Supporting Information

Diketopyrrolopyrrole-based Semiconducting Polymer Nanoparticles for *In Vivo* Photoacoustic Imaging

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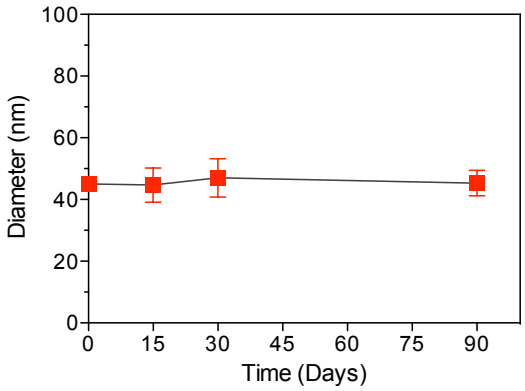
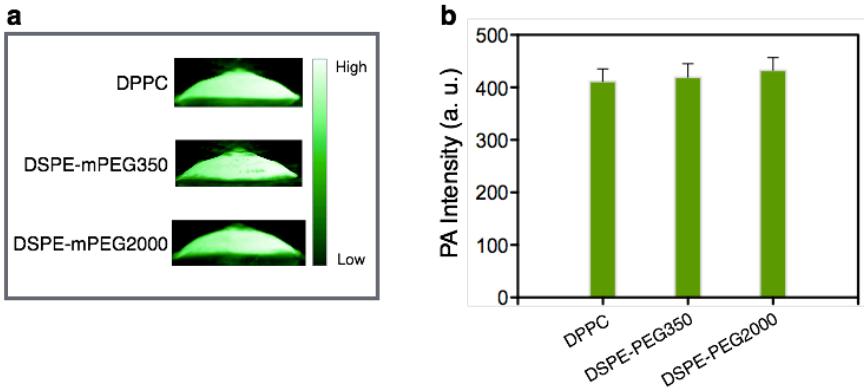
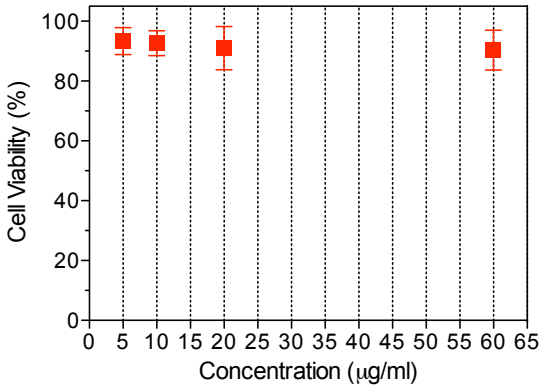
Supporting Figures



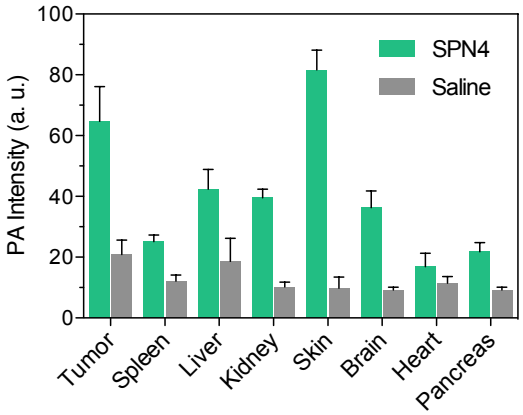
Supporting Figure S1. DLS data of SPN4 (1 μg/mL) after storage at 4 °C under dark over a period of 90 days.



Supporting Figure S2. PA images (**a**) and intensities (**b**) of SPN3 prepared from different lipids: DPPC, DSPE-mPEG350 and DSPE-mPEG2000. The hydrodynamic diameters for DPPC-SPN3, DSPE-mPEG350-SPN3 and DSPE-mPEG200-SPN3 are 36±2, 42±3 and 47±3, respectively. [SPN3] = 20 μg/mL in 1× PBS at pH = 7.4.



Supporting Figure S3. Cytotoxicity studies of SPN4. *In vitro* viability of HeLa cells treated with SPN4 solutions at concentrations of 5, 10, and 20 μg/mL for 24 h. The percentage cell viability of treated cells is calculated relative to that of cells treated with the same volume of PBS (viability was arbitrarily defined as 100%). Error bars represent standard deviations of three separate measurements.



Supporting Figure S4. *Ex vivo* PA quantification of major organs of mice 24 h after systemic administration of SPN4 or saline.

Experimental Section

Chemicals. All chemicals were obtained from Sigma-Aldrich unless otherwise stated. 1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-

2000] (DSPE-mPEG2000), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-350] (DSPE-mPEG350) was purchased from Avanti Lipids. Poly[cyclopentadithiophene-*alt*-benzothiadiazole] (SP1) (M_W = 20872) was purchased from Sigma-Aldrich.

Materials Characterization. ^{1}H NMR spectra were recorded on a Varian Mercury console spectrometer (400 MHz) under 100 °C using deuterated 1,1,2,2-tetrachloroethane (TCE-d_2). Chemical shifts are given in parts per million (ppm) with respect to TCE as internal standard. High temperature size exclusion chromatography (SEC) using 1,2,4-trichlorobenzene was performed under 180 °C on Tosoh High-temperature EcoSEC equipped with a single column which was calibrated by monodiperse polystyrene standards to evaluate the number average molecular weight (M_n), weight average molecular weight (M_w), and polydispersity index (PDI) of SPs. TEM images were obtained on a JEM 1230 transmission electron microscope with an accelerating voltage of 200 kV. Dynamic light scattering (DLS) was performed on the Malvern ZetaSizer Nano S. UV-Vis spectra were recorded on an Agilent spectrophotometer. Tecan safire microplate reader was used to measure the optical absorption or fluorescence from well plates. Fluorescence measurements were carried out on a wavelength-calibrated FluoroMax-3 fluorometer (Horiba Jobin Yvon). Fluorescence images of SPNs solution were acquired with xenograft IVIS spectrum imaging system. The quantum yield of SPNs was measured using indocyanine green (ICG) as the standard with a known quantum yield of 1% in H_2O.

PA Instrumentation. PA spectra and PA/US coregistered images were acquired with a LAZR instrument (Visualsonics, 2100 High-Resolution Imaging System). It is equipped

with a MS-250 linear array transducer (21 MHz, 70%-6 dB two-way bandwidth, 256 elements) to detect US and PA signals, and a tunable Nd:YAG laser system (OPOTEK Inc., Carlsbad, CA, 680-950 nm, 20 Hz repetition rate, 5 ns pulse width, 50 mJ pulse peak energy) was used to trigger the system acquisition, and excite tissue with optical pulses to generate the PA effect. The spot size is 1×24 mm, and the full field of view is 14–23 mm wide. The spatial resolution for PA images was measured to be at least 340 μm. The axial and lateral resolutions for US images are 75 and 165 μm, respectively. Acquisition rate of 5 frames per second was used for all the experiments. A commercial Endra Nexus128 PA tomography system (Endra Inc., Ann Arbor, Michigan) was also used in this study. The system houses a tunable nanosecond pulsed laser (7 ns pulses, 20 Hz pulse repetition frequency, 7 mJ/pulse on the animal surface, wavelength range (680–950 nm), 128 unfocused ultrasound transducers (with 5 MHz center frequency and 3 mm diameter) arranged in a hemispherical bowl filled with water, animal tray on top of the bowl, data acquisition/reconstruction console, servo motors for 3D rotation of the bowl, and a temperature monitor of the water bath.

Photothermal Heating. SPNs solution (20 μg/mL in 1× PBS at pH = 7.4.) was irradiated along with 100 μL of a control PBS solution. The solutions were irradiated with a 808 nm laser diode with the power intensity of 0.24 W/cm^2 for 4 min. A MikroShot thermal camera (Mikron) was used to collect thermal images and quantify solution temperature.

Synthesis of SP2. In a 25 mL flame-dried Schlenk flask, 2,5-bis(2-hexyldecyl)-3,6-bis(5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)thiophen-2-yl)-2,5-dihydropyrrolo[3,4-c] pyrrole-1,4-dione (250.0 mg, 0.25 mmol), dioctyl 2-(2,7-dibromo-9H-fluoren-9-ylidene)-1,3-dithiole-4,5-dicarboxylate (184.3 mg, 0.25 mmol),

tris(dibenzylideneacetone)dipalladium ($Pd_2(dba)_3$) (4 mg), and tri(*o*-tolyl)phosphine (3 mg) were dissolved in 5.0 mL of degassed toluene and degassed 20% aqueous tetraethylammonium hydroxide (1 mL). The reaction mixture was vigorously stirred at 60 °C for 24 h and then heated up to 95 °C for 6 h. The solution turned from red to dark purple, and blue after an hour. The polymer was purified by precipitation in methanol/water (10:1), filtered through 0.45 μm nylon filter, and purified on a Soxhlet apparatus with methanol, hexanes, and chloroform. To the chloroform fraction was added palladium removal regent (E)-*N*,*N*-diethyl-2-phenyldiazenecarbothioamide (5 mg). The resulting solution was stirred for 2 h. The solution was concentrated under reduced pressure, precipitated in methanol (300 mL), filtered through 0.45 μm nylon filter, washed with methanol, and dried under vacuum at 60 °C overnight. M_n=3.45 kDa, M_w=6.87 kDa, PDI=2.0. ^{1}H NMR (400 MHz, TCE-d_2, δ/ppm): 8.97-7.32 (brm, ArH, 10H), 4.44 (br, O-CH_2-, 4H), 4.08 (br, >N-CH_2-, 4H), 2.08-1.87 (br, N-CH_2-CH< & O-CH_2-CH_2- 6H), 1.49-1.31 (br, -CH_2-, 68H), 0.89 (br, -CH_3, 18H).

Synthesis of SP3 and SP4. To a microwave vessel (8 mL) charged with a string bar were added $Me_3SnArSnMe_3$ (0.25 mmol) and Br-DPP-Br (0.25 mmol), followed by the addition of degassed toluene (6 mL). The resulting solution was bubbled with argon for 15 min. tris(dibenzylideneacetone)dipalladium ($Pd_2(dba)_3$) (4 mg), and tri(*o*-tolyl)phosphine (3 mg) were quickly added under argon. The vessel was then sealed with a snap cap and immediately subjected to the following reaction conditions in a microwave reactor (Microwave Setups: CEM Discover Automatic Microwave Reactor; power cycling; power = 250 W; power cycles = 40; temperature = 150-180 °C; heating = 150 s; cooling = 30 s; pressure = 150 psi; stirring = high). After the completion, the

reaction was automatically cooled to room temperature and the obtained solids were suspended into acetone. The solids were collected by filtration through a high-quality glass thimble. The thimble was placed inside a Soxhlet extractor, and the solids were successively extracted with acetone, hexane, and chloroform. To the chloroform fraction was added palladium removal regent (E)-*N*,*N*-diethyl-2-phenyldiazenecarbothioamide (5 mg). The resulting solution was stirred for 2 h. The chloroform solution was concentrated and precipitated into acetone. The polymer was filtered and dried *in vacuo* at 60 °C overnight. SP3: M_n=42.2 kDa, M_w=114 kDa, PDI=2.7. ^{1}H NMR (400 MHz, TCE-d_2, δ/ppm): 8.86 (br, ArH, 2H), 7.50-7.13 (brm, ArH, 22H), 4.06 (br, >N-CH_2-, 4H), 2.64 (br, Ph-CH_2-, 8H), 2.01 (br, N-CH$_2$-CH<, 2H), 1.67 (br, Ph-CH$_2$-CH_2-, 8H), 1.49-1.27 (br, -CH_2-, 88H), 0.94 (br, -CH_3, 24H. SP4: M_n=13.9 kDa, M_w=117 kDa, PDI=8.4. ^{1}H NMR (400 MHz, TCE-d_2, δ/ppm): 8.94 (br, ArH, 2H), 7.13 (br, ArH, 4H), 4.08 (br, >N-CH_2-, 4H), 2.04 (br, N-CH$_2$-CH<, 2H), 1.48-1.31 (br, -CH_2-, 48H), 0.92 (br, -CH_3, 12H).

Preparation of SPNs. SPs (1 mg) were dissolved in THF (1 mL) by bath sonication. The resulted solutions were filtered through a polyvinylidene fluoride (PVDF) syringe driven filter (0.22 μm) (Millipore). The concentrations of SPs were determined by UV-Vis absorption. Then, a mixed THF solution (1 mL) containing SP (0.25 mg/mL) and DPPC, DSPE-mPEG350 or DSPE-mPEG2000 (2.5 mg/mL) was used to prepare SPNs by rapidly injecting it into distilled-deionized water (9 mL) under continuous sonication with a microtip-equipped probe sonicator (Branson, W-150) at a power output of 6 watts RMS for 2 min. After sonication for additional 1 min, THF was evaporated at 65 °C under nitrogen atmosphere. The aqueous solution was filtered through a polyethersulfone (PES) syringe driven filter (0.22 μm) (Millipore), and washed three times using a 30 K

centrifugal filter units (Millipore) under centrifugation at 3,500 rpm for 15 min at 4 °C. The concentrations of SPNs solutions were determined by UV-Vis absorption according to their absorption coefficients. The SPN solutions were finally concentrated to 0.08 mg/mL (based on the mass of SP) by ultrafiltration and stored in dark at 4 °C.

Cell culture. HeLa cervical adenocarcinoma epithelial cells were purchased from the American Type Culture Collection (ATCC). HeLa cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) (GIBCO) supplemented with 10% FBS (fetal bovine serum) (GIBCO). The cells were maintained in an atmosphere of 5% CO_2 and 95% humidified air at 37 °C.

Cytotoxicity test. Cells were seeded in 96 well plates (1000 cells in 100 μL per well) for 24 h, and then SPN4 (final concentration 5, 15, and 20 μg/mL) was added to the cell culture medium. Cells were incubated with SPN4 or saline for 24 h, followed by the addition of MTT (20 μL, 5 mg/mL) for 3 h. The media was removed and DMSO (200 μL) was added into each well and gently shaken for 10 min at room temperature to dissolve all formed precipitates. The absorbance of MTT at 550 nm was measured by using a Tecan microplate reader. Cell viability was expressed by the ratio of the absorbance of the cells incubated with SPN4 solution to that of the cells incubated with culture medium only.

Imaging nanoparticle solution in matrigel *in vivo*. All animal experiments were performed in compliance with the Guidelines for the Care and Use of Research Animals established by the Stanford University Animal Studies Committee. In order to compare the *in vivo* signal evolution at equal mass concentrations of SPNs, nanoparticles were suspended in Matrigel (BD Biosciences) to different concentrations. The final

concentration of Matrigel was 50% v/v for all solutions. Matrigel-nanoparticle mixtures (30 μL) were injected subcutaneously on the dorsal aspects of female nude mice (Charles River Inc.) to form inclusions of nanoparticles, and PA imaging was performed using a Vevo LAZR imaging system. Three separate inclusions of each concentration as well as of Matrigel alone were imaged.

Tumor mouse model. To establish tumors in six-week-old female nu/nu mice, two million HeLa cells suspended in 50 mL of 50% v/v mixture of Matrigel in supplemented DMEM (10% fetal bovine serum, 1% pen/strep (100 U/ml penicillin and 100 μg/mL streptomycin) were injected subcutaneously in the shoulders of the mouse. Tumors were grown until a single aspect was 7–9 mm (approximately 10–15 days) before used for PA imaging experiments.

Tumor imaging. HeLa tumor xenografted nude mice were anesthetized using 2% isoflurane in oxygen, and a catheter was applied to the tail vein. They were placed in the Endra Nexus128 PA imaging system, and were scanned to determine the endogenous signal of tumors at 750 before systemic administration with SPN4 (30 μg in 120 μL) (n=4) or saline (120 μL) (n=4) through catheter. The first and last PA images were recorded 5 min and 24 h post-injection, respectively. Data was acquired through a continuous model that took 12 s to obtain one data set. The real-time PA spectra were recorded with a LAZR instrument (Visualsonics, 2100 High-Resolution Imaging System). After imaging, mice were sacrificed by CO_2 asphyxiation, and necropsy was performed by a midline incision ventrally from caudal to rostral aspects of the mouse through both the skin and peritoneum. Organs were subsequently removed, rinsed in saline. For *ex vivo* PA imaging, organs were embedded in agar phantom and acquired

immediately with Endra Nexus128 PA imaging system. Three-dimensional PA image was reconstructed off-line using data acquired from all 128 transducers at each view and a back-projection algorithm. The algorithm corrects for pulse-to-pulse variations in the laser intensity and small changes in the temperature that affect acoustic velocity in the water. The reconstructed raw data is analyzed using OSiriX software.

Data Analysis. PA signal intensities were measured by region of interest (ROI) analysis using the Vevo LAZR imaging system software package or OsiriX. Results are expressed as the mean±SD deviation unless otherwise stated. All statistical calculations were performed using GraphPad Prism v. 5 (GraphPad Software Inc., CA, USA).