Electronic Supplementary Material (ESI) for Chemical Science.

Supporting Information

Semiconducting Polymer Dots with Bright Narrow-Band Emission at 800 nm for Biological Applications

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Experimental Section

General information

All the chemicals for polymer synthesis were purchased from Sigma-Aldrich and TCI America company unless indicated elsewhere. The fluorescent semiconducting poly{[2,7-(9,9-bis-(2-octyl)-fluorene)]-alt-[5,5-(4,7-di-2-hexylthienyl-2,1,3-benzothiadiazole)]} (PFDHTBT, average Mw =20,751, polydispersity= 2.4) and PS-PEG-COOH were synthesized by our group. The amphiphilic functional polymer poly(styrene-co-maleic anhydride) (PSMA, cumene-terminated, average MW ~1,700, styrene content = 68%), solvent tetrahydrofuran (THF, anhydrous, 99.9%), HEPES, poly(ethylene glycol) (PEG) and streptavidin were purchased from Sigma-Aldrich (Shanghai, China). 1-Ethyl-3-(3-(dimethylamino)propyl)carbodiimide hydrochloride (EDC) was purchased from Thermo Scientific. Methylene blue was purchased from J&K Scientific Ltd. (Beijing, China).

^{1}H (500 MHz) NMR spectra ^{13}C (125 MHz) NMR spectra were recorded on Bruker AV500 spectrometers. ^{1}H NMR ^{13}C NMR spectra used tetramethylsilane as an internal standard in $CDCl_3$. The molecular weight of polymers was measured by the GPC method (Shimadzu GPC), where polystyrene was used as the standard (THF as eluent). The particle size and zeta-potential of Pdots in bulk solution were characterized by dynamic light scattering (Malvern Zetasizer NanoS). TEM measurements were recorded on a transmission electron microscope (H-600, Hitachi, Japan). UV-Vis absorption spectra were recorded with DU 720 scanning spectrophotometer (Beckman Coulter, Inc., CA USA) using a 1-cm quartz cuvette. Fluorescence quantum yields were measured using a Hamamatsu photonic multichannel analyzer C10027 equipped with CCD and integrating sphere. In quantum yield calibration, we used the solvent as the reference. We also used dyes in different solvents for calibration and found the measured QY values were very close to the reported values for the dyes.

Synthesis of $Br_2SiNc[OSi(n\text{-}C_6H_{13})_3]_2$

1*H*-benzo[*f*]isoindole-1,3(2*H*)-diimine. The synthesis procedure was following Esposito's group (*Inorganic Chemistry, 1965*, 4, 128). We mixed 2,3-naphthalene dicarbonitrile (5.1 g) and sodium methoxide (750 mg) with dry MeOH (45 mL). Then, anhydrous ammonia was bubbled into reaction for 45 min. The reaction was turned to reflux for 3 h under NH_3 bubbling. After cooling down to room temperature, the product was filtrated and washed with ether, dried, and we obtained 4.5 g, 80% yield. ^{1}H NMR (500 MHz, $(CD_3)_2SO$) = 8.33 (s, 2H), 8.08 (m, 2H), 7.77 (m, 2H). ^{13}C NMR $(CD_3)_2SO$) =170.3, 136.5, 127.2, 125.6, 124.3, 123.5. HRMS (ESI): (M^+, $C_{12}H_9N_3$) calcd 195.0796; found 195.0792.

6-bromo-1*H*-benzo[*f*]isoindole-1,3(2*H*)-diimine. We followed the same method as above but used 6-bromonaphthalene-2,3-dicarbonitrile (200 mg) as a starting material. We obtained 165 mg (78% yield). ^{1}H NMR (500 MHz, $(CD_3)_2SO$) = 8.32 (m, 3H), 8.03 (m, 1H), 7.76 (m, 1H). ^{13}C NMR $(CD_3)_2SO$) = 171.5, 135.5, 134.7, 132.8, 131.9, 131.7, 121.3, 120.9, 120.2. HRMS (ESI): (M^+, $C_{12}H_8BrN_3$) calcd 272.9902; found 272.9900.

Br_2NcCl_2. $SiCl_2$ (97 mg), 1*H*-benzo[*f*]isoindole-1,3(2*H*)-diimine (71 mg) and 6-bromo-1*H*-benzo[*f*]isoindole-1,3(2*H*)-diimine (100 mg) were dissolved in a mixture with tetrahydronaphthalene (8 mL) and tri-*n*-butylamine (5 mL) and then refluxed for 2.5 h. After cooling to room temperature and diluting with 5-mL MeOH, the product was collected by filtration, washed and dried.

$Br_2Nc(OH)_2$. Br_2NcCl_2 (1 g) was mixed with conc. H_2SO_4 (30 mL) and stirred for 2 h. Then the reaction was poured onto ice. The filtrate was collected and washed with water and acetone. The solid was refluxed in conc. NH_4OH for 1 h. After cooling down, the product was filtered and dried. We moved to next step without further purification.

$Br_2SiNc[OSi(n\text{-}C_6H_{13})_3]_2$. A mixture of $Br_2Nc(OH)_2$ (30 mg), tri-*n*-hexylchlorosilane (0.15 mL), tri-*n*-butylamine (0.1mL) and dry 3-picoline (3.5 mL) were refluxed 1.5 h. After cooling to room temperature, the reaction was filtered. The filtrate was reacted with 5 mL of ethanol-1:1water solution. The solid was collected by filtration and purified by column chromatography (Hexane: Toluene = 3:1). Although there are two regioisomers, it did not affect their physical properties. ^{1}H NMR (500 MHz, $(CDCl_3)$ = 10.11 (m, 3H), 10.01(m, 1H), 8.82 (m, 1H), 8.68~8.65 (m, 3H), 7.93~7.91 (m, 3H), 0.64 (m, 6H), 0.44 (m, 9H), 0.21 (m, 6H), 0.06~-0.19 (m, 18H). HRMS (ESI): (M^+, $C_{84}H_{100}Br_2N_8O_2Si_3$) calcd 1494.5644; found 1494.5642.

Synthesis of 6-(6-(2,7-dibromo-9-hexyl-9*H*-fluoren-9-yl)hexyl)-4,8-di(thiophen-2-yl) -6,7-dihydro-5*H*-[1,2,3]triazolo[4',5':4,5]benzo[1,2-*c*][1,2,5]thiadiazole

4,8-bis(thiophen-2-yl)-5*H*-[1,2,5]thiadiazolo[3,4-*f*]benzotriazole (440 mg) dissolved in dry DMF (10 mL), 1M *t*-BuOK (1.54 mL) was added to the reaction and refluxed for 30 mins. 2,7-dibromo-9-(6-bromohexyl)-9-hexyl-9*H*-fluorene (883 mg) (following the literature) 57-58 was added to 10-mL DMF and then added drop-wise into reaction and refluxed for 24 h. After cooling to room temperature, washed by water and dried, the product was purified by column chromatography. The yield was 58% (742 mg). ^{1}H NMR (500 MHz, $(CDCl_3)$ = 8.84 (m, 2H), 7.63 (m, 2H), 7.49~7.39 (m, 6H), 7.32-7.29 (m, 2H), 4.86 (t, 2H), 2.14 (m, 2H), 1.92 (m 4H), 1.87 (m, 2H), 1.84-0.74 (m, 12H), 0.56 (t, 3H). HRMS (ESI): (M^+, $C_{39}H_{37}Br_2N_5S_3$) calcd 829.0578; found 829.0574.

Synthesis of NIR800 Polymer

The NIR800 polymer was synthesized by Suzuki coupling by using the following feeding ratios: 9,9-Doctylfluorene-2,7-diboronic acid bis(1,3-propanediol)ester (0.5 eq.), 4,7-dibromobenzo[*c*][1,2,5]oxadiazole (0.41 eq.), 4,7-bis(5-bromothiophen-2-yl)benzo [*c*][1,2,5]thiadiazole (0.017 eq.), 6-(6-(2,7-dibromo-9-hexyl-9*H*-fluoren-9-yl)hexyl)-4,8-di (thiophen-2-yl)-6,7-dihydro-5*H*-[1,2,3]triazolo[4',5':4,5]benzo[1,2-*c*][1,2,5]thiadiazole (0.06 eq.) and $Br_2SiNc[OSi(n\text{-}C_6H_{13})_3]_2$ (0.007 eq.), TBAB (0.008 mmol), and $Pd(PPh_3)_4$ (3.5 mol%) was added in toluene/2N Na_2CO_3 (1:1). The mixture was degassed and refilled with N_2 then refluxed for 2 days. Phenylboronic acid (100 mg) dissolved in THF was added to reaction. After 2 h, bromobenzene (0.5 mL) was added and further stirred for 3 h. The mixture was poured into methanol, and the precipitate was filtered, washed with methanol, water and acetone to remove monomer, small oligomers, and inorganic salts. The crude product was dissolved in DCM (7 mL), filtered through 0.2 μm membrane and re-precipitated in methanol (75 mL). The powder was stirred in acetone (100 mL) for 4 h and collected by filtration and dried in vacuum. (Yield: 82%) Mn = 27652, PDI = 2.9.

Preparation of Pdots

The NIR800 Pdots were prepared using a reprecipitation method. The 1 mL mixture of NIR800 polymer and 20 wt% PSMA in THF was quickly injected into water (10 mL) under sonication to prepare the small-sized Pdots. Blended NIR800 Pdots with different NIR800 polymer to PFDHTBT polymer ratio (wt%) were prepared following the same procedure. 5mL mixture of PFDHTBT, 20% PSMA and different weight ratio of NIR 800 polymer was quickly injected into 10 mL water under sonication to prepare the blended NIR800 Pdots. For the PEGylated Pdots, a THF mixture containing 100 μg/mL of PFDHTBT, 20 μg/mL of PS-PEG-COOH and 25 μg/mL of NIR800 polymer was quickly injected into 10 mL water under sonication. The prepared Pdots was evaporated by N_2 flow at 70 °C to remove THF and the solution was concentrated, followed by filtration through a 0.2-μm filter.

Single-particle Brightness Measurement

For the measurement of single-particle fluorescence brightness, samples were diluted in Milli-Q water, then were dropped on cleaned glass coverslips (previously functionalized with (3-aminopropyl)trimethoxysilane), followed by drying under nitrogen. Then samples were imaged on a custom built Total Internal Reflection Fluorescence (TIRF) microscope. Fluorescence intensity emitted per frame for a given particle was estimated by integrating the CCD signal over the fluorescence spot. The wide-field microscope was constructed by directing the 488-nm laser beam into an inverted microscope (Olympus IX71, Japan). Laser excitation power was measured at the nosepiece before the objective. The objective used for illumination and light collection

was 1.49-NA UAPON 100× TIRF objective (Olympus, Japan). The fluorescent signal was filtered by a 593-nm long-pass filter (HQ500LP; Chroma, Rockingham, VT, USA) and imaged onto an Andor iXon3 frame transfe EMCCD camera (Andor Technology, UK).

Bioconjugation of NIR800 Pdots

Bioconjugation was performed by utilizing the EDC-catalyzed reaction between carboxyl groups on Pdots' surface and amine groups on streptavidin. In a typical bioconjugation reaction, 20 μL of polyethylene glycol (5% w/v PEG, MW 3350) and 20 μL of HEPES buffer (1 M, pH7.3) were added to 1 mL of NIR800 polymer Pdots solution (50 μg/mL in MilliQ water), resulting in a Pdots solution in 20 mM HEPES buffer with a pH of 7.3. Then, 60-μL streptavidin (Invitrogen, 1mg /mL) was added to the solution and mixed well on a vortex. 15 μL of freshly prepared EDC solution (5 mg/mL in MilliQ water) was added to the solution. The mixture was left on a rotary shaker for 4 h at room temperature. Finally, the reaction mixture was centrifuged five times in a centrifugal ultrafiltration tube (Amicon Ultra-4, Millipore, MWCO: 100 kDa) to remove free streptavidin. Adding 80 μL Triton-X 100 (2.5 wt %) for each centrifugation prevent Pdots' aggregation. Finally, the Pdots bioconjugate was purified by gel filtration using Sephacryl HR-300 gel media.

Cell Culture

The breast cancer cell line MCF-7 was ordered from American Type Culture Collection (ATCC, Manassas, VA, USA). All the agents for cell culture were purchased from Gibco unless indicated otherwise. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Biological industries), 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified 5% CO_2 atmosphere at 37 °C. The culture medium was changed every other day. Cells were detached with 0.25% trypsin-EDTA, then subcultured into culture flasks when cell reached 80% confluency. The cell concentration was determined by microscopy using a hemacytometer.

Cellular and Subcellular Labeling

For specific cell labeling, a million cells in 100-μL labeling buffer (PBS containing 1% bovine serum albumin, or BSA) were incubated with biotinylated primary anti-EpCAM antibody (Biolegend, 324216) on a rotary shaker for 30 mins, followed by a washing step using labeling buffer to remove free antibody. Then the cells were incubated with Pdots-streptavidin in BlockAid buffer (Molecular Probes, B10710) or PE-Cy7-streptavidin for 30 minutes, followed by two washing steps using labeling buffer. Finally, the cells were fixed in 0.5-mL 4% (v/v) paraformaldehyde (PFA) solution. For the negative control labeling, no biotinylated primary anti-

EpCAM antibody was added. Flow cytometry measurements inflow cytometers LSR II (BD Bioscience, San Jose, CA USA) were performed on fresh samples with a concentration of 10^6 cells/0.5mL. Corresponding detection channel for fluorescence emission of Pdots was collected with PE-Cy7 channel (735nm LP + 780/60) under the excitation source of a 488-nm laser. Scattered light and fluorescence emission were detected by PMT arrays. Representative populations of cells were chosen by selection of appropriate gates. Detection of cell-scattered and fluorescent light was continued until at least 104 events had been collected in the active gate. Data were analyzed using FlowJo Software (Tree Star, Inc., Ashland, OR USA). Fluorescence images were acquired on an inverted fluorescence microscope (Olympus IX71, Japan) with a 40× (0.45 NA LUCPLFLN) objective. The excitation light was generated from a mercury lamp, filtered by a 445/45-nm band-pass filter. The fluorescent signal was filtered by a 593-nm long-pass filter and imaged on an Andor iXon3 frame transfer EMCCD (Andor, UK).

For microtubulin labeling, MCF-7 cells were plated on glass-bottomed dishes and cultured in DMEM medium supplemented with 10% FBS. After reaching 80% confluence, the cells were pre-extracted with extracting buffer containing 0.1M PIPES, 1mM EGTA, 1mM $MgCl_2$, and 0.2% TritonX-100 for 1min, followed by rinsing with PBS for once. Then cells were fixed with 4% v/v PFA and 0.1% glutaraldehyde for 15 min at room temperature. After fixation, cells were permeabilized with 0.5% Triton-X 100 for 5min, followed by washing with PBS for three times. Then the cells were blocked with 5% BSA and 0.1% Triton-X 100 for 30 min, followed by incubation with a biotinylated anti-tubulin-α antibody (Biolegend, 627904) in blocking buffer for 1 hour at room temperature. Then cells were washed with PBS and 0.1% Triton-X 100 for three times to remove free primary antibody. Streptavidin-Pdots were diluted in blocking buffer and incubated with cells for 1 hour at room temperature. After washing with PBS and 0.1% Triton-X 100, fluorescence imaging was performed as described above.

***In Vitro* Cytotoxicity Analysis of blended NIR800 Pdots**

Cytotoxicity analysis of NIR800 Pdots was evaluated by MTT assay. MCF-7 cells were seeded into 96-well plates and cultured in a humidified 5% CO_2 atmosphere at 37°C. Culture medium containing different concentrations of Pdots (0, 12.5, 25, 50, 100 μg/mL) were added to each well of plates. After a 24-hour incubation, 20 μL of MTT (5 mg/mL in PBS) was added into plates. The plates without MTT were controls to subtract the absorbance of Pdots. 4 hours later, the culture medium was removed carefully, then 150-μL DMSO was added into each well. The plates were shaken gently for 10 min to fully dissolve the precipitates. Then absorbance of formazan at 570 nm was recorded on a microplate reader (Cytation3, Biotek).

Animals

All animal experiments were performed under the approval of Jilin University's Administrative Panel on Laboratory Animal Care. Eight-week-old male nude mice and ICR mice were obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and Changsheng Biotechnology Co., Ltd. (Shenyang, China), respectively. All mice were anaesthetized using 2% isoflurane in oxygen in a rodent anaesthesia machine.

***In Vivo* Fluorescent Imaging with blended NIR800 Pdots**

The ICR mice were anesthetized with isoflurane and were treated with a depilatory cream to remove fur. Next, 100 μL of PSMA-NIR Pdots or PEG-NIR Pdots (1mg/mL) were intravenously injected into a mouse. After injection, the mice were immediately imaged with a small-animal imaging system. Then fluorescence images were acquired at designated time points. The organs, including heart, liver, spleen, lung, kidney, axillary lymph nodes and inguinal lymph nodes, were resected for fluorescence imaging analysis.

For tumor imaging, 100 μL of MCF-7 cells suspended in sterilized PBS (1×10^7 cells/mL) were implanted subcutaneously into the right shoulder of female nude mice. Two weeks after implantation, the tumor-bearing mice were anesthetized with isoflurane and systematically injected with 100 μL of PEG-NIR Pdots (1mg/mL) through the tail vein. Whole-body fluorescent imaging was performed at designated time points within 24 hours. At 24 hours after Pdots injection, the skins of the mice were removed to expose the tumor and the lymph nodes to image with the small-animal imaging system. The peritoneums were further dissected to expose liver tissue so that the fluorescent images could be acquired. The mice were killed under deep isoflurane anesthesia. Then the tumor, heart, liver, spleen, lung, kidneys and lymph nodes were harvested for *ex vivo* fluorescence imaging to estimate the tissue distribution.

For lymph-node imaging, 20 μL (0.5 mg/mL) PEG-NIR Pdots were subcutaneously injected into the paws of mice. One minute after injection, the mice were placed in a light-sealed area for whole-body fluorescent imaging. The imaging was performed at designated time points. 20 μL methylene blue dye at a concentration of 1% was intradermally injected into the paw of mice after PEG-NIR Pdots injection. Photographs of the lymph nodes were obtained using a camera.

Whole-body fluorescent imaging was performed by using a small animal imaging system equipped with an Andor frame transfer CCD (Andor iKon-M 934, UK) and xenon light source (Asahi Spectra MAX-303, Japan) (excitation: 543±22 nm; emission: 809±81 nm) with an exposure time of 5 seconds.

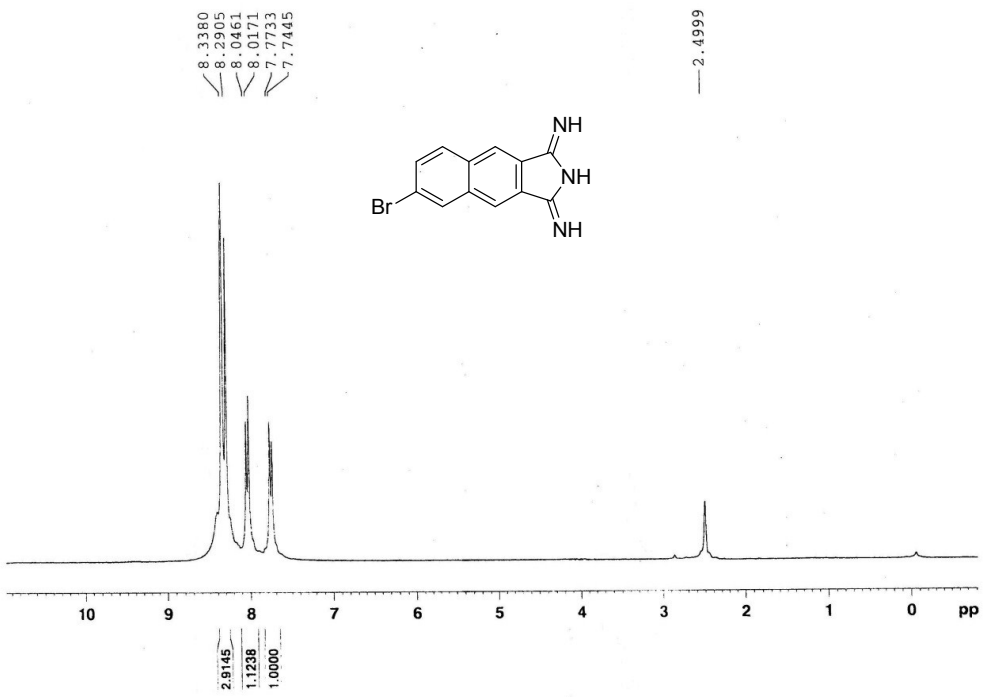

Fig. S1 ^{1}H NMR of 6-bromo-1*H*-benzo[*f*]isoindole-1,3(2*H*)-diimine

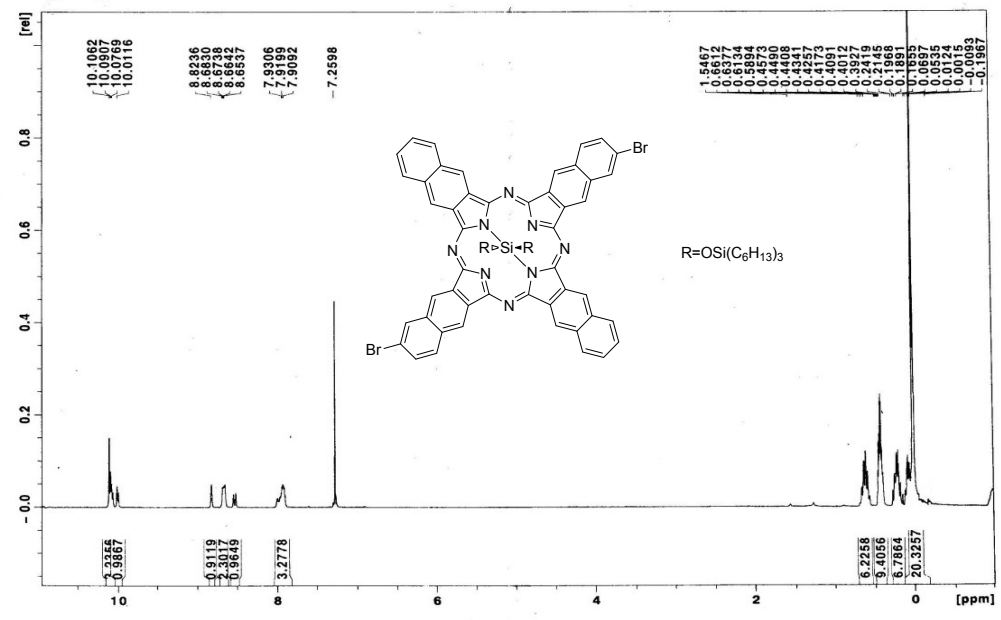

Fig. S2 ^{1}H NMR of $Br_2SiNc[OSi(n\text{-}C_6H_{13})_3]_2$

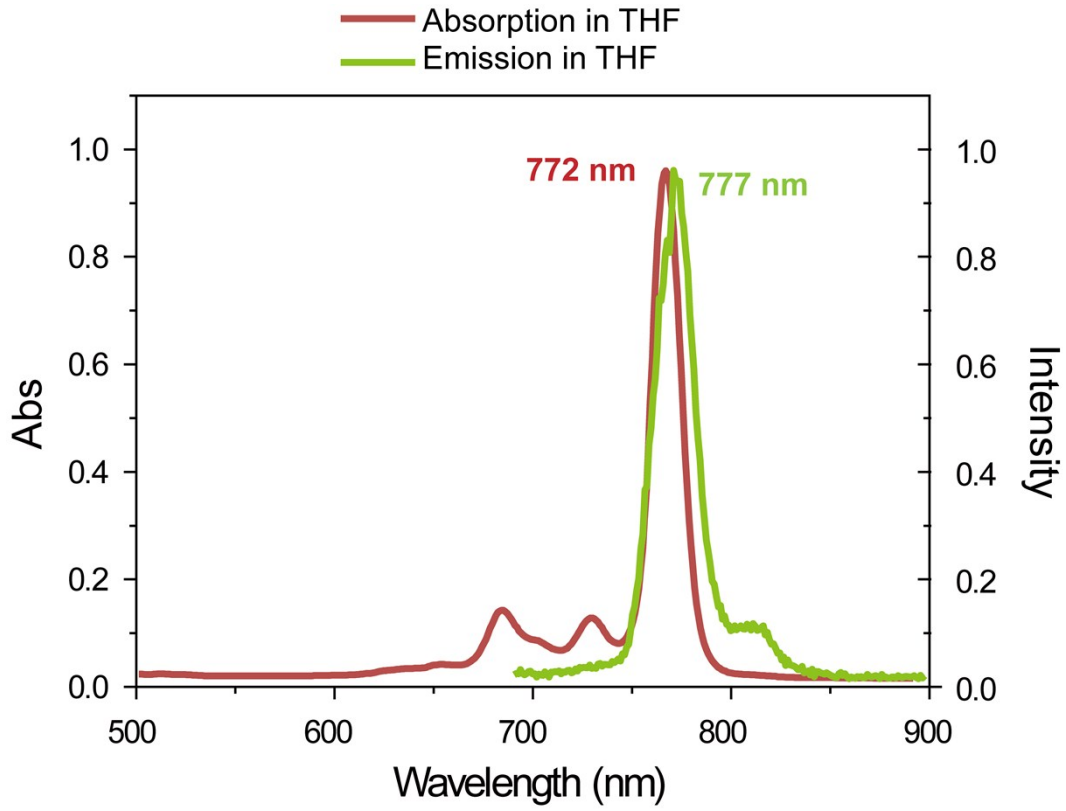

Fig. S3 Spectra characterization of $Br_2SiNc[OSi(n\text{-}C_6H_{13})_3]_2$. Red line represents absorption spectrum, and green line represents emission spectrum.

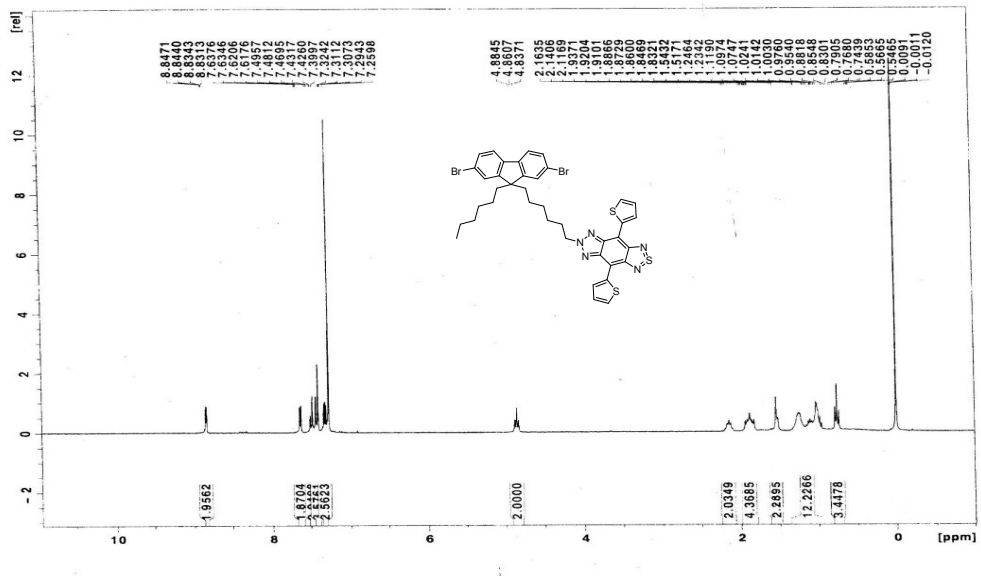

Fig. S4 ^1H NMR of 6-(6-(2,7-dibromo-9-hexyl-9*H*-fluoren-9-yl)hexyl)-4,8- di(thiophen-2-yl)-6,7-dihydro-5*H*-[1,2,3]triazolo[4',5':4,5]benzo[1,2-*c*][1,2,5] thiadiazole

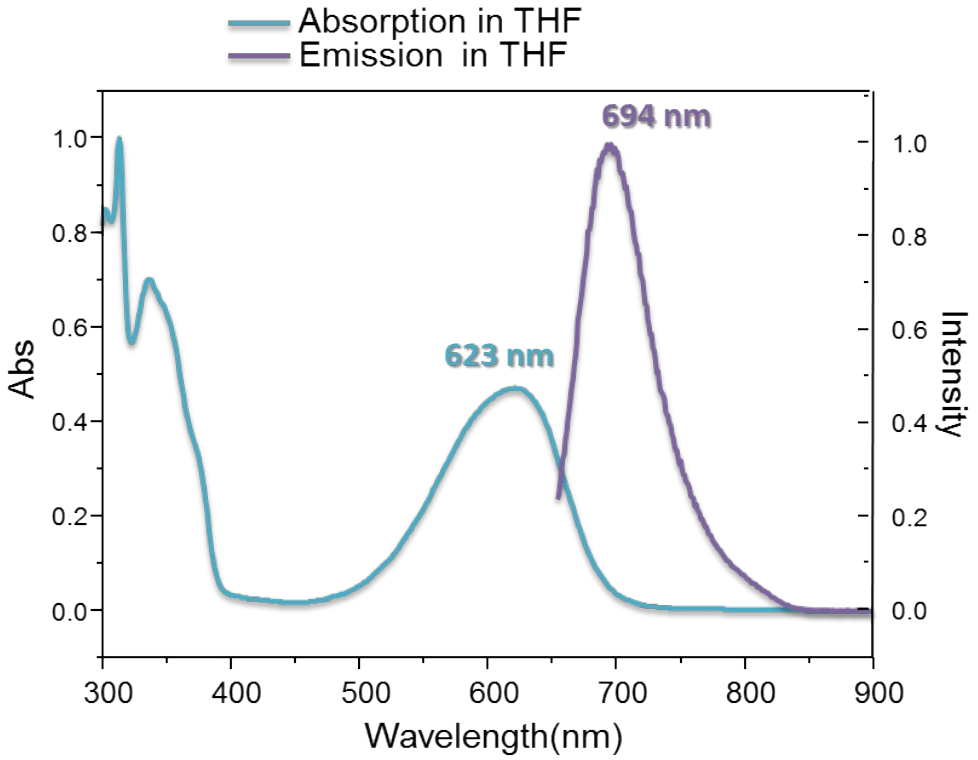

Fig. S5 Absorption spectrum and emission spectrum of 6-(6-(2,7-dibromo-9-hexyl-9*H*-fluoren -9-yl)hexyl)-4,8-di(thiophen-2-yl)-6,7-dihydro-5*H*-[1,2,3]triazolo[4',5':4,5]benzo[1,2-*c*][1,2,5]thiadiazole.

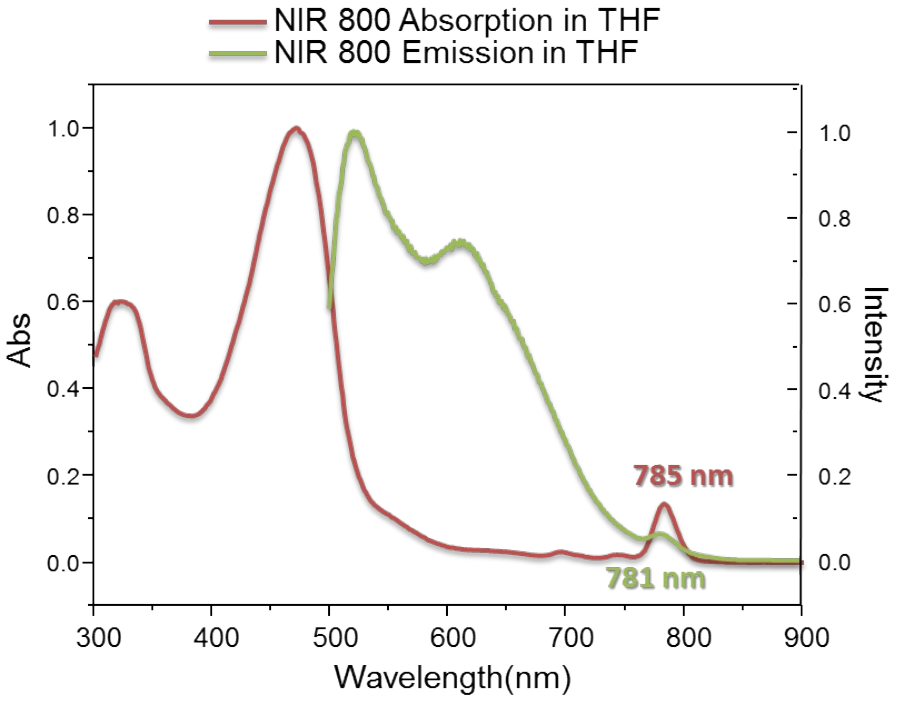

Fig. S6 Absorption and emission spectrum of NIR800 polymer.

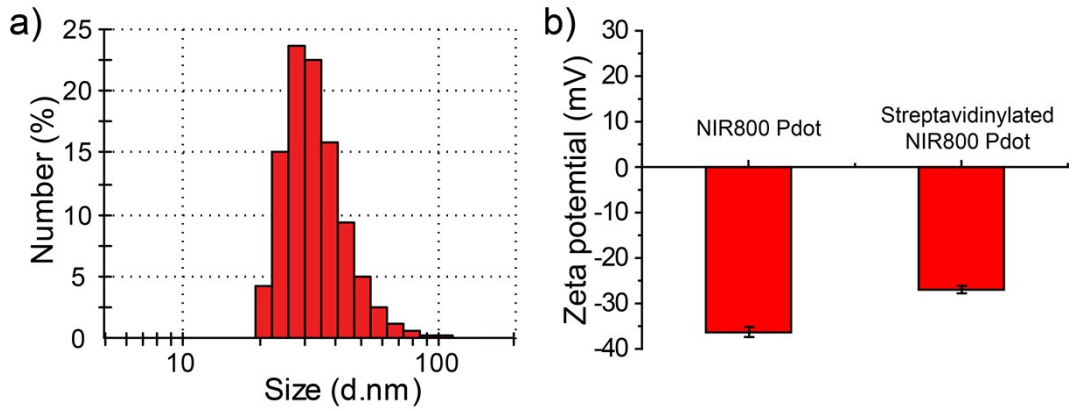

Fig. S7 a) Hydrodynamic diameter of streptavidin-conjugated NIR800 Pdots. b) Zeta potential measurement of NIR800 Pdots and streptavidin-conjugated NIR800 Pdots.

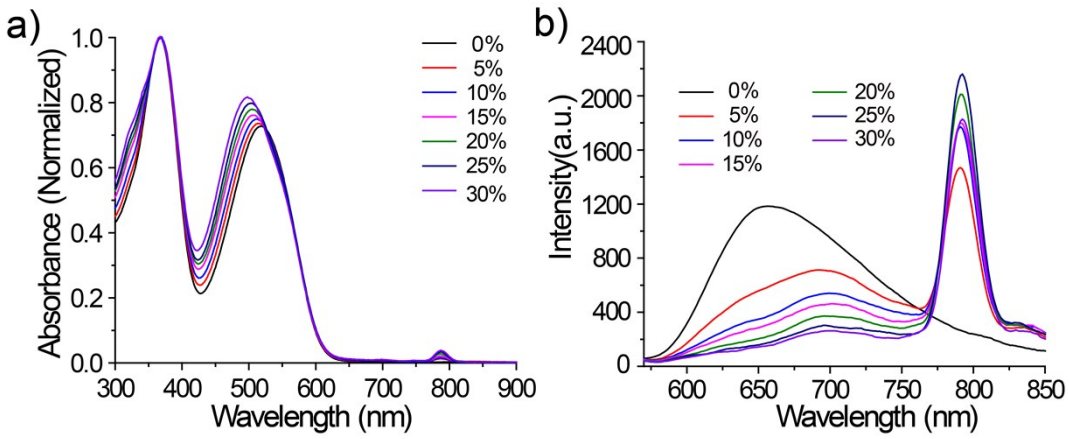

Fig. S8 a) Absorption spectra and b) emission spectra of NIR800 Pdots at variable NIR800-to-PFDHTBT ratios (0 to 30 wt%).

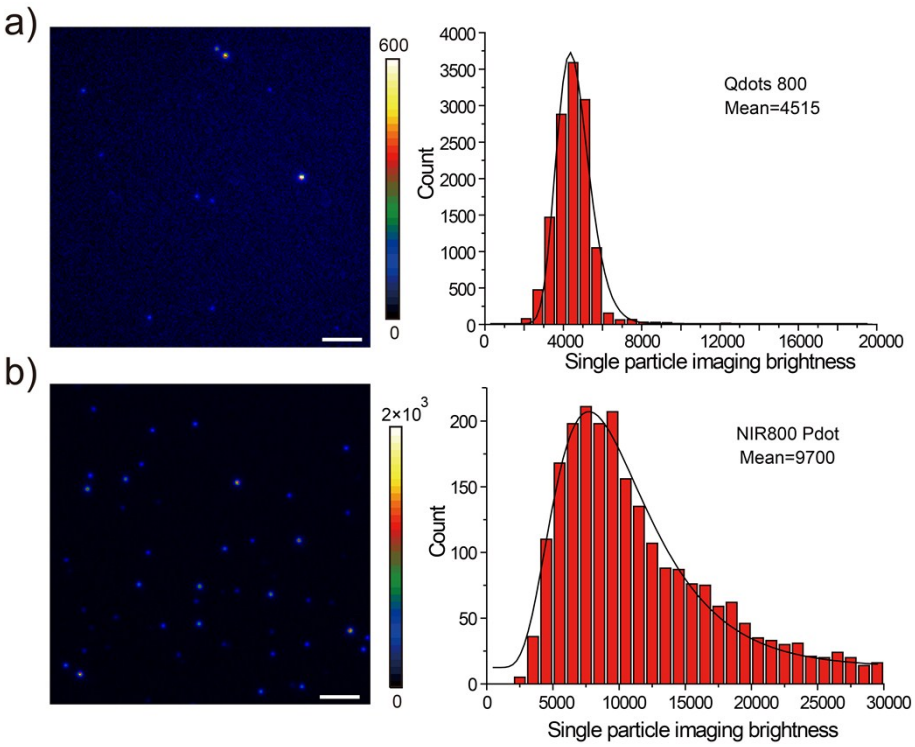

Fig. S9 a) Single-particle imaging of NIR800 Pdots (left) and the corresponding histograms showing the intensity distributions (right). b) Single-particle imaging of Qdot800 (left) and the corresponding histograms showing the intensity distributions (right). The black curves were obtained by fitting a log-normal distribution to the histogram, resulting in mean brightness of 4515 and 9700 counts for Qdots800 and NIR800 Pdots, respectively. Both images were obtained with a 488-nm laser under the same excitation power and identical detection conditions.