Electronic Supplementary Information for

A 1064 nm Excitable Semiconducting Polymer Nanoparticle for Photoacoustic Imaging of Gliomas

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Materials

1,2-Distearoyl-*sn*-glycero-3-phosphoetanolamine-*N*-[methoxy(polyethylene glycol)-2000] (DSPE-PEG_{2000}) was obtained from Laysan Bio, Inc. Milli-Q water (18.2 MΩ) was supplied by a Milli-Q Plus System (Millipore Corporation, Bedford, USA) and used for all the experiments requiring aqueous medium. 2,5-bis(2-butyloctyl)-3,6-bis(5-(trimethylstannyl)thiophen-2-yl)-2,5-dihydropyrrolo[3,4-c]pyrrole-1,4-dione[1] was purchased from Derthon Optoelectronic Materials Science Technology Co LTD. All other Chemicals were purchased from Sigma-Aldrich or Energy Chemical (China) and used as received. 4,8-Dibromo-6-(2-ethylhexyl)-[1,2,5]thiadiazole[3,4-*f*]benzotriazole was prepared according previous reports.[1]

Characterization

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance III 400 MHz NMR spectrometer (400 MHz for ^1H, referenced to TMS at δ = 0.00 ppm and 100 MHz for ^{13}C, referenced to $CDCl_3$ at 77.0 ppm). The hydrodynamic diameter and zeta potential of PDPPTBZ NPs were recorded on Micromeritics Nanoplus-3 (US). Transmission electron microscopy (TEM) images were obtained on a JEOL JEM-2100 electron microscope with an accelerating voltage of 200 KV. TEM images of U87 cells were performed on a JEOL JEM-1200EX electron microscope with an accelerating voltage of 120 KV. UV-vis-NIR spectra were measured on a Shimadzu UV-1750 spectrometer. Photolumunescence (PL) spectra were

recorded on an Edinburgh instruments FLS980, using Xe lamp as excitation source and a liquid nitrogen cooled InGaAs diode detector for signal detection. All photoacoustic measurements were carried out with our custom-built acoustic-resolution photoacoustic microscopy (AR-PAM) system, and the detailed PA imaging system information will be described in the following section.

PA imaging system

PA imaging system consists of a tunable pulsed optical parametric oscillator (OPO) laser (Vibrant 355 II HE, Opotek, Carlsbad, USA) for photoacoustic excitation, a focused ultrasound transducer (V315-SU, Olympus IMS, Waltham, USA; central frequency: 25 MHz) for both ultrasonic firing and photoacoustic/ultrasonic detection, and a precision motorized 3D scanning stage (PSA2000-11, Zolix, Beijing, China) to scan the imaging head across the x–y plane for 3D imaging. Note that, as the depth resolution comes from the time of arrival of the received acoustic signals, the 3D stage's z axis is used for positioning the imaging head only. During experiments, the OPO laser was operated primarily from 730 nm to 880 nm, and 1064 nm, with a repetition rate of 20 Hz and a pulse width of 5 ns.

For light delivery, the OPO laser was reshaped by an iris of 6 mm in diameter, attenuated by a neutral density filter, and then focused by a convex lens before coupling to an optical fiber via an assembled fiber coupler. A beam sampler and a photodiode (SM05PD1A, Thorlabs, Newton, USA) were used to monitor the fluctuation of laser energy per pulse. The laser beam coming out of the fiber was collimated by another convex lens and focused by a conical lens to create a cone shaped beam, which was able to pass through the ultrasound transducer without being blocked. This beam was further reflected by a custom-made mirror to illuminate the imaging objects, with the illumination area overlapping with the focal region of the ultrasound transducer. A water tank sealed with a thin low-density polyethylene (LDPE) film was used to couple the photoacoustic/ultrasonic waves from the imaging target to the ultrasound transducer.

For system control, the trigger-out signal from the OPO laser was used to trigger the data acquisition board (DAQ) (CS1422, Gage Applied Technologies Inc., Lockport, USA) for synchronization. Following the triggering of each laser pulse, photoacoustic signals were acquired; a user-defined delay of 30 ms was implemented, after which an ultrasonic pulser-receiver (5073 PR, Olympus, Tokyo, Japan) was triggered to emit an ultrasound pulse and subsequently receive the echo signals by the transducer. Both the photoacoustic and the ultrasound signals were amplified 39 dB using the ultrasonic pulser-receiver, and then digitized with the DAQ in a personal computer. The photoacoustic and ultrasound images were reconstructed as follows: the A-line signal from each scanning position was divided into the photoacoustic segment and the ultrasound segment. The photoacoustic segment was compensated for laser intensity variation first, and then Hilbert transformed for envelope detection. The ultrasound segment was processed for envelope detection only. In the end, photoacoustic and ultrasound images were displayed separately or coregistered using Amira software.

Synthesis of PDPPTBZ

A Schlenk tube was charged with 2,5-bis(2-butyloctyl)-3,6-bis(5-(trimethylstannyl)thiophen-2-yl)-2,5-dihydropyrrolo[3,4-*c*]pyrrole-1,4-dione (96.2 mg, 0.1 mmol), 4,8-dibromo-6-(2-ethylhexyl)-[1,2,5]thiadiazolo[3,4-f]benzotriazole (44.7 mg, 0.1 mmol), $Pd_2(dba)_3$ (5.0 mg, 5.4 μmol), P(*o*-tolyl)$_3$ (3.2 mg, 10.8 μmol) and toluene (10 mL) before it was sealed with a rubber septum. The reaction system was degassed with three freeze-pump-thaw cycles to remove air. After the reaction mixture was stirred at 100 ^{o}C for 24 h, the reaction was stopped and cooled down to room temperature. The mixture was dropped slowly into methanol (100 mL) to precipitate the crude polymer followed by centrifugation. The crude polymer was subsequently re-dissolved in dichloromethane (200 mL), washed with water 3 times, and dried over $MgSO_4$. After filtration with filter paper, the organic phase was concentrated to ~10 mL, which was added slowly to methanol (100 mL). Then PDPPTBZ (65 mg, yield:

71%) was collected as a black solid by centrifugation. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 9.10 (br), 5.08 (br), 4.09 (br), 2.59 (br), 2.03 (br), 1.55–1.16 (m, br), 0.88 (br).

Preparation of PDPPTBZ NPs

PDPPTBZ (5 mg) and DSPE-PEG_{2000} (10 mg) were dissolved well in tetrahydrofuran/chloroform (4 mL/1 mL), followed by filtration through a 0.45 μm syringe driven filter. Afterward, the filtrate was added to Milli-Q water (50 mL) under sonication for 2 min using a microtip sonicator at 195 W output. The obtained mixture was stirred vigorously at room temperature in fumehood to remove organic solvents. Then filtration was carried out using a 0.2 μm syringe driven filter to afford PDPPTBZ NPs. The obtained NPs were concentrated to a concentration of 1 mg mL^{-1} in 1 × PBS for further use. The concentration of PDPPTBZ NPs was determined by absorption spectrum.

Photoacoustic performance

Different concentrations of PDPPTBZ NPs (1, 0.5, 0.25, 0.125, 0.0625 mg mL^{-1}), mixed with agarose gel (1.5%) at 1:1 ratio, were imaged using AR-PAM under 1064 nm pulsed laser irradiation to characterize the dependence of photoacoustic signal on the PDPPTBZ NPs concentration. The PDPPTBZ NPs solution was irradiated under 4000 pulse laser with 4 mJ cm^{-2} at 1064 nm wavelength. In addition, different concentrations of PDPPTBZ NPs, mixed with agarose gel (1.5%) at 1:1 ratio were covered by different thickness chicken tissue, and then were imaged by AR-PAM under 1064nm pulsed laser irradiation. The signal-to-noise ratio (SNR) was calculated using equation: SNR = 20 × lg (S/N), where S is signal and N is noise.

Cell lines and animal models

Human brain glioma cell line U87 was obtained from American Type Culture Collection (ATCC), and cultured in DMEM media supplemented with10% FBS and 1% penicillin-streptomycin solution in a humidified incubator (5% CO_2 at 37 °C).

Balb/c nude mice (4-6 weeks) were obtained from the Medical Experimental Animal Center of Guangdong Province (Guangzhou, China). All animal experiment procedures were performed in compliance with the Animal Study Committee of Shenzhen Institutes of Advanced Technology, Chinese Academy of Sciences.

For orthotopic transplantation tumor model, 1×10^6 U87 cells in 6 μL PBS were injected into the striatum: Bregma 1.0 mm, left lateral 2.0 mm, depth 3.0 mm. The growth of the brain tumor was monitored by MRI (3T Magnetom Trio, Erlangen, Germany).

TEM imaging of U87 cells

U87 cells incubated with PDPPTBZ NPs (100 μg mL^{-1}) were trypsizined, centrifuged, and washed three times with PBS buffer for 5 min at each step. The cell pellet was fixed with paraformaldehyde when trypsin was removed. After 10 min, the pellet was washed again with PBS buffer to remove the fixatives, then dehydrated in an alcohol series, embedded in Epon, and sliced to thickness of 70 nm. Images of the slices were performed with JEOL JEM-1200EX transmission electron microscope.

In vitro biocompatibility

For cytotoxicity assay, 5×104 U87 per well were plated in a 96-well plate and cultured for 24 h. Afterward, the cells were treated with 10, 50, 100, 200 and 400 μg/mL of PDPPTBZ NPs for further 24 h. Relative cell viability (RCV) was assessed by CCK-8 assay, and then determined by a 96-well plate reader (BioTek SynergyTM 4) at an absorbance value of 450 nm:

$$RCV\ (\%) = \frac{A_t - A_{nc}}{A_{pc} - A_{nc}} \times 100\%$$

Where A_t, A_{pc} and A_{nc} represent the absorbance of the tested groups, positive and negative controls, respectively.

In vitro hemolysis test.

For hemolysis test, the blood from Balb/c nude mouse was collected, centrifuged (1500 rpm, 3 min), and washed 3 times with PBS to separate the red blood cells (RBC) from the plasma. 10% RBC (v/v) in PBS was incubated with different concentrations of PDPPTBZ NPs (10, 50, 100, 200 and 400 μg mL^{-1}) for 3 h at 37 °C. The mixture was then centrifuged (10,000 rpm, 1 min) and the supernatant of the suspensions was collected and analyzed by a UV-Vis-NIR spectrometer using 541 nm wavelength. The hemolytic ratio (HR) was calculated using the following equation:

$$HR\ (\%) = \frac{OD_t - OD_{nc}}{OD_{pc} - OD_{nc}} \times 100\%$$

Where OD_t, OD_{pc} and OD_{nc} are the absorbance of the tested samples, positive (deionized water) and negative (PBS) controls, respectively.

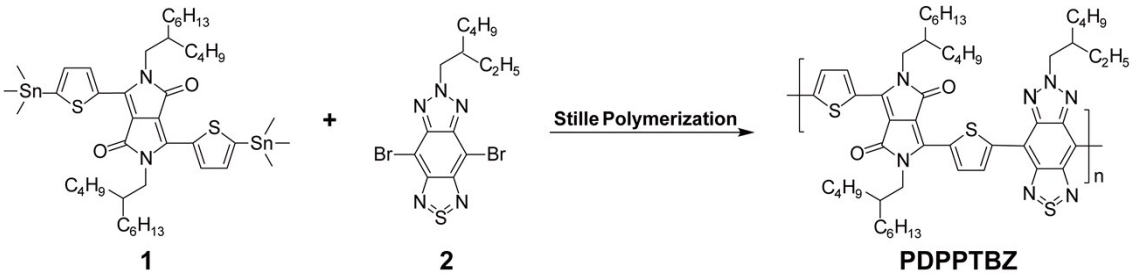
In vivo PA imaging of orthotopic transplantation brain tumor.

To investigate the in vivo PA performance of PDPPTBZ NPs for U87 orthotopic brain tumor imaging, mice bearing orthotopic U87 tumors were anesthetized with 2% isoflurane in oxygen, and placed with prone position. Before PDPPTBZ NPs injection, the PA and US images of the tumor area were obtained first as control using the AR-PAM system. The PA and US images of the tumor area were captured before and 3, 18, 36 hours after i.v. injection of PDPPTBZ NPs (2.5 mg kg^{-1}).

Toxicology evaluation

PDPPTBZ NPs (15 mg kg^{-1}) were i.v. injected into healthy Balb/c mice (5 mice per group), respectively. Before and 7 and 30 days post injection of PDPPTBZ NPs, the mouse blood was collected via orbit for blood biochemistry assay and complete blood panel test, which was performed at Shenzhen center for disease and prevention. Moreover, major organs (including heart, liver, kidney, lung and spleen) of the PDPPTBZ NPs treated mice were harvested. Through a series of processes, including fixation in 10% neutral buffered formalin,

embedding into paraffin and sectioning at 5 mm thickness, the tissues were stained with H&E and examined by a digital microscope.



Scheme S1 Synthetic route to PDPPTBZ. Reaction conditions. Reagents and conditions: $Pd_2(dba)_3$, P(*o*-tolyl)$_3$, toluene, 100 ^oC, 24 h.

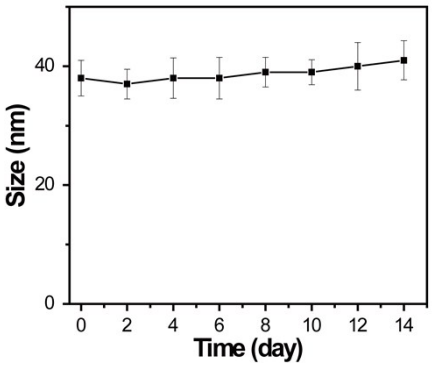


Fig. S1 Stability study of PDPPTBZ NPs in water over 14 days. Bars show means ± standard deviation (n = 3).

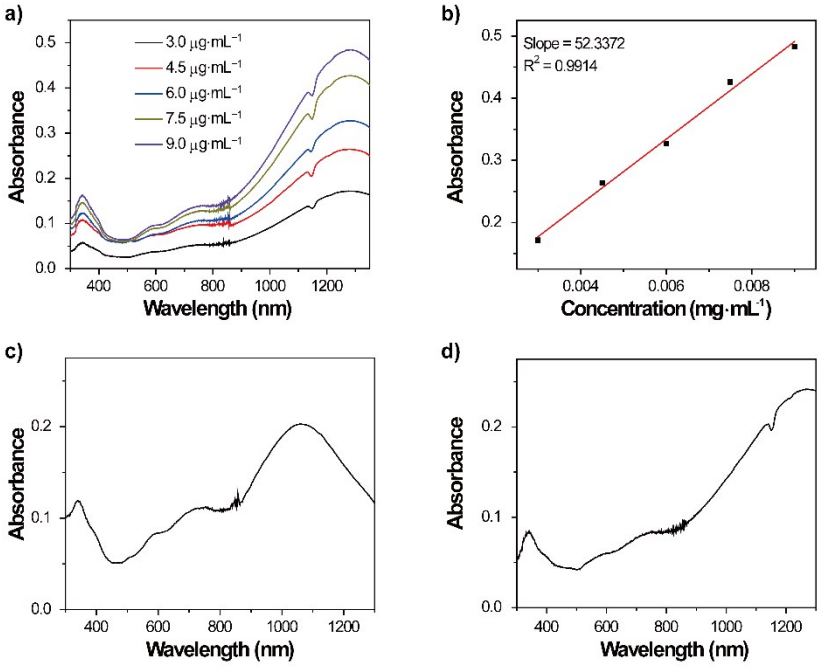


Fig. S2 Measurement of the mass extinction coefficient of PDPPTBZ NPs in water. a) Absorption spectra of PDPPTBZ in chloroform with different concentration. b) Optical density of PDPPTBZ at 1282 nm plotted as a function of concentration of PDPPTBZ. The slope was used to determine the mass extinction coefficient (52 mL mg^{-1} cm^{-1}). c) Absorption spectra of PDPPTBZ NPs in water, and (d) its corresponding absorption spectrum in chloroform. To make sure the same concentration of BPTB molecules in (c) and (d), the aqueous solution used in (c) was lyophilized followed by drying in vacuum oven. The obtained powder was redissolved in chloroform with the same concentration in (c), which was used to measure the corresponding absorption spectrum (d). According to the absorption intensity at 1282 nm in (d) and the mass extinction coefficient of PDPPTBZ, we can calculate the actual concentration of PDPPTBZ molecules in (c). Based on (c) and the obtained actual concentration of PDPPTBZ molecules, the mass extinction coefficient of PDPPTBZ NPs was determined to be 43 mL mg^{-1} cm^{-1}.

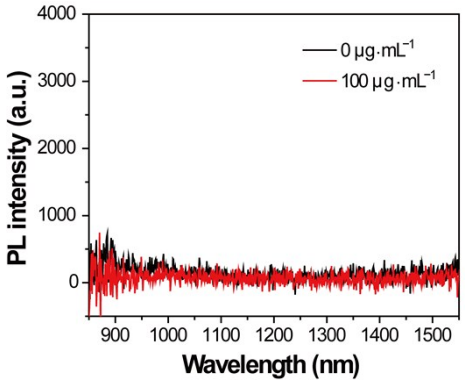


Fig. S3 PL spectrum of PDPPTBZ in toluene using InGaAs detection system. No fluorescence signal can be detected, indicating that PDPPTBZ is non-fluorescence.

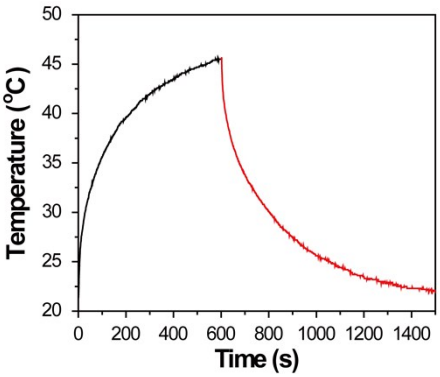


Fig. S4 Photothermal effect of the PDPPTBZ NPs under irradiation of a 1064 nm laser (300 mW cm^{-2}), which was turned off after irradiation for 600 s.

Table S1 The mass extinction coefficient at 1064 nm and photothermal conversion efficiency comparison of reported SPNs.

SPNs	Mass extinction coefficient (mL mg^{-1} cm^{-1})	Photothermal conversion efficiency (%)	Ref[a]
SPN-II	~22.5	N/A	32
P1 NPs	22.6	30.1	38
TSPNs	~30	N/A	39
PIGD	37.5	N/A	40
SPN-PT	N/A	53	41
SPN-DT	N/A	49	41
SPN-OT	N/A	36	41
PPy nanosheets	27.8	64.6	44
DPPTBZ NPs	43	67	This work

[a] The reference number referred to the corresponding number in the manuscript.

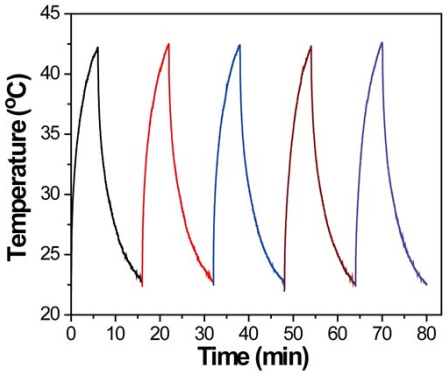


Fig. S5 Temperature elevation of PDPPTBZ NPs at the concentration of 70 μg mL^{-1} over 5 laser ON/OFF cycles of 1064 nm laser irradiation.

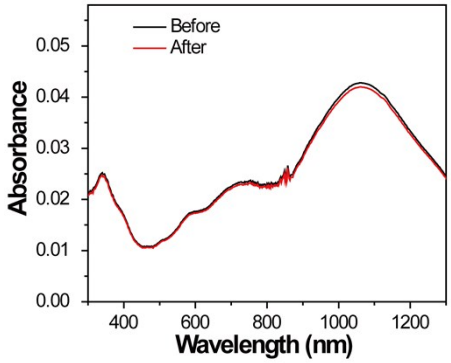


Fig. S6 Absorption spectra of PDPPTBZ NPs in water before and after exposure to 1064 nm laser (laser power fluence: 300 mW cm^{-2}) for 1 h.

Table S2 The penetration depth comparison of NIR-II PA contrast agents using chicken breast muscle for tissue mimicking and 1064 nm pulse laser as light source.

NIR-II PA contrast agents	Laser fluence (mJ cm^{-2})	Concentration (mg mL^{-1})	Penetration depth (cm)	Ref[a]
SPN-II	20	1	5	32
P1 NPs	10	0.01	0.8	38
TSPNs	55	0.04	5.3	39
PIGD	25	0.25	5	40
CTN	20	0.5	5	42
CuS	100	0.025	5	43
P-Pc	56	50	11.6	49
DPPTBZ NPs	4	0.05	4	This work

[a] The reference number referred to the corresponding number in the manuscript.

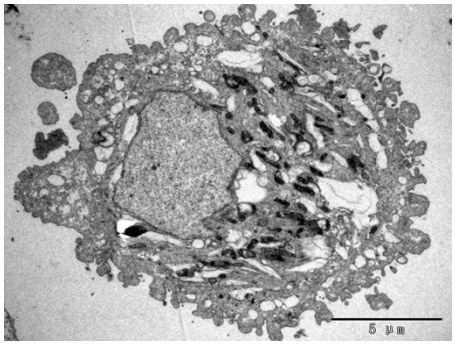


Fig. S7 Representative TEM image of PDPPTBZ NPs-treated U87 cells.

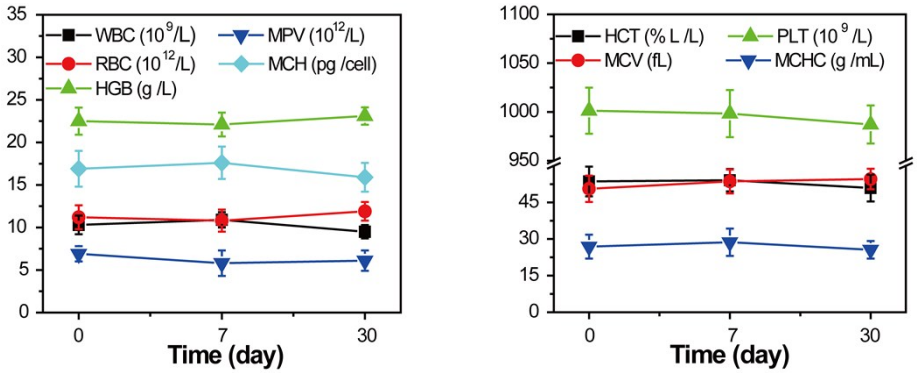


Fig. S8 Blood analysis of mice 0, 7, 30 days after injection of NPs (2 mg mL^{-1}, 150 μL). WBC: number of white blood cells; RBC: number of red blood cell; MPV: mean platelet volume; MCH: mean corpuscular hemoglobin; HGB: the concentration of hemoglobin; HCT: Hematocrit; MCV: mean corpuscular volume; MCHC: mean corpuscular hemoglobin concentration; PLT: Platelets.

References

1 Y. Dong, W. Cai, M. Wang, Q. Li, L. Ying, F. Huang and Y. Cao, *Org. Electron.*, 2013, **14**, 2459.