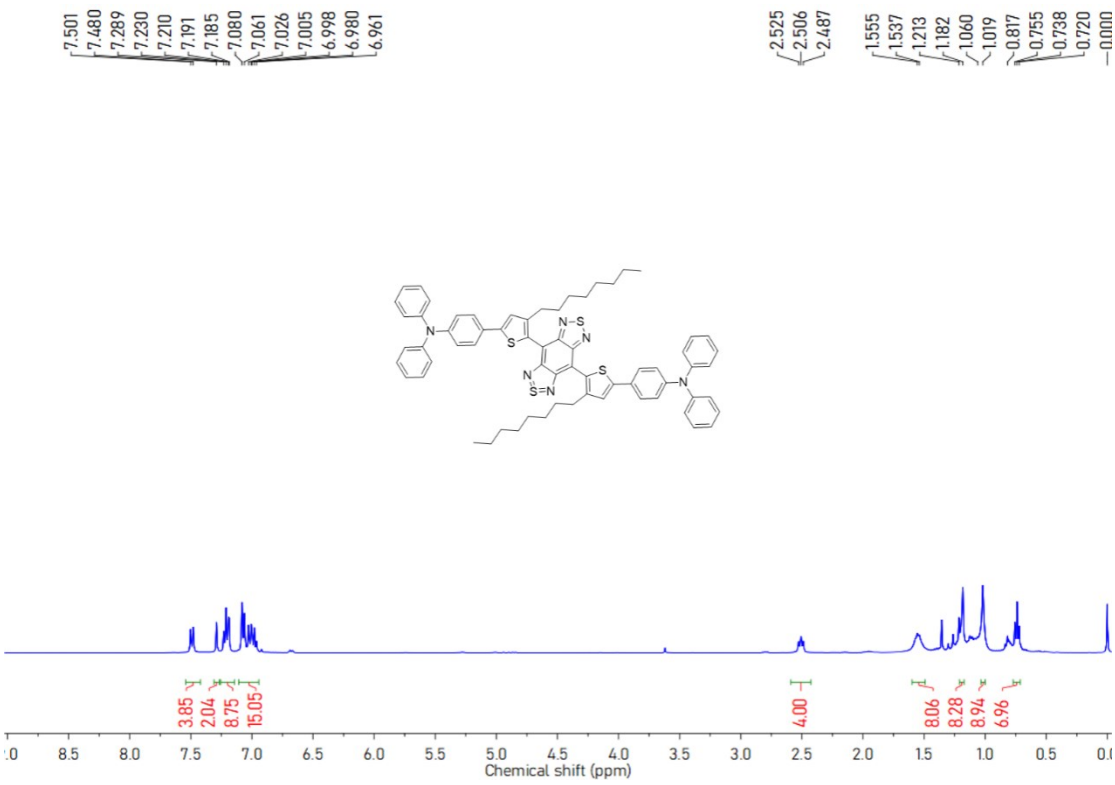

Figure S2. ^{1}H NMR spectrum of TTB in $CDCl_3$.

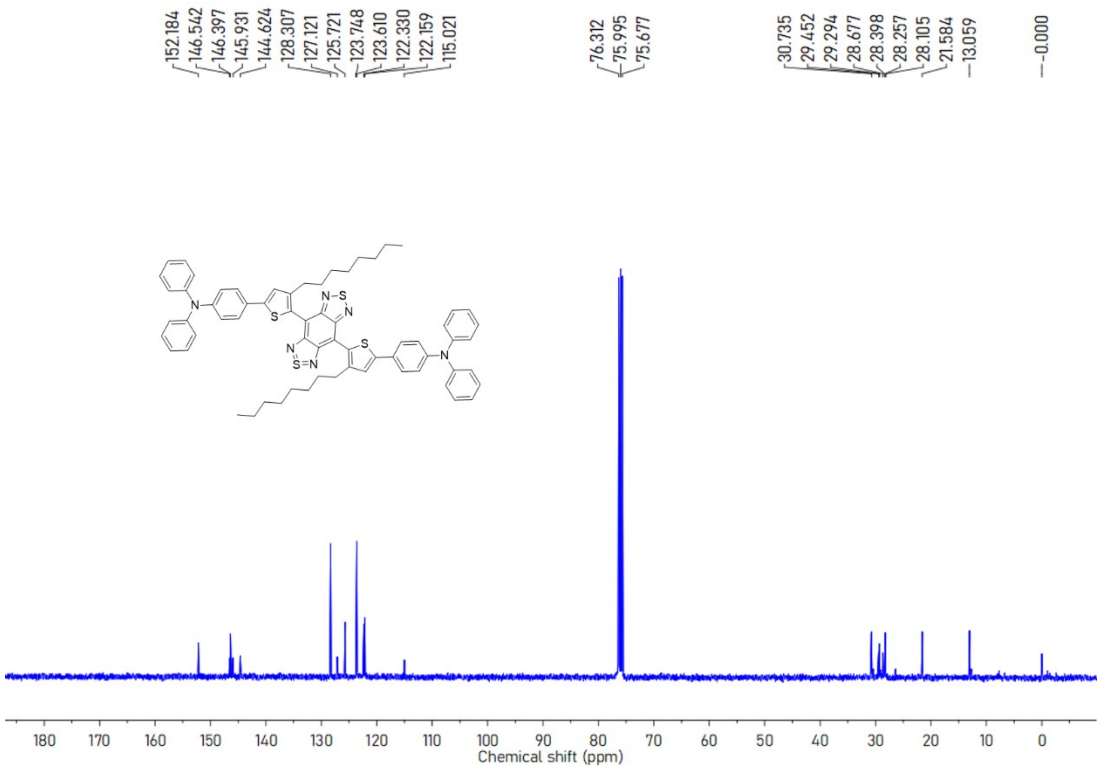

Figure S3. ^{13}C NMR spectrum of TTB in $CDCl_3$.

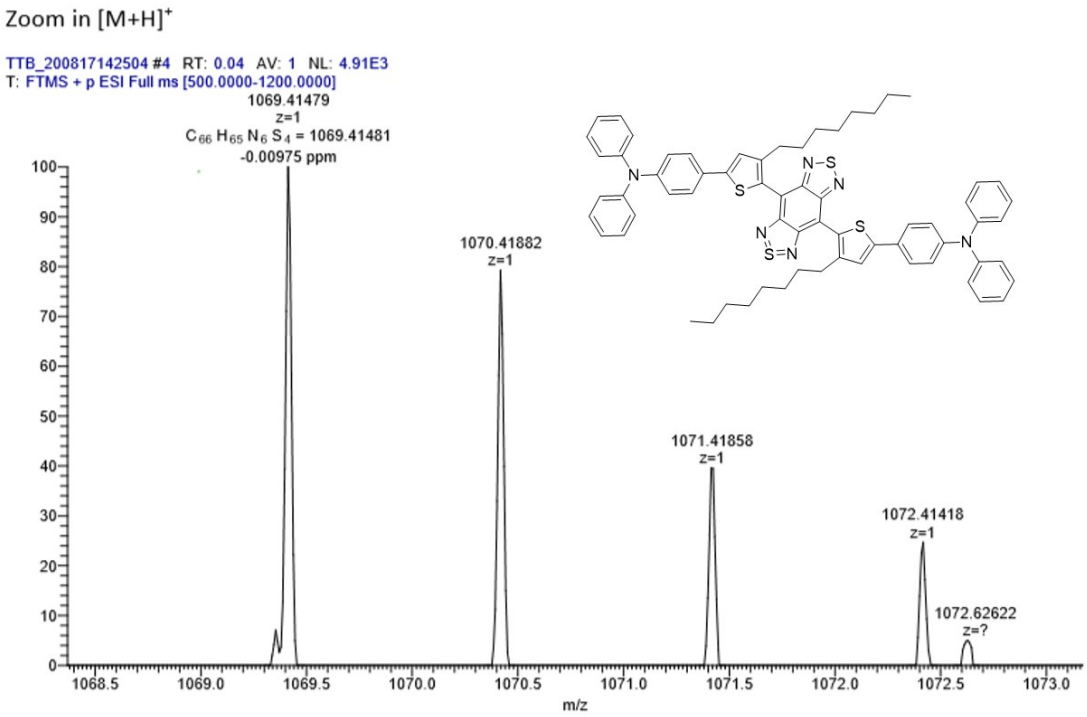

Figure S4. High-resolution mass spectrum of TTB.

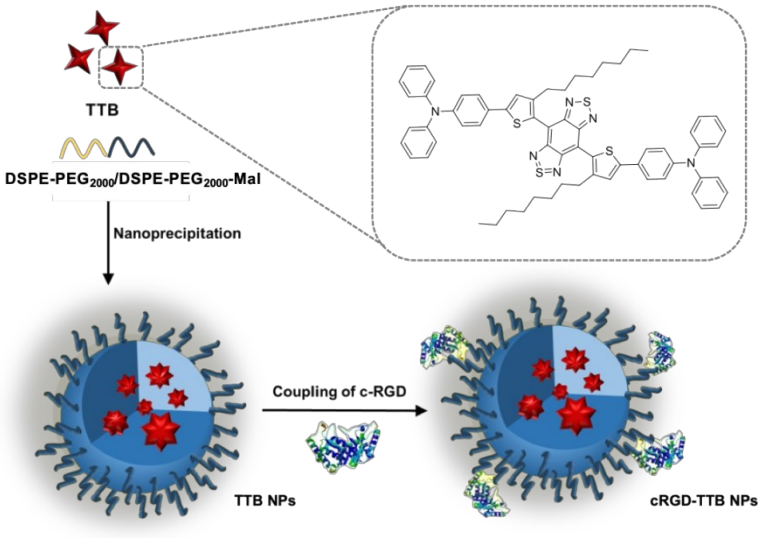

Figure S5. The synthetic route of cRGD-TTB NPs.

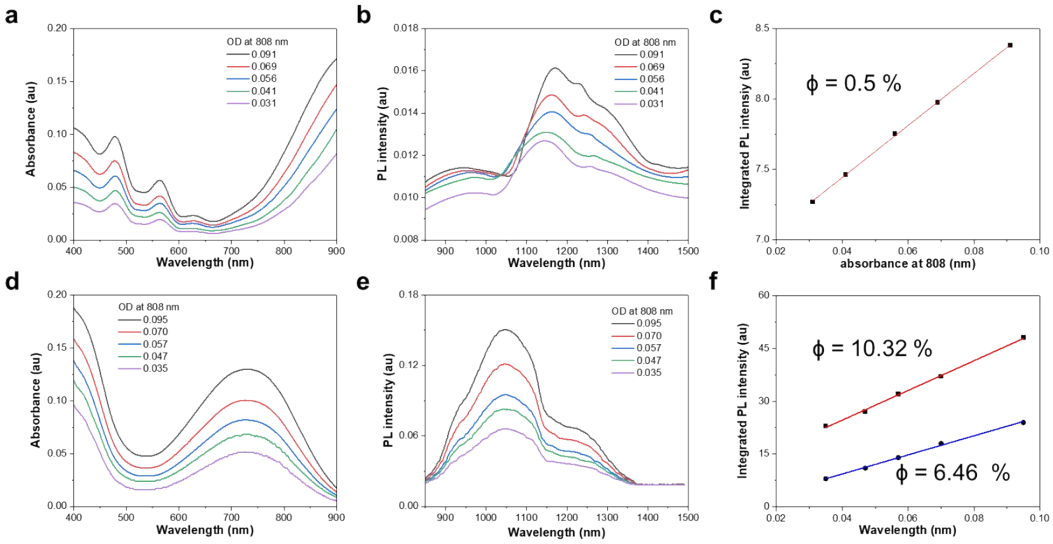

Figure S6. NIR-II quantum yield (ϕ) measurement of cRGD-TTB NPs. (The red and blue fit lines of **f** are calculated in 850-1500 nm range and in 1000-1500 range from **e**, respectively.)

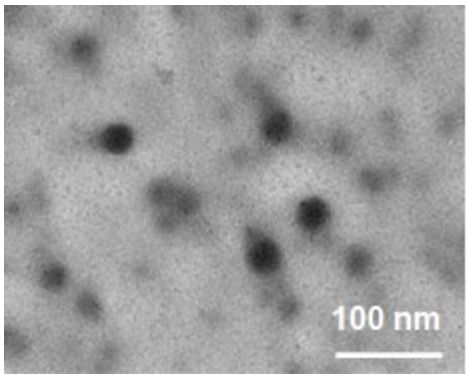

Figure S7. TEM image of cRGD-TTB NPs.

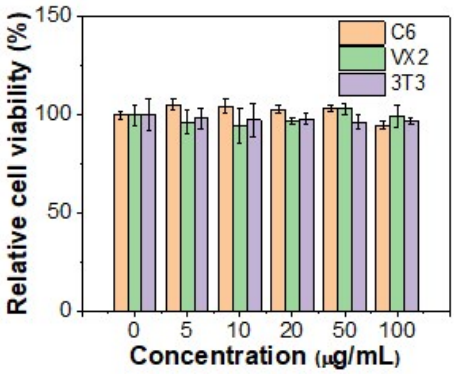

Figure S8. Viability of C6, VX2 and 3T3 cells after incubation with cRGD-TTB NPs at varied concentrations for 24 h.

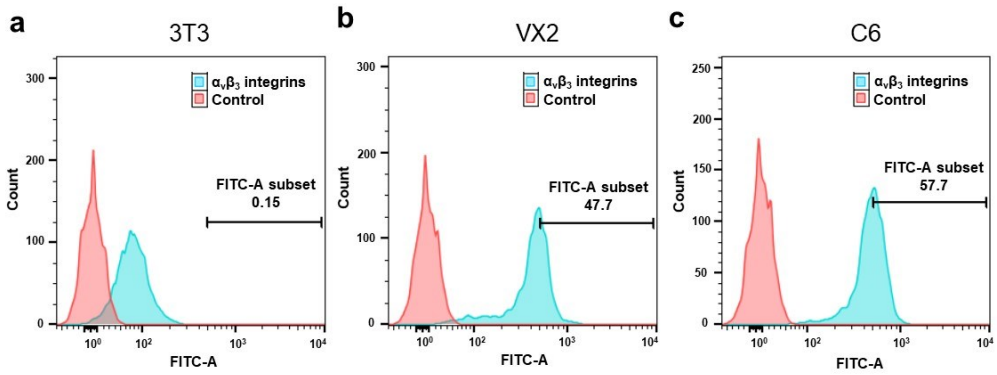

Figure S9. Flow cytometry histograms of a) 3T3 cells b) VX2 cells and c) C6 cells after incubation with rabbit anti-integrin $\alpha_v\beta_3$ (CD51+CD61) antibody and then secondary antibody Alexa Fluor® 488 goat anti-rabbit IgG (H+L) (Excitation: 488 nm; Filter: 500-550 nm).

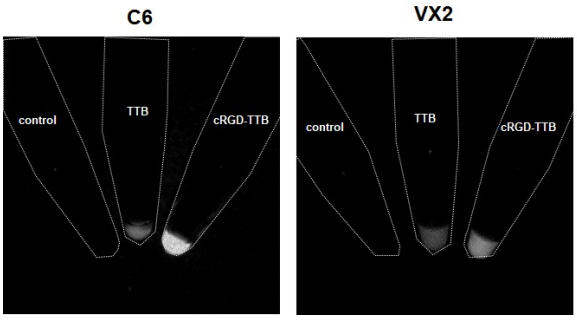

Figure S10. NIR-II fluorescence imaging (1319LP, 2000ms) of C6 and VX2 cells before and after incubation with TTB and cRGD-TTB NPs (40 μg/mL).

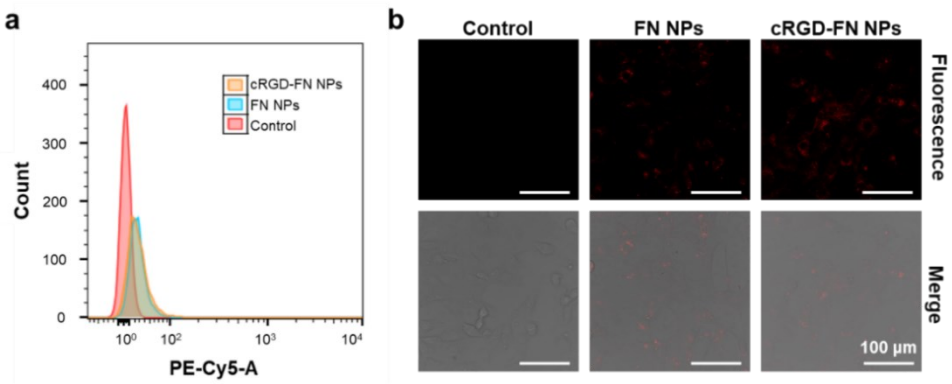

Figure S11. a) Flow cytometry histograms of 3T3 cells after incubation with 40 μg/mL of cRGD-FN or FN NPs for 4 h (Excitation: 561 nm; Filter: 640-700 nm). b) The corresponding confocal images of 3T3 cells treated with cRGD-FN or FN NPs (Excitation: 488 nm; Filter: 620-700 nm). Scale bar = 100 μm.

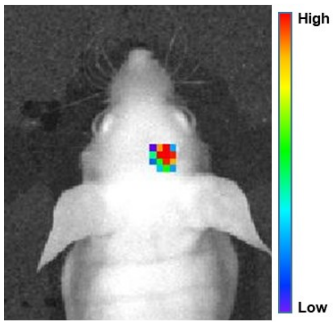

Figure S12. The bioluminescence imaging of representative C6 glioma tumor-bearing mouse at day 7 post transplantation.

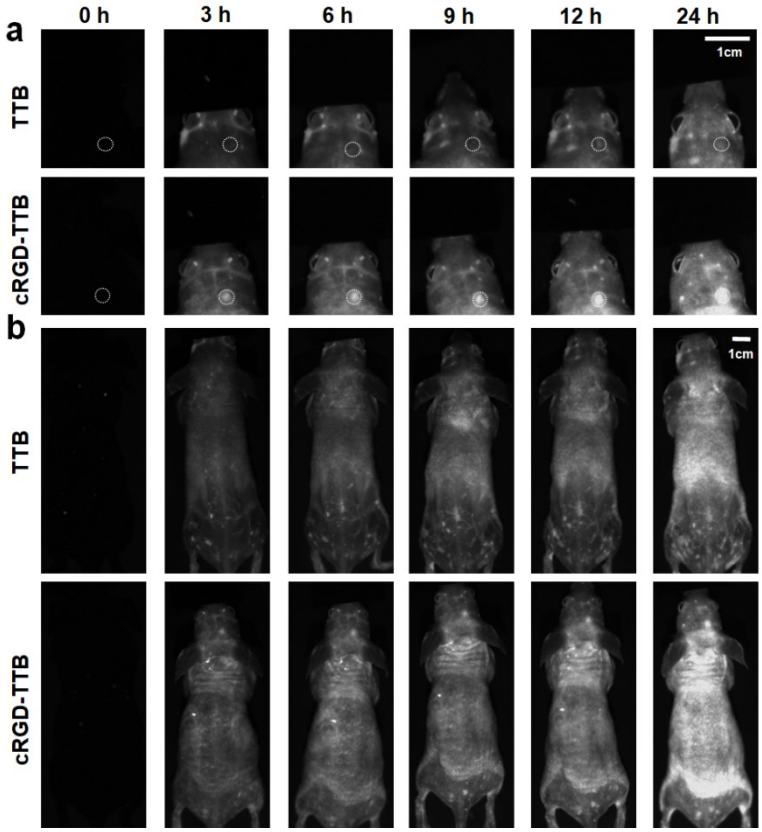

Figure S13. The supplementary NIR-II fluorescence imaging of mice brains and whole bodies at different time points after intravenous injection of TTB and cRGD-TTB NPs (10 mg/kg), respectively. (Excitation: 808 nm with a power density of 30 mW/cm^2; Filter: 1319 nm long-pass; Exposure time: 500 ms)

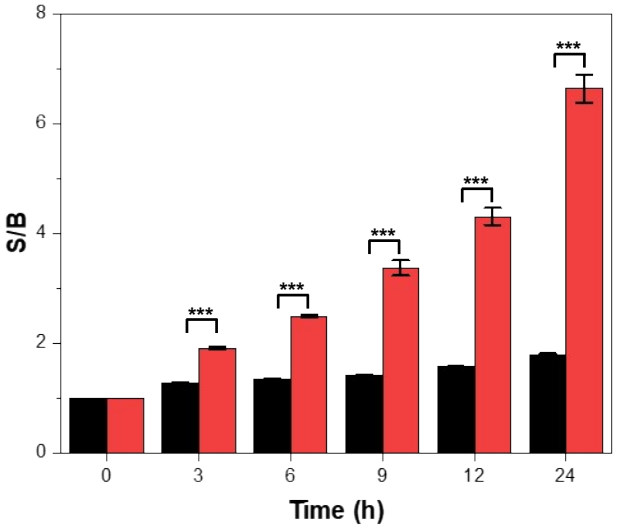

Figure S14. Quantitative analysis of S/B ratios in tumor-bearing mice treated with TTB NPs (black) and cRGD-TTB NPs (red). (n = 3 per group. ***$p < 0.001$.)

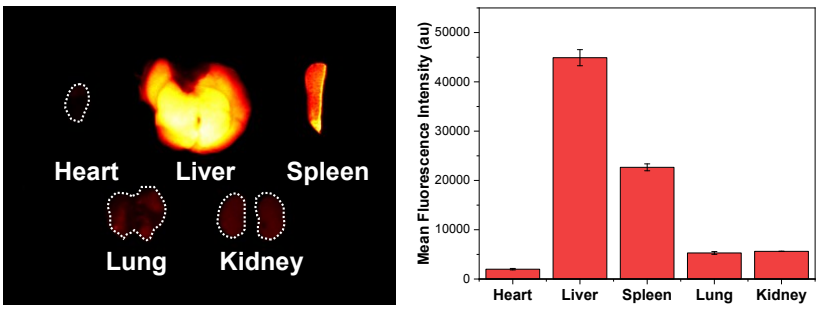

Figure S15. The representative ex vivo NIR-II fluorescence imaging of different organs collected from mice injected with cRGD-TTB NP at 24 h and the quantitative analysis of fluorescence intensities. Excitation: 808 nm; Filter: 1319 nm long-pass.

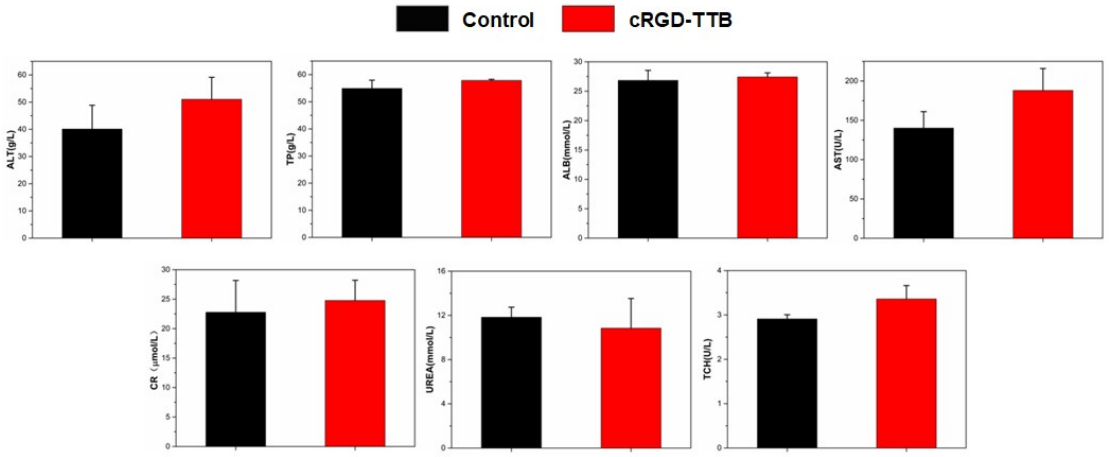

Figure S16. Blood biochemical analysis of BALB/c female mice treated with 1× PBS (pH = 7.2-7.4) or cRGD-TTB NPs, including alanine amiotransferase (ALT), total protein (TP), albumin (ALB), aspartate aminotransferase (AST), creatinine (CR), urea (UREA) and total cholesterol (TCH). (n = 3 per group.)

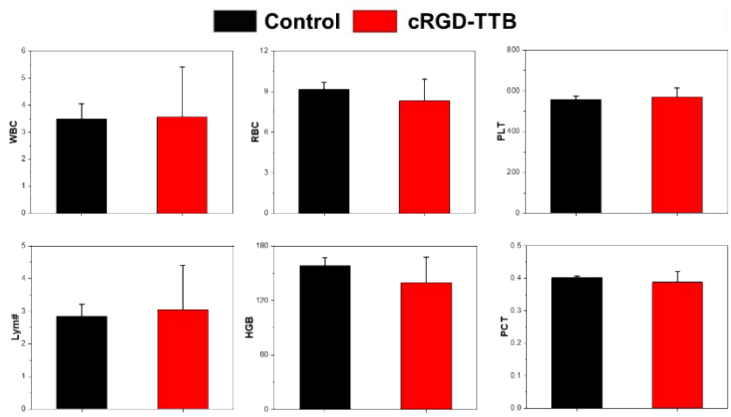

Figure S17. Blood routine analysis of BALB/c female mice treated with 1× PBS (pH = 7.2-7.4) or cRGD-TTB NPs, including white blood cell (WBC), red blood cell (RBC), platelet (PLT), lymphocyte (Lym#), hemoglobin (HGB), procalcitonin (PCT). (n = 3 per group.)

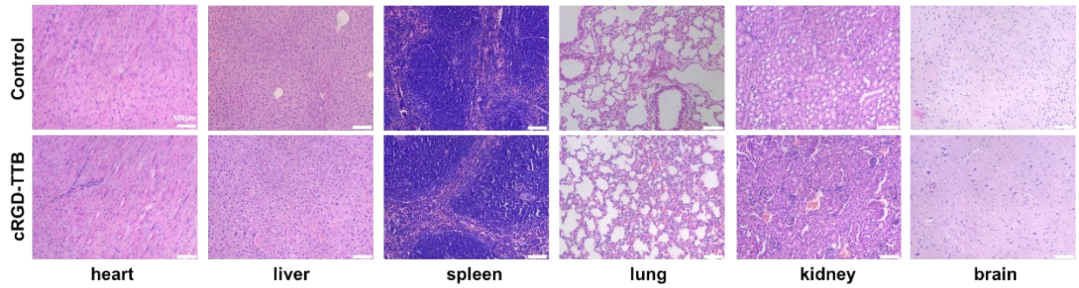

Figure S18. H&E stained images of the tissues from major organs, including the heart, liver, spleen, lung, kidney and brain of the BALB/c female mice after injection of 1× PBS (pH = 7.2-7.4) or cRGD-TTB NPs. Scare bar: 100 μm.

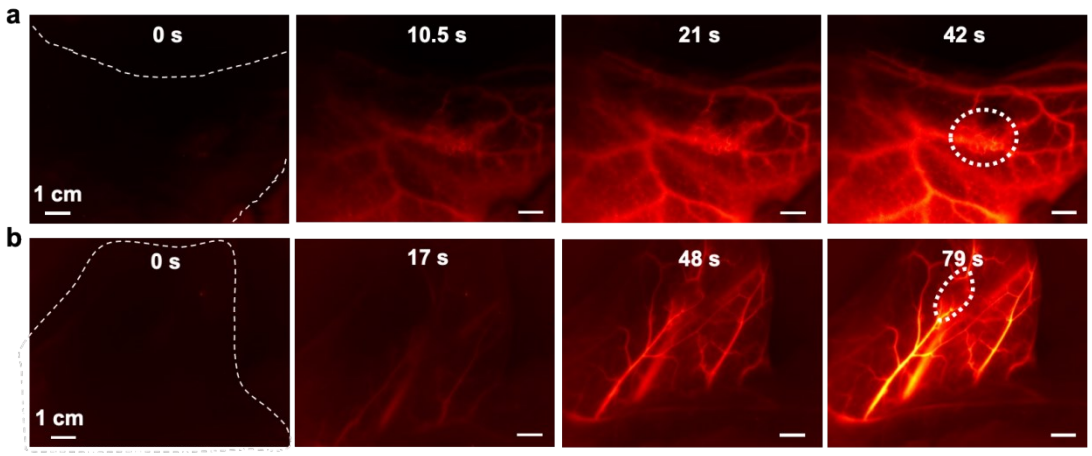

Figure S19. Time-dependent NIR-II fluorescence imaging of rabbit hindlimb with tumors upon injection of a) TTB NPs and b) cRGD-TTB NPs at 2 mg/kg. (White circles show tumor regions.)

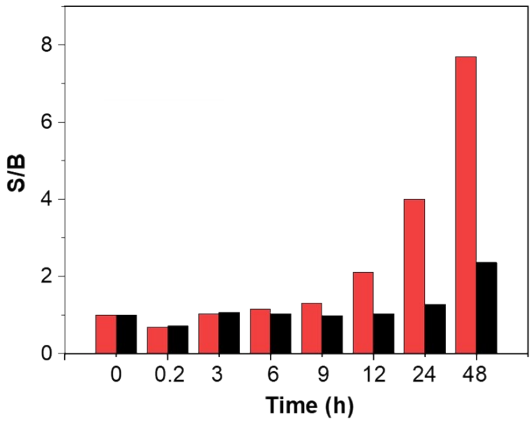

Figure S20. Representative quantitative analysis of S/B ratios in tumor-bearing rabbits treated with TTB NPs (black) and cRGD-TTB NPs (red).

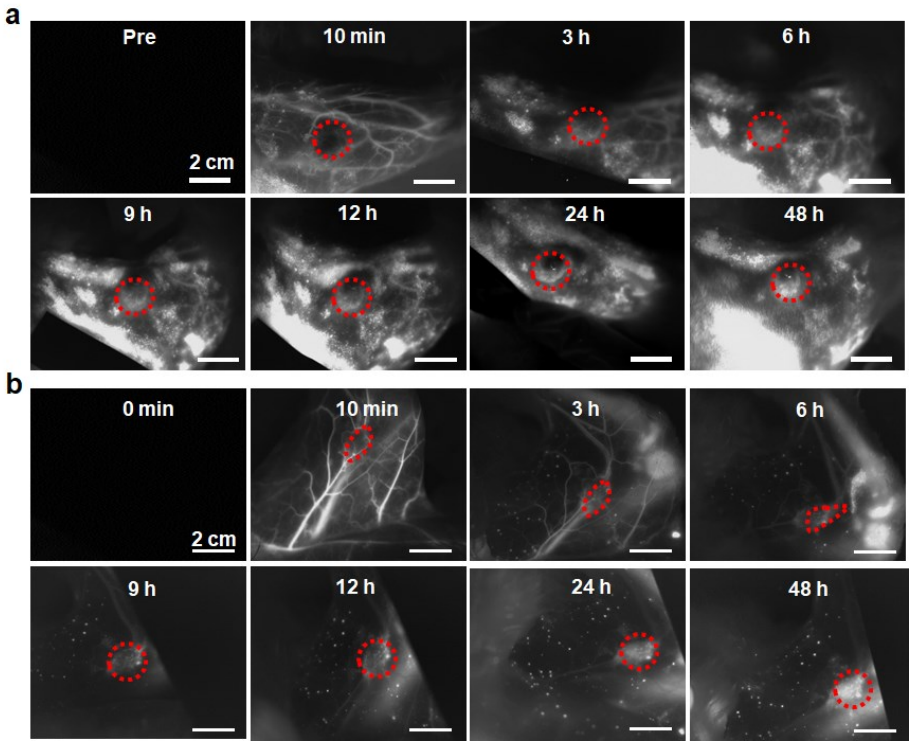

Figure S21. Time-dependent NIR-II fluorescence images of subcutaneous VX2 tumor-bearing rabbit after injection of TTB NPs (a) and cRGD-TTB NPs (b). Red circles show tumor regions.