Supporting Information

Engineering Biocompatible Benzodithiophene-Based Polymer Dots with Tunable Absorptions as High-Efficiency Theranostic Agents for Multiscale Photoacoustic Imaging-guided Photothermal Therapy

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MATERIALS AND METHODS

Materials. 4,9-Bis(5-bromo-2-thienyl)-2,7-bis(2-octyldodecyl)-benzo[lmn][3,8]phenanthroline-1,3,6,8(2H,7H)-tetrone (NDI) and 4,8-Bis[5-(2-ethylhexyl)thiophen-2-yl]-2,6-bis(trimethylstannyl)benzo[1,2-b:4,5-b']dithiophene (BDT) were purchased from Derthon Optoelectronic Materials Science Technology Co LTD (Shenzhen, China). The functional copolymer poly (styrene-co-maleic anhydride) (PSMA, average MW ~1,700, styrene content 68%), Acridine orange (AO), Ethidium bromide (EB), Phosphate buffered saline (PBS), and Tetrahydrofuran (THF, anhydrous, 99.9%) were purchased from Sigma-Aldrich (Shanghai, China). (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (MTT) were purchased from BioSharp (Hefei, China). All chemicals were used directly without further purification. Deionized (DI) water (18.25 MΩ cm, 25 °C) was used in all experiments.

Synthesis of BDT-NDI and BDT-TH polymers. In a 25 mL flask, the mixture of monomer NDI (0.3 mmol, 271.4 mg) and monomer BDT (0.3 mmol, 345.0 mg) was dissolved in 10 mL toluene. And then the solution was degassed and recharged with nitrogen (N_2) five times before and after addition $Pd(PPh_3)_4$ (5 mg, 0.004 mmol). Under the protection of N_2, the solution was stirred at 110 °C for 48 h. In addition, the hot solution was cooled down to room temperature and subsequently dropped slowly into methanol (150 mL). Next, the acetone (200 mL) was added to the solution and then it was stirred overnight. Further, D-A-type BDT-NDI polymer was obtained after removing solvents. The BDT-TH polymer was prepared under the same conditions which are used monomer TH instead of monomer NDI.[1]

Preparation of BDT-NDI and BDT-TH Pdots. BDT-NDI/PSMA and BDT-TH Pdots were prepared using a precipitation method. Briefly, BDT-NDI or BDT-TH and PSMA (4:1 w/w) were dissolved in THF at a total polymer concentration of 50 μg/mL. The mixture of the polymer was injected swiftly into water under sonication for 3 min. And subsequently, the THF was removed by nitrogen (N_2) stripping under heating. The solution was further filtered through a 0.22-micrometer membrane to remove larger aggregates, and these solutions were further concentrated by rotary evaporation. Finally, the resulting Pdots were purified by anion exchange chromatography and gel filtration chromatography for further characterization and application.

Characterization of BDT-NDI and BDT-TH Pdots. Transmission electron microscope (TEM) images of the samples were obtained using a Hitachi H-600 transmission microscope at 200 kV. Dynamic light scattering was performed with Zetasizer NanoZS (Malvern, UK). The absorption spectra were measured using a Uv-Vis 1700 spectrophotometer with a 1.0 cm optical path length quartz cuvette. The ^1H NMR spectra were recorded on a 500 MHz Bruker Avancein using $CDCl_3$ as solvent and tetramethylsilane (TMS) as an internal standard (δ = 0.00 ppm). Number-average (Mn) and weight-average (Mw) molecular weights of polymers were quantified by using a Waters Gel Permeation Chromatography (GPC) 2410 in trichloromethane ($CHCl_3$) based on a calibration curve of polystyrene standard.

Photothermal Performance of the BDT-NDI Pdots. The temperature of the BDT-NDI Pdots dispersions (100 μg/mL, 2 mL), PSMA solution (100 μg/mL, 2mL), and PBS solution (100 μg/mL, 2mL) were measured upon 660 nm light-emitting diode (LED) irradiation (200 mW/cm^2) for 600 s, respectively. DI water was served as a control. The BDT-NDI Pdots dispersions with different concentrations (50, 100, 150, 200, 250 μg/mL) were irradiated with 660 nm LEDs under different power densities (50, 100, 150, 200 mW/cm^2) for 600 s, respectively. During these measurements, a thermocouple probe was inserted into the aqueous solution of these samples in a position perpendicular to the LEDs path. Temperatures were acquired every 10 s.

The photothermal conversion efficiency of the BDT-NDI Pdots was quantified by a relationship that was generated by recording the change of temperature of the Pdots aqueous solution as a function of time (t) under continuous irradiation of 660 nm LEDs (200 mW/cm^2) till the temperature of solution reached steady state after 600 s (t). Then the photothermal conversion efficiency (η is calculated using the following equation,

$$\eta = [hA(T_{Max}-T_{Surr})-Q_{Dis}]/I\ (1-10^{-A660}) \quad (1)$$

in which h is the heat transfer coefficient, A is the surface area of the container, T_{Max} is representative of the maximum steady-state temperature, T_{Surr} stands for the ambient temperature of the environment, Q_{Dis} is the heat dissipation from the light absorbed by the solvent and the quartz sample cell, I indicates the incident LEDs power (200 mW/cm^2), and A_{660} is the absorbance of the sample at 660 nm. Here, hA is calculated by

$$\tau_s = m_D c_D / hA \quad (2)$$

in which τ_s is the time constant for heat transfer of the system, which is determined to be 127.03 s from Figure S9; m_D and c_D, is the mass and heat capacity of the DI water used to disperse the Pdots, respectively. Hence, hA was determined to be 0.0165 W. Meanwhile, Q_{Dis} was measured to be 0.241 W. Based on the Equations (1) and (2), the photothermal conversion efficiency of the BDT-NDI Pdots was determined to be 40 %.

In vitro Cytotoxicity by MTT Assay. MCF-7 cells were respectively seeded on 96-well plates in DMEM supplemented with 10 % FBS (Gibco) and 1% penicillin/streptomycin (Gibco) overnight. The original medium was then totally removed from each well. DMEM (100 μL) containing different concentrations of the Pdots was added to the designated wells, respectively. After incubation in the dark at 37 °C for 12 h, the cells incubated with Pdots were irradiated with or without 660 nm LEDs (200 mW/cm^2) for 600 s, respectively. The final concentration of Pdots on each well plate was ranged from 12.5 to 200 μg/mL. In addition, plates of MCF-7 cells were irradiated under 660 nm LEDs with different power densities ranging from 50 to 200 mW/cm^2 for 600 s, which were performed to demonstrate the LEDs irradiation itself having no cytotoxicity. All these plates were then incubated at 37 °C in the dark for another 24 h. Subsequently, we remove the old medium and add DMEM (100 μL without FBS) containing 10% MTT stock solution (5 mg/ml in sterile PBS) to each well. After incubation for 4h, the medium was removed completely, followed by adding dimethyl sulfoxide (DMSO, 100 μL) to each well. In vitro cell viabilities were measured by a BioTek Powerwave XS microplate reader (read the absorbance at 490 nm). The cells incubated with DMEM without any treatment represented 100 % cell survival.

***In vivo* PAI.** The animal and phantom tests were performed by using the home-made PAT and OR-PAM system. For PAT system, a pulsed light from an Nd:YAG laser (wavelength range from 680 to 1064 nm; pulse duration: 5-10 ns; frequency rate: 20 Hz; Surelite I-10, Continuum) was adopted as a laser source to illuminate the phantom or the animals through an optical subsystem. To generate PA signals, a 1M transducer was circularly rotated by a rotary stage at 360 positions (1MHz central frequency; bandwidth range from 0.65~1.18MHz; V303-SU, Olympus-NDT). The complex wave field signal was first amplified by a Pulser/Receiver (5073R, Olympus) and subsequently converted into digital. Finally, the images were reconstructed by the delay-and-sum beam forming algorithm. For the OR-PAM system, 532 nm laser with a pulse width of 1 ns and a repetition rate up to 5 KHz was adopted as the light source.

Schematic illustration of the home-made system was shown in Figure S8. The detailed description can be found in our previous report.[2]

For the phantom experimental tests, the targets with different concentrations of BDT-NDI and BDT-TH Pdots were placed into the solid phantom, respectively. For the phantom materials, the agar powder (1-2%) solution was used to solidify the Intralipid as scattered and India ink as absorber. Finally, the object-bearing solid phantom was immersed in water to measure the PA properties of Pdots.

For the animal experiment, all protocols were approved by the Animal Management and Ethics Committee of the University of Macau. The animal tumor model was developed by subcutaneous injection of MCF-7 cells onto the back of the mice. In vivo experiments were performed when the tumor size was about 80~140 mm^3. The MCF-7 tumor-bearing mice were intravenously injected with Pdots at a dose of 1.5 mg/kg (body weight). PAI was performed at wavelength 700 nm and OR-PAM was carried out at the wavelength of 532 nm under a water system of 37.5 °C.

AO/EB Tests. The MCF-7 cells were cultured in four 35 mm dishes with the same cell density. After overnight culturing, the cells were respectively treated with PBS, LEDs, BDT-NDI Pdots, and Pdots with LEDs irradiation (660 nm, 200 mW/cm^2, 600 s). And then the cells were further incubated at 37 °C for 24 h. After staining with a mixture of AO/EB for 10 min and washing with PBS three times, images of the four samples were generated by using fluorescence microscopy.

In Vivo Antitumor Activity and Biosafety. For the *in vivo* experiments, BALB/c mice (5-6 weeks of age), were purchased from the Shanghai Slac Laboratory Animal Co. Ltd (Shanghai, China). 5×10^6 MCF-7 cells were subcutaneously injected into the mice about two weeks before commencing treatment. Mice were monitored daily for the appearance of tumors. When the mean tumor size reached a volume of about 80~140 mm^3, the mice were randomly divided into four groups with five mice in each group. The mice then received intratumor injections of different formulations including PBS, PBS + LEDs, Pdots, and Pdots + LEDs, respectively (660 nm, 200 mW/cm^2 for 600 s;

concentration of various formulations: 1 mg/mL, 300 μL). The final injected dose was 1.5 mg/kg per mouse. Both tumor volumes and mouse body weights were monitored every other day during the period of treatment (28 d). After 28 d from drugs administration, the mice were sacrificed and then the tumor tissue and major organs were dissected for H&E staining.

Hemolysis assay. Blood sample (1 mL) was obtained from the BALB/c mice. RBCs were further separated from the sample by being centrifugated at 2000 rpm for 10 min. The cells were then diluted by 10 mL PBS. Afterwards, 500 μL of the cell suspension was added to 1 mL of PBS (negative control), deionized water (positive control), and different concentrations of BDT-NDI Pdots, respectively. After being incubated for 3 h at 37 °C, the samples were centrifugated at 12000 rpm for 10 min. Absorbance at 540 nm of the supernatant was further recorded by using UV–vis spectrometer. The hemolysis ratio (HR) was calculated as followed:

$$HR(\%) = (A_{sample} - A_{negative}) / (A_{positive} - A_{negative}) \times 100\ \%$$

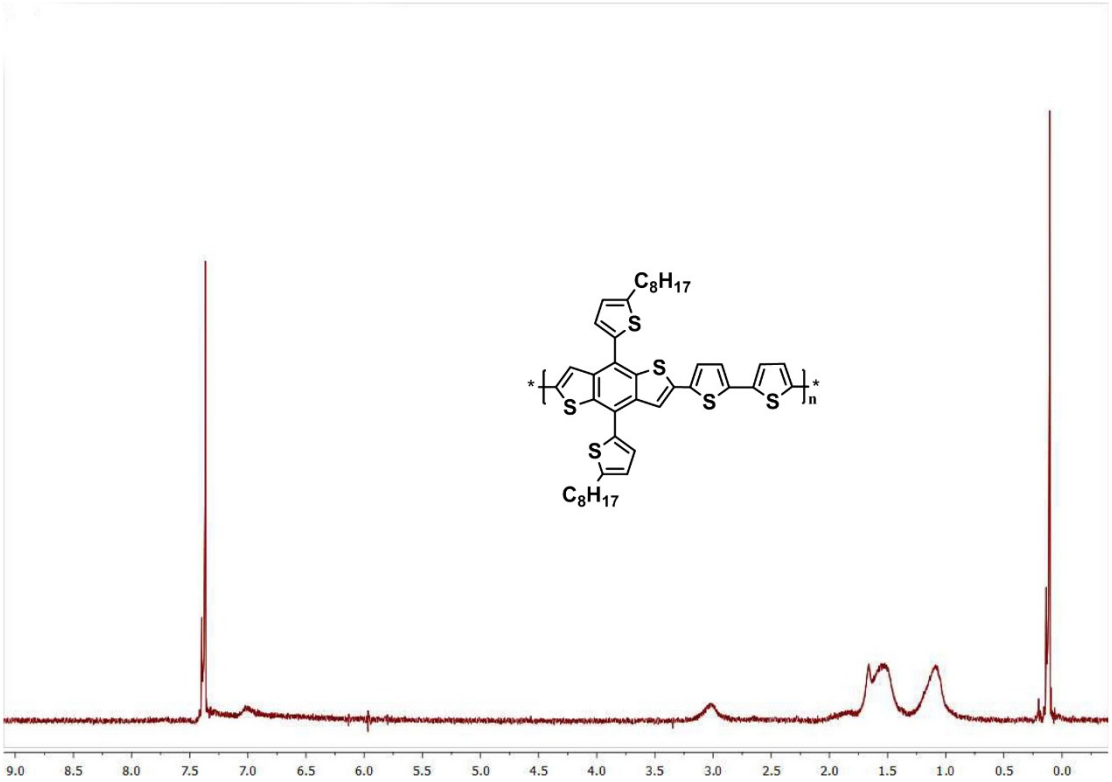

Figure S1. ^{1}H-NMR spectra of BDT-TH polymer.

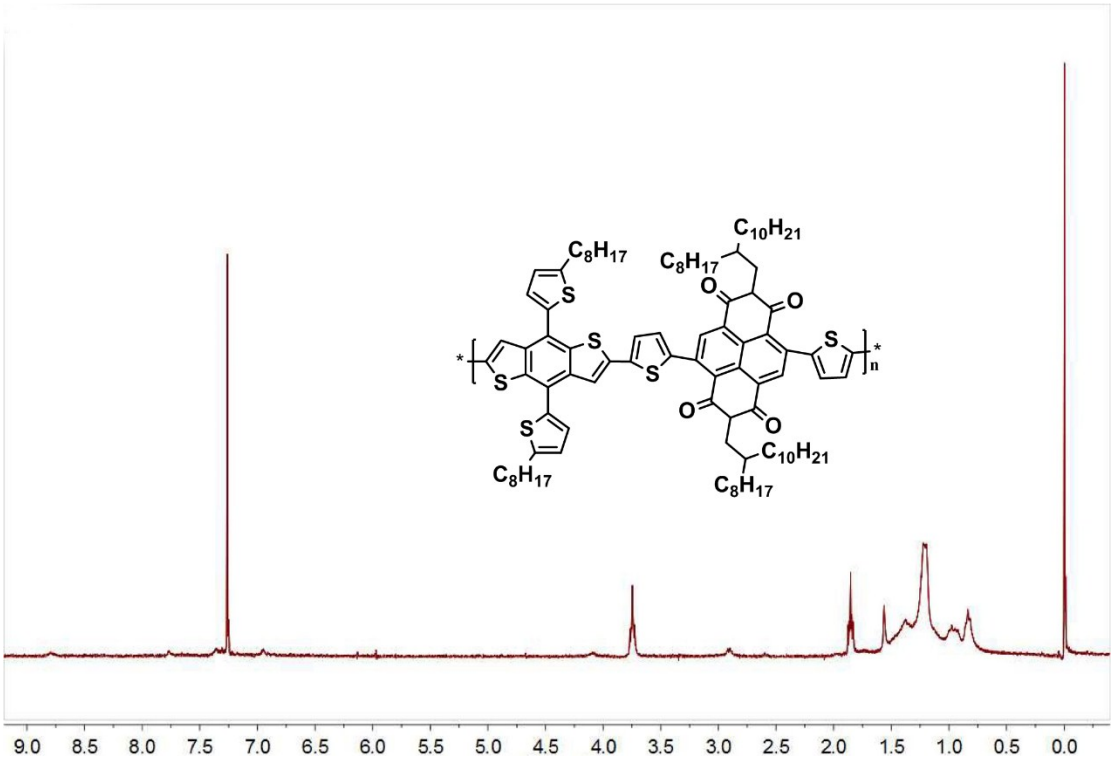

Figure S2. ^{1}H-NMR spectra of BDT-NDI polymer.

GPC Results

	Dist Name	Elution Volume (ml)	Retention Time (min)	Adjusted RT (min)	Mn	Mw	MP	Mz	Mz+1	Mz/Mw	Mz+1/Mw
1		10.579	10.579	10.579	498249	505218		511822	518028	1.013071	1.025355

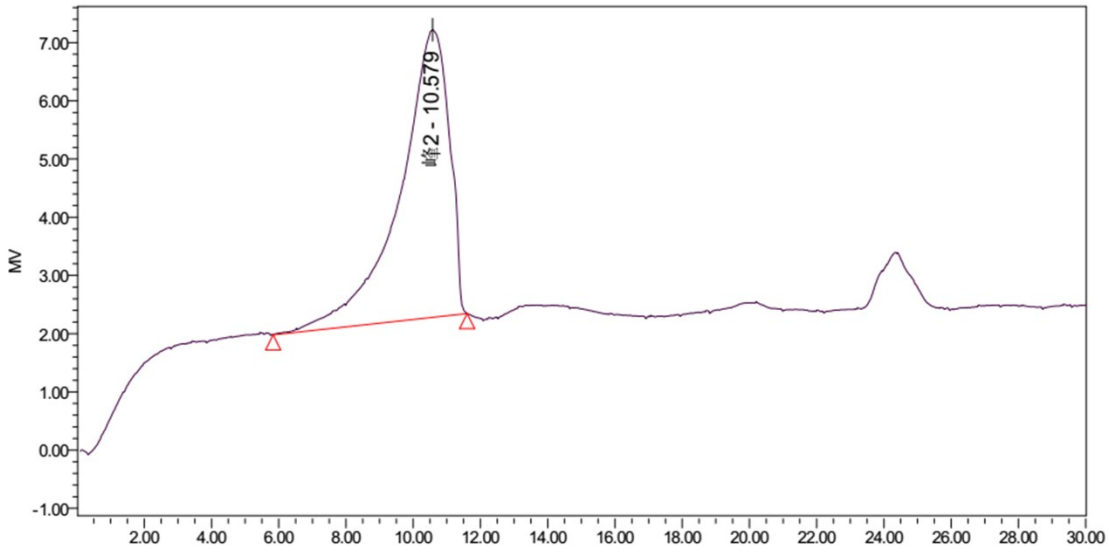

Figure S3. Gel permeation chromatography (GPC) profile of BDT-TH polymer.

GPC Results

	Dist Name	Elution Volume (ml)	Retention Time (min)	Adjusted RT (min)	Mn	Mw	MP	Mz	Mz+1	Mz/Mw	Mz+1/Mw
1		8.880	8.880	8.880	542336	544753		547094	549351	1.004297	1.008441

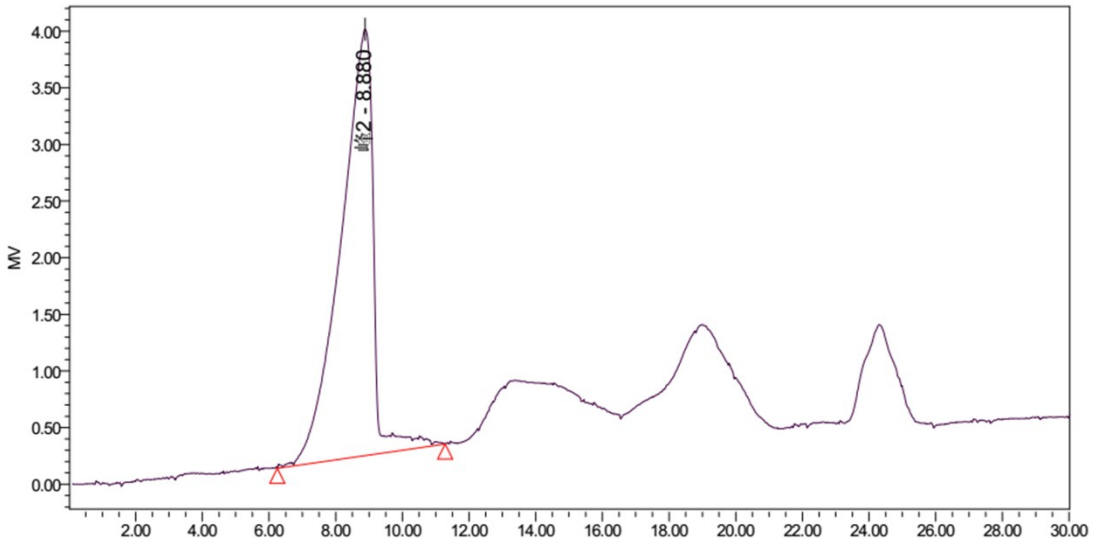

Figure S4. Gel permeation chromatography (GPC) profile of BDT-NDI polymer.

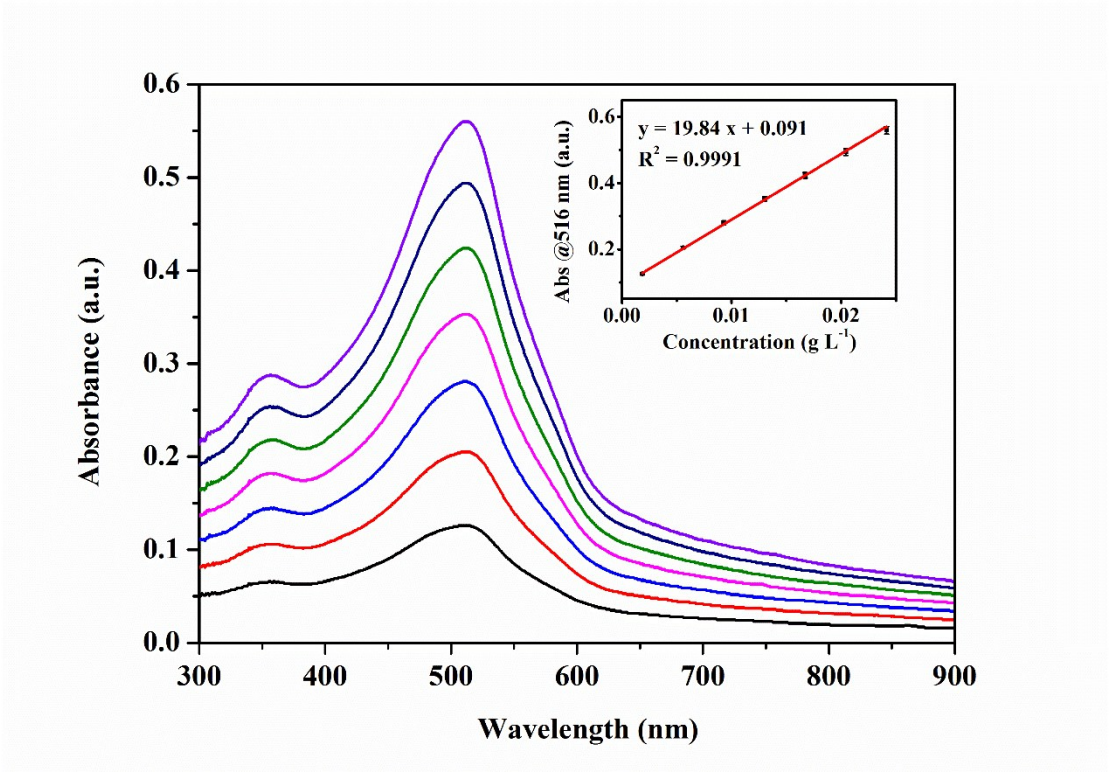

Figure S5. Uv-vis-NIR absorption spectra and linear relationship between the absorption at 516 nm and the concentrations (insert) of the BDT-TH dissolved in THF.

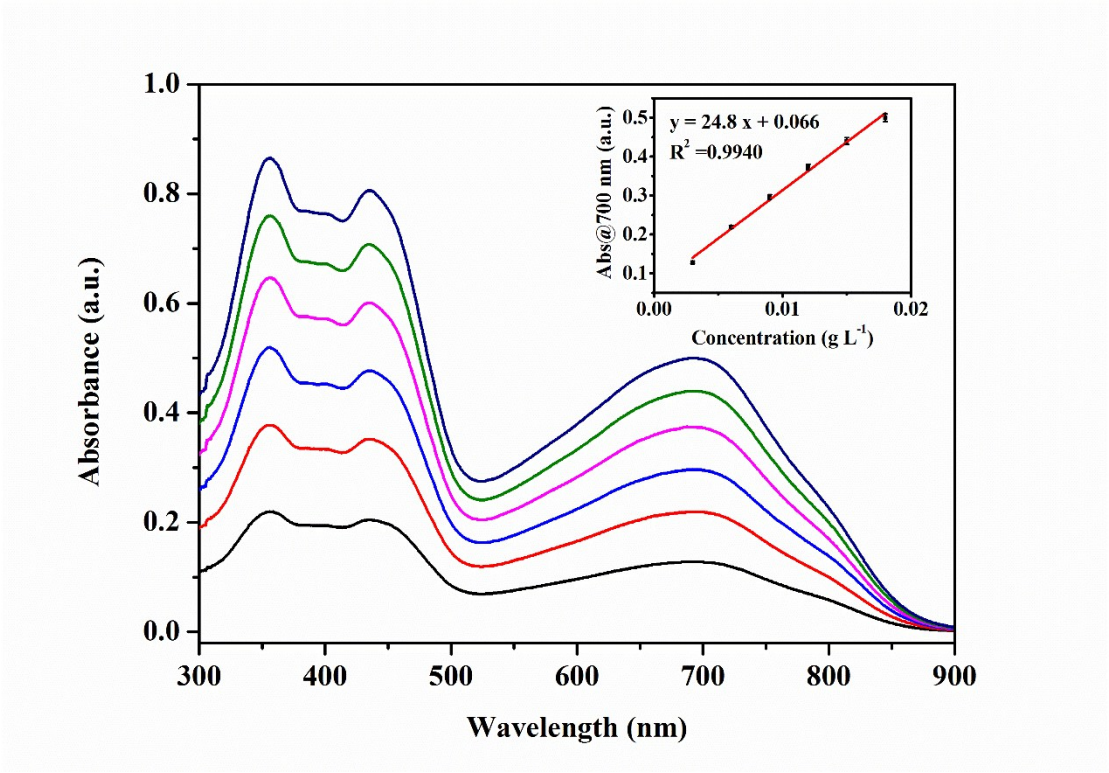

Figure S6. Uv-vis-NIR absorption spectra and linear relationship between the absorption at 700 nm and the concentrations (insert) of the BDT-NDI dissolved in THF.

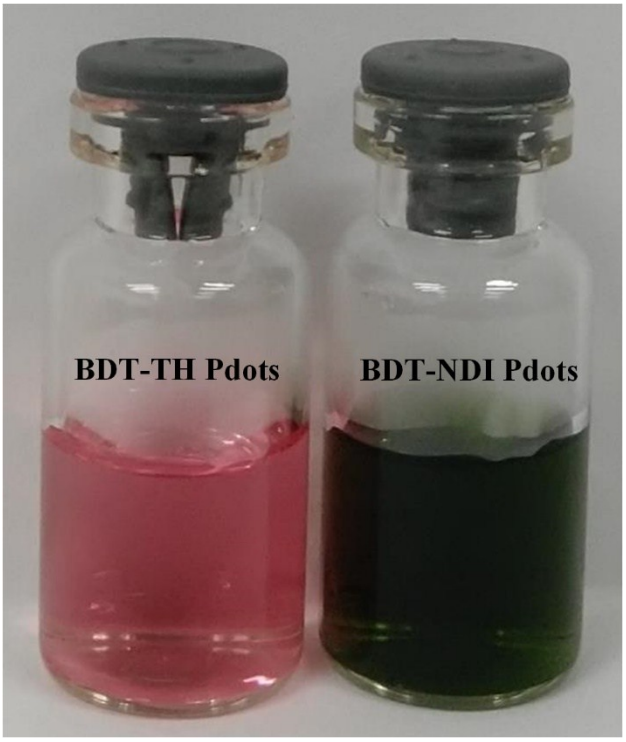

Figure S7. Photograph of the as-synthesized BDT-based Pdots dispersed in H_2O.

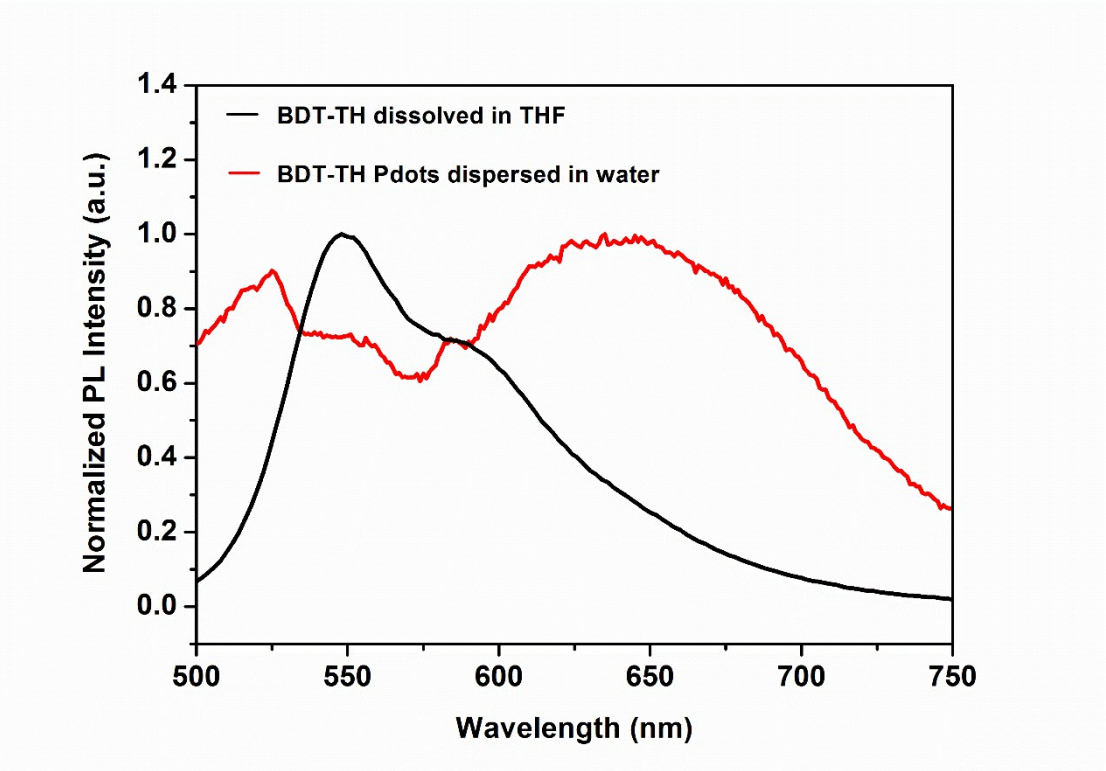

Figure S8. Fluorescence spectra of BDT-TH dissolved in THF and BDT-TH Pdots dispersed in water

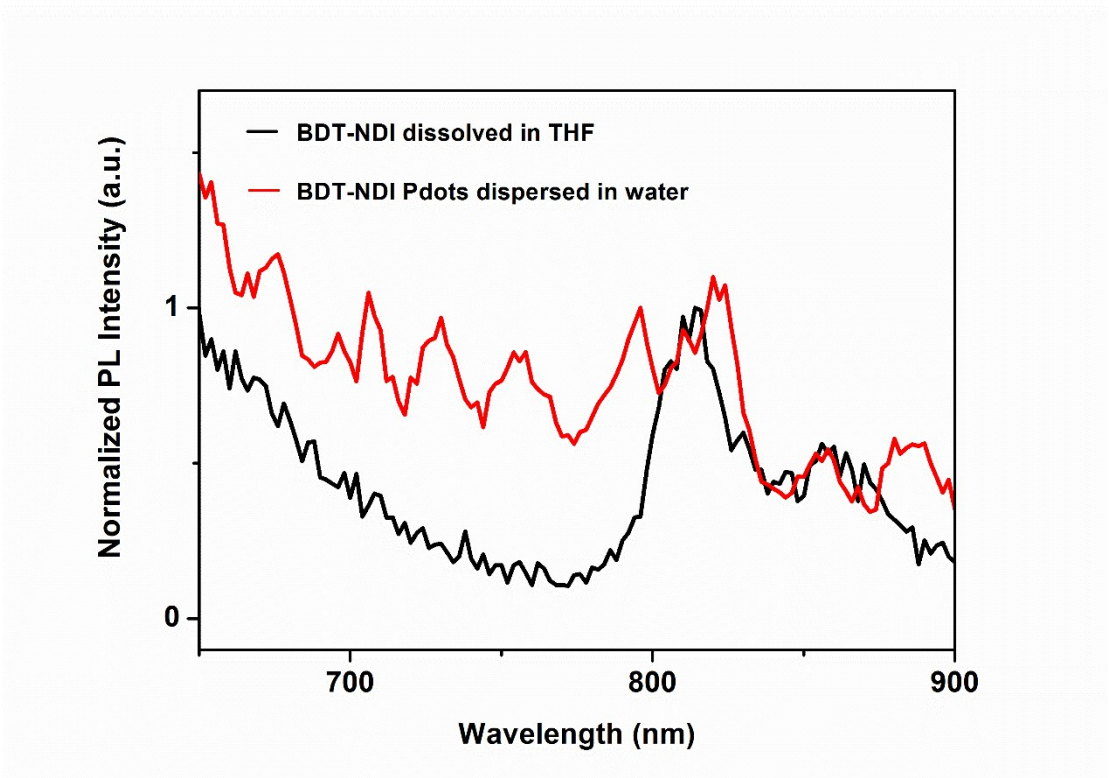

Figure S9. Fluorescence spectra of BDT-NDI dissolved in THF and BDT-NDI Pdots dispersed in water

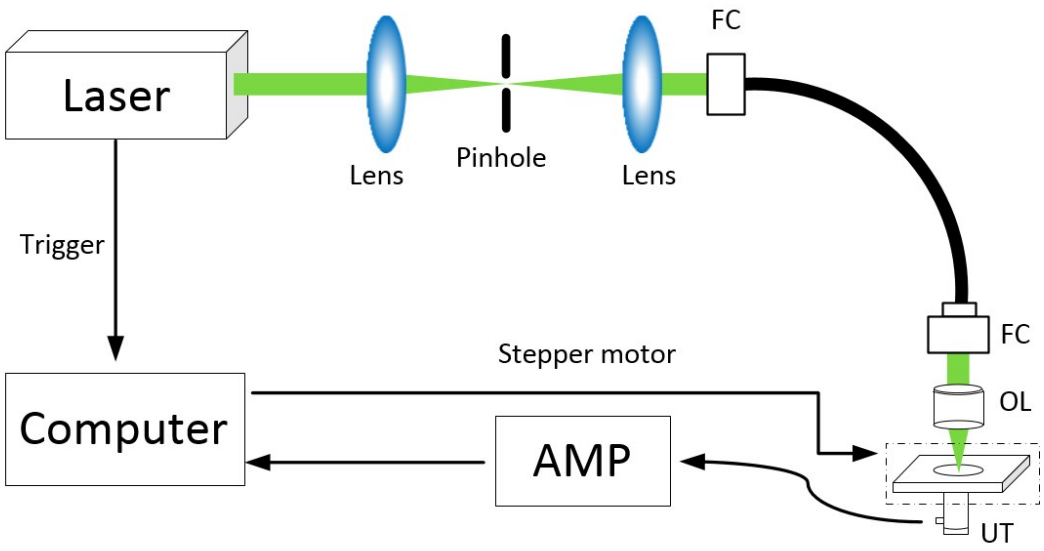

Figure S10. Schematic of the experimental setup for OR-PAM imaging. FC: Fiber Collimator; OL: Objective Lens; UT: Ultrasound Transducer; AMP: Amplifier.

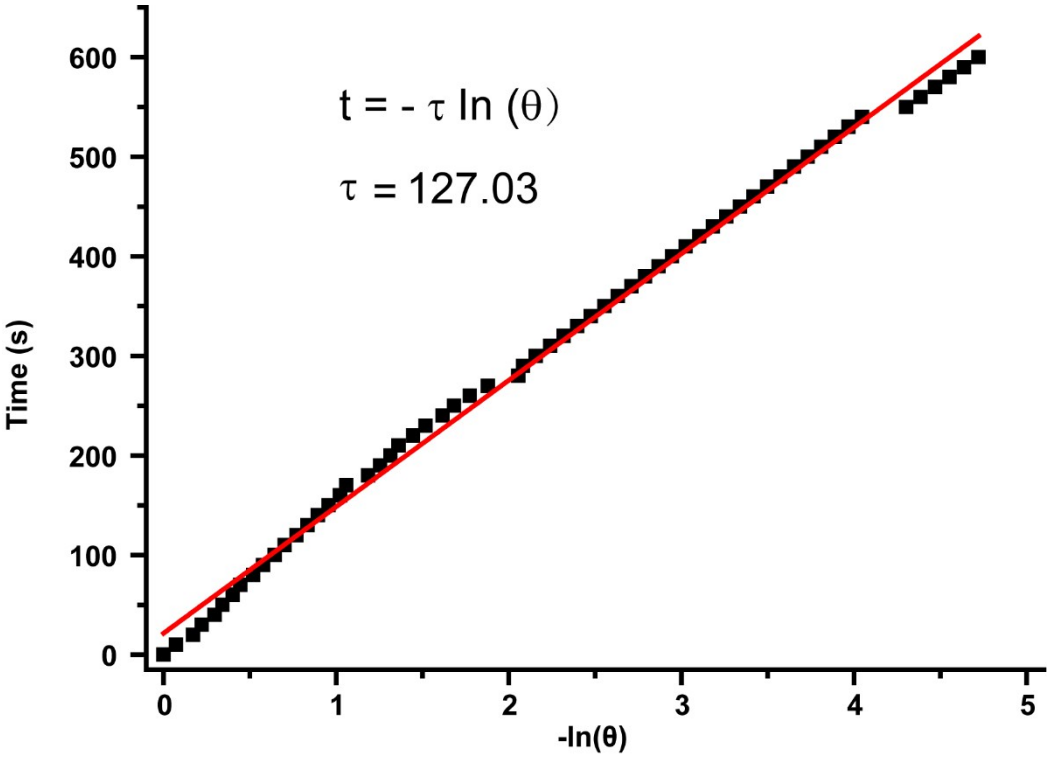

Figure S11. Time constant for heat transfer was determined to be 127.03 by applying the linear time data from the cooling period (after 600 s) versus negative natural logarithm of driving force temperature, which was obtained from the cooling stage of Figure 4c.

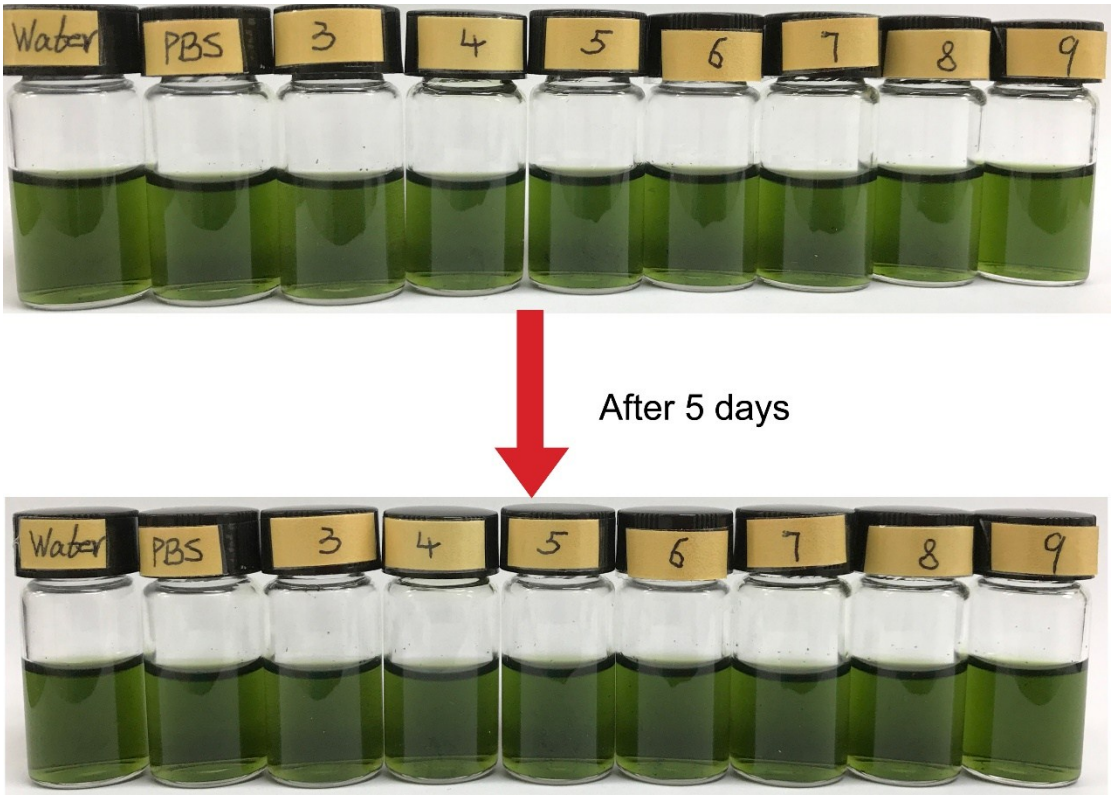

Figure S12. Stability of the BDT-NDI Pdots under different pH values.

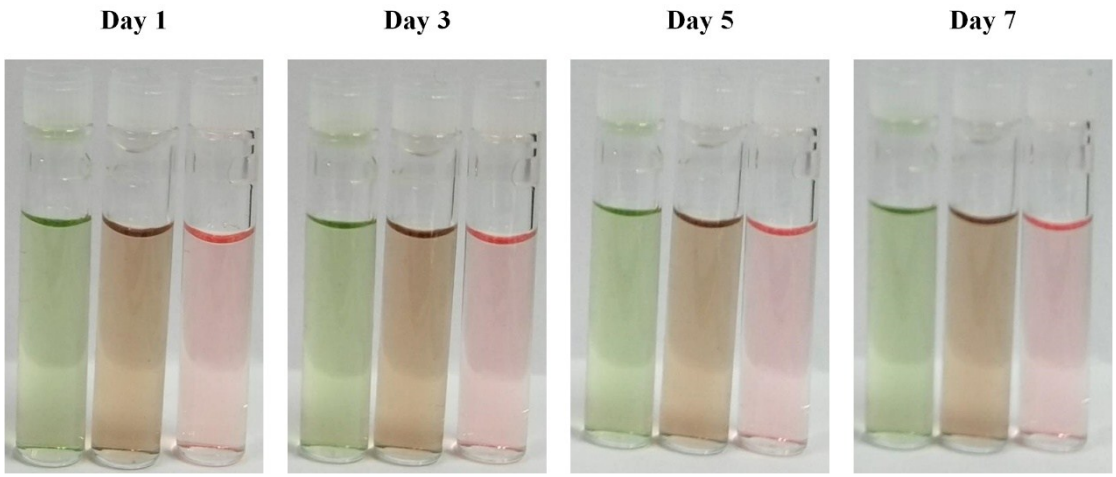

Figure S13. stability of the BDT-NDI Pdots dispersed in DMEM with 10% FBS (left: BDT-NDI Pdots dispersed in PBS; middle: BDT-NDI Pdots dispersed in DMEM with 10% FBS; right: DMEM with 10% FBS)

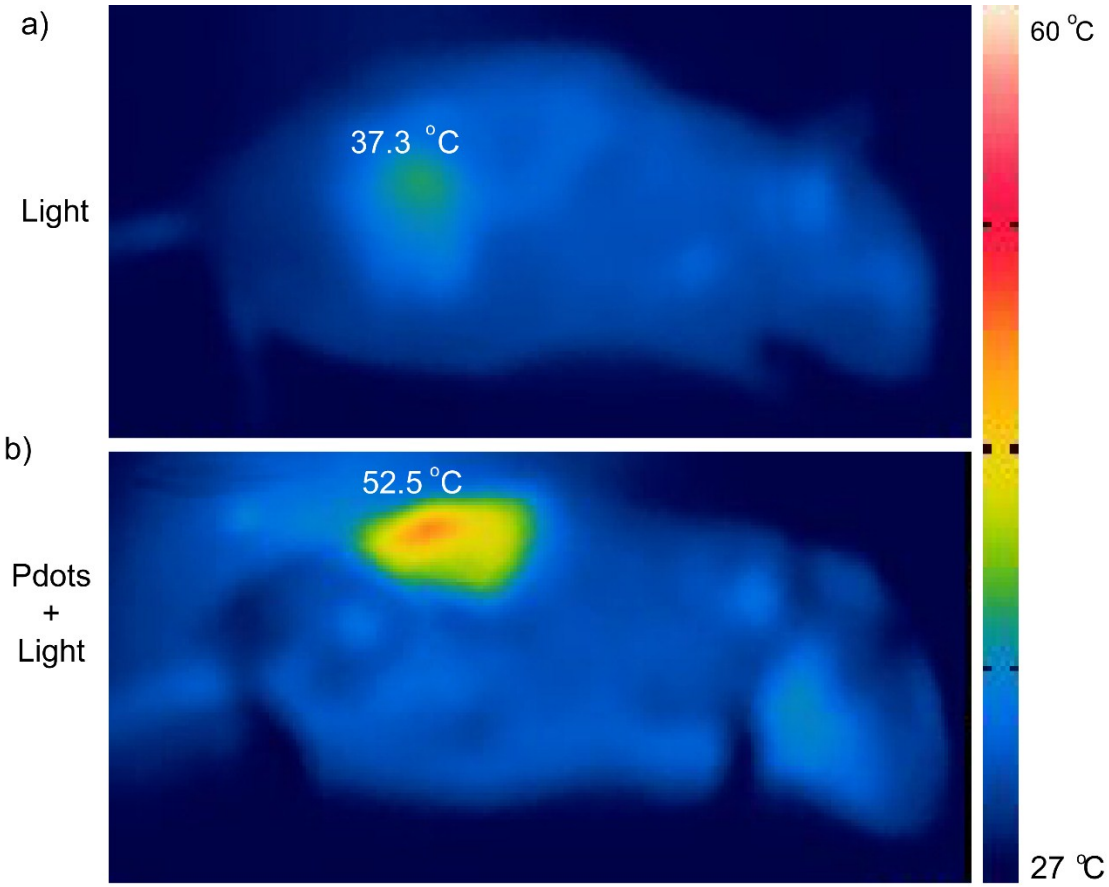

Figure S14. Temperature variation of the tumor treated without (a) and with BDT-NDI (b) Pdots under LEDs irradiation.

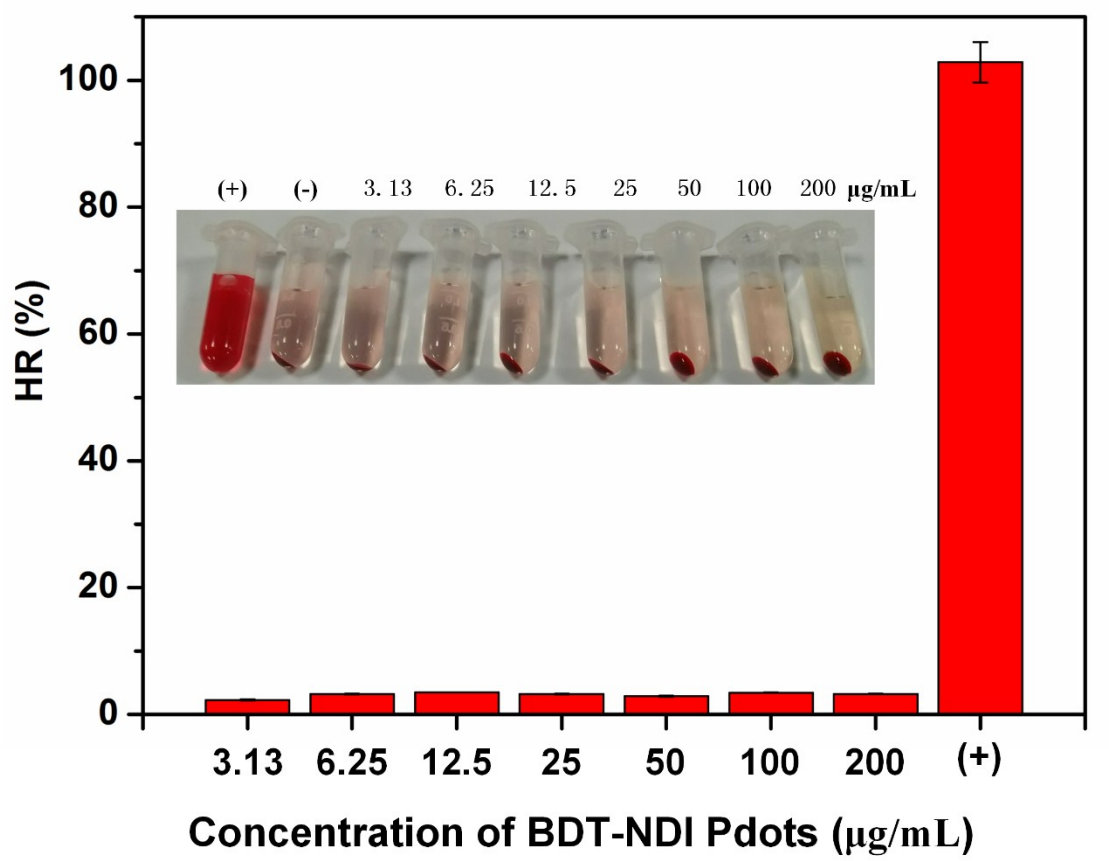

Figure S15 Hemolysis percentage of RBCs incubated with BDT-NDI Pdots at various concentrations (3.13, 6.25, 12.5, 25, 50, 100 and 200 μg/mL) for 3 h, using deionized water (+) and PBS (−) as the positive and negative controls, respectively. The inset picture shows a photograph of hemolysis.

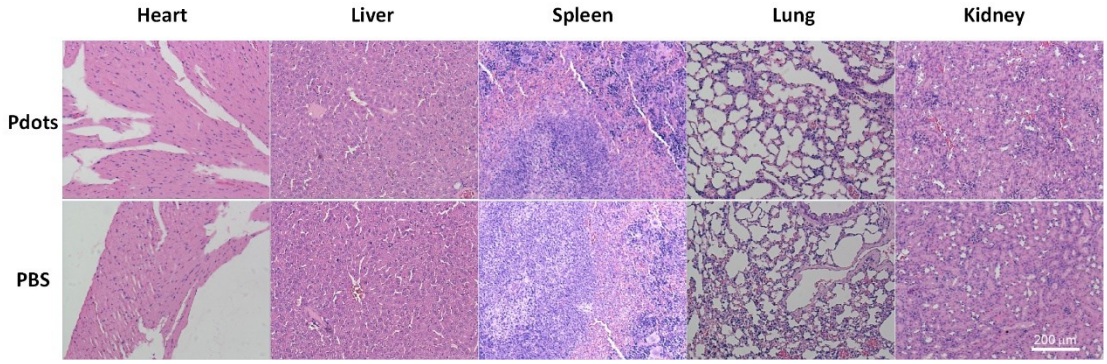

Figure S16. Representative H&E-stained images of major organs including the heart, liver, spleen, lung, and kidney collected from mice sacrificed 7 days after intravenous injection of BDT-NDI Pdots.

REFERENCES

1. Chang, K.; Liu, Y.; Hu, D.; Qi, Q.; Gao, D.; Wang, Y.; Li, D.; Zhang, X.; Zheng, H.; Sheng, Z.; Yuan, Z., Highly Stable Conjugated Polymer Dots as Multifunctional Agents for Photoacoustic Imaging-Guided Photothermal Therapy. *ACS Appl. Mater. Inter.* **2018,** *10*, 7012-7021.

2. Gao, D.; Liu, Y.; Wang, Y.; Yuan, Z., Protein-Modified Ultra-Small Gold Clusters for Dual-Modal in Vivo Fluorescence/Photoacoustic Imaging. *Quant. Imag. Med. Surg.* **2018,** *8*, 326-332.