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Stable Functionalization of Small Semiconducting Polymer Dots via Covalent Cross-linking and Their Application for Specific Cellular Imaging

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Synthesis of monomer 2,7-dibromo-9,9-bis(3-(tert-butyl hexylcarbamate)fluorene. A mixture of 2, 7-dibromofluorene (5 mmol, 1.62 g), tert-butyl 6-bromohexylcarbamate (11 mmol, 3.05 g), sodium hydroxide solution (40%, 25 mL), Bu_4NBr (1 mmol, 0.32 g), toluene (40 mL) was stirred at 85 °C overnight. The organic phase was separated, washed with water and dried over $MgSO_4$. After evaporation of the solvent, the residue was purified by column chromatography (DCM). ^{1}HNMR (500 MHz, Acetone-*d6*): δ = 7.72 (d, J = 8.5 Hz, 2H), 7.62 (s, 2H), 7.48 (d, J = 9.5 Hz, 2H), 5.74 (s, broad, 2H), 2.88 (q, J = 6.5 Hz, 4H), 2.76 (d, J = 17.5Hz, 2H), 2.02-2.08 (m, 4H), 1.31 (s, 18H), 1.20-1.27 (m, 4H), 1.00-1.09 (m, 8H), 0.50-0.59 (m, 4H). ^{13}CNMR (125 MHz, $CDCl_3$) δ = 155.81, 152.20, 138.97, 130.17, 126.00, 121.43, 121.17, 55.50, 40.36, 40.01, 29.83, 29.41, 28.34, 26.32, 23.49. MS (MALDI-TOF): *m*/*z* Cal. 722.2; Found,

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745.1 ($M + Na^+$). Anal. Calcd (%) for $C_{35}H_{50}Br_2N_2O_4$: C, 58.18; H, 6.97; N, 3.88. Found: C, 58.24; H, 6.92; N, 3.79.

Synthesis of PFBT-3.5%NH_2 polymers. Polymers were synthesized by copolymerization of monomers 2,7-dibromo-9,9-bis(3-(tert-butyl hexylcarbamate)fluorene (**A**), 9,9-dioctylfluorene-2,7-diboronic acid bis(1,3-propanediol) ester (**B**), and 4,7-dibromobenzo[c][1,2,5]thiadiazole (**C**) by Suzuki coupling with different monomer feed ratios. In a 100 mL flask, monomer **A** (0.1 mmol, 71.8 mg), **B** (1 mmol, 558.4 mg) and **C** (0.9 mmol, 264.6 mg) were dissolved in toluene (20 mL), Bu_4NBr (0.04 mmol, 12.5 mg) and Na_2CO_3 (2M, 12 mL) were also added. The mixture was degassed and refilled with N_2 (repeated 4 times) before and after addition of $Pd(PPh_3)_4$ (0.035 mmol, 40 mg). The reactants were stirred at 90°C for 40 hours and phenylboronic acid (100 mg) dissolved in THF (1 mL) was added. After two hours, bromobenzene (1 mL) was added and further stirred for 3 hours. The mixture was poured into methanol (200 mL). The precipitate was filtered, washed with methanol, water, and acetone to remove monomers, small oligomers, and inorganic salts. The crude product was dissolved in DCM (15 mL), filtered through 0.2μm membrane and re-precipitated in methanol (150 mL). The powder was then stirred in acetone (200 mL) for 4 hours and then collected by filtration, and dried in vacuum. Yield: 76%. ^{1}H-NMR (500 MHz, $CDCl_3$): δ = 8.15 - 8.07 (m, 2 H), 8.03 - 7.72 (m, 6 H), 7.68 - 7.38 (m, 5H), 3.03 (m, 4H), 2.18 (m, 4H), 1.59 (m, 8 H), 1.45 (m, 18H), 1.28 - 1.13 (m, 40 H), 0.85-0.83 (m, 6H). In the synthesis, the feed ratio of Monomer A to other monomers was 5%, and the actual molar ratio was 3.5% according to the ^{1}HNMR spectra. The polymer (100 mg) was dissolved in DCM (20 mL) and TFA (1.5 mL) was added to remove protecting groups and generate amine groups. The mixture was stirred at room temperature overnight (in dark) and then washed with 10% NaOH water solution three times. DCM phase was separated, concentrated to ca. 5mL, and then added into methanol (50 mL) to precipitate the final

polymer. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.10 - 8.04 (m, 2 H), 7.97-7.95 (m, 6 H), 7.80 - 7.38 (m, 10H), 3.01 (br, 4H), 2.15 (br, 8 H), 1.39 (br, 4 H), 1.17 - 0.96 (br, 26 H), 0.81 (br, 6H) . ^{13}C NMR (125MHz, $CDCl_3$): δ = 154.60, 152.01, 141.12, 136.70, 133.83, 128.55, 128.21, 124.25, 120.29, 55.66, 40.46, 32.06, 30.34, 29.49, 28.59, 24.28, 22.84, 14.30. Molecular weight was measured by GPC as M_n = 12390, M_w = 23072, PDI = 1.86.

Synthesis of PFO-5%NH_2 polymers. In a 50 mL flask, 2,7-dibromo-9,9-bis(3-(tert-butyl hexylcarbamate)fluorene (0.1 mmol, 72.2 mg), 9,9-dioctylfluorene-2,7-dibromofluorene (0.4 mmol, 219 mg) and 9,9-dioctylfluorene-2,7-diboronic acid bis(1,3-propanediol) ester (0.5 mmol, 279.2 mg) were dissolved in toluene (20 mL), Bu_4NBr (0.02 mmol, 6.3 mg) and Na_2CO_3 (2M, 7 mL) was added. The mixture was degassed and refilled with N_2 (repeated 4 times) before and after addition of $Pd(PPh_3)_4$ (0.02 mmol, 23 mg). The reactants were stirred at 90°C for 40 hours and phenylboronic acid (60 mg) dissolved in THF (0.6 mL) was added. After two hours, bromobenzene (0.6 mL) was added and further stirred for 3 hours. The mixture was poured into methanol (150 mL). The precipitate was filtered, washed with methanol, water, and acetone to remove monomers, small oligomers, and inorganic salts. The crude product was dissolved in DCM (10 mL), filtered through 0.2 μm membrane and re-precipitated in methanol (150 mL). The powder was then stirred in acetone (150 mL) for 4 hours and then collected by filtration, and dried in vacuum. Yield: 72%. ^{1}HNMR (500 MHz, $CDCl_3$): δ = 7.89 - 7.62 (m, 6 H), 7.54 - 7.39 (m, 5H), 3.03 (m, 4H), 2.18 (m, 4H), 1.59 (m, 8 H), 1.45 (m, 18H), 1.28-1.13 (m, 40 H), 0.85 - 0.83 (m, 6H). The polymer (100 mg) was dissolved in DCM (15 mL) and TFA (2 mL) was added to generate amine groups. The mixture was stirred at room temperature overnight (in dark) and then washed with 10% NaOH water solution two times. DCM phase was separated, concentrated to ca. 5mL, and then added into methanol (50 mL) to precipitate the final polymer. ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.85 (m, 2H), 7.68 (m, 4H), 7.51 - 7.39 (m, 10H), 3.02 (br, 4H), 2.14 (br, 8H),

1.16 (br, 26H), 0.83 (br, 6H). ^{13}C NMR (125MHz, $CDCl_3$): δ = 152.24, 140.93, 140.46, 126.59, 122.13, 120.70, 55.76, 41.06, 32.21, 30.64, 29.64, 24.33, 23.02, 14.49. Molecular weight was measured by GPC as M_n = 7763, M_w = 11048, PDI = 1.423.

Bioconjugation. We performed bionconjugation by utilizing the EDC-catalyzed reaction between carboxyl groups on Pdot surface and amine groups on biomolecules. In a typical bioconjugation reaction, 80 μL of polyethylene glycol (5% w/v PEG, MW 3350) and 80 μL of concentrated HEPES buffer (1 M) were added to 4 mL of functionalized Pdot solution (50 μg/mL in MilliQ water), resulting in a Pdot solution in 20 mM HEPES buffer with a pH of 7.4. Then, 240 μL of streptavidin (purchased from Invitrogen (Eugene, OR, USA)) was added to the solution and mixed well on a vortex. 80 μL of freshly-prepared EDC solution (5 mg/mL in MilliQ water) was added to the solution, and the above mixture was left on a rotary shaker. After 3 h at room temperature, BSA (2% (w/v), 20 μL) and Triton-X 100 (0.25% (w/v), 20 μL) were added and stirred for 30 mins. The mixture was then left on rotary shaker for one hour. Finally, the resulting Pdot bioconjugates were separated from free biomolecules by gel filtration using Sephacryl HR-300 gel media.

Cell culture. The breast cancer cell line MCF-7 and the cervical cancer cell line HeLa were ordered from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured at 37 °C, 5% CO_2 in Eagles minimum essential medium supplemented with 10% Fetal Bovine Serum (FBS), 50 U/mL penicillin, and 50 μg/mL streptomycin. The cells were cultured prior to experiments until confluence was reached. The cells were harvested from the culture flask by briefly rinsing with culture media followed by incubation with 5 mL of Trypsin-EDTA solution (0.25 w/v % Trypsin, 0.53 mM EDTA) at 37°C for 5-15 minutes. After complete detachment, the cells were rinsed, centrifuged, and resuspended in 1 × PBS buffer. The cell concentration was determined by microscopy using a hemocytometer.

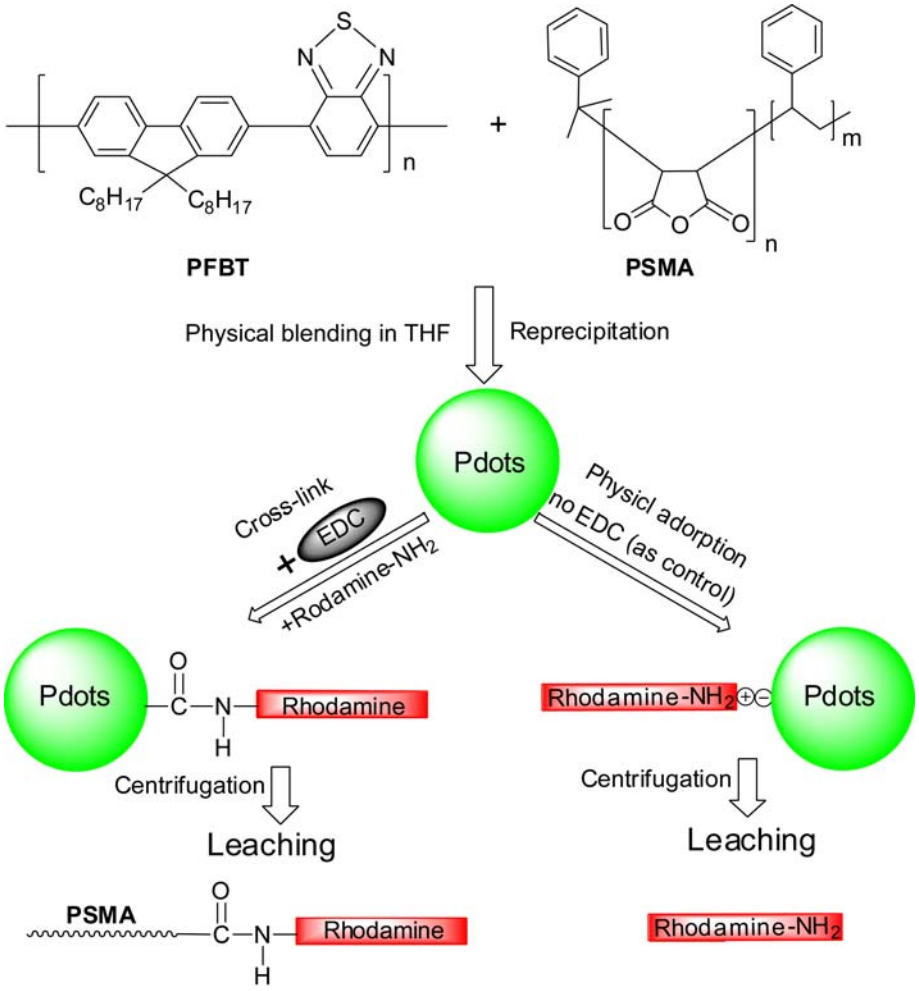
Specific labeling for flow cytometry. For specific cell labeling with cross-linked Pdot-streptavidin (Pdot-SA), a million cells were blocked with BlockAid blocking buffer (Invitrogen, Eugene, OR, USA) and then were incubated sequentially with biotinylated primary anti-EpCAM antibody (used to label the cell-surface EpCAM receptors on MCF-7 cells) and 20 μg/mL (based on Pdots) cross-linked Pdot-SA for 30 minutes each, followed by two washing steps using labeling buffer. Finally, the specifically labeled cells were fixed in 0.6 mL 4% (v/v) paraformaldehyde solution. For the control labeling, no biotinylated primary anti-EpCAM antibody was added. Flow cytometry was operated on a BD FACS Canto flow cytometer (BD Biosciences, San Jose, CA, USA). A 488 nm laser was used for excitation and emission was collected through FITC channel equipped with a 500 nm long-pass filter and a 530/30 nm band-pass filter. Data was analyzed using the FACSDiva1 software.

Specific labeling for cellular surface imaging. For labeling cellular surface with cross-linked Pdot-SA conjugates, live MCF-7 cells were plated on a 22×22-mm glass coverslip and cultured until the density reach 60-70% confluence. The cells were fixed with 4% paraformaldehyde for 15 minutes and were blocked with BlockAid blocking buffer for 30 mins. Then the MCF-7 cells were incubated sequentially with biotinylated primary anti-EpCAM antibody (were used to label the cell-surface EpCAM receptors on MCF-7 cells) and 5 μg/mL cross-linked Pdot-SA for 40 minutes each, followed by two washing steps after each incubation. For the control, no biotinylated primary anti-EpCAM antibody was added. The cross-linked Pdot-tagged cells were then counterstained with Hoechst 34580 and imaged immediately on a fluorescence confocal microscope (Zeiss LSM 510).

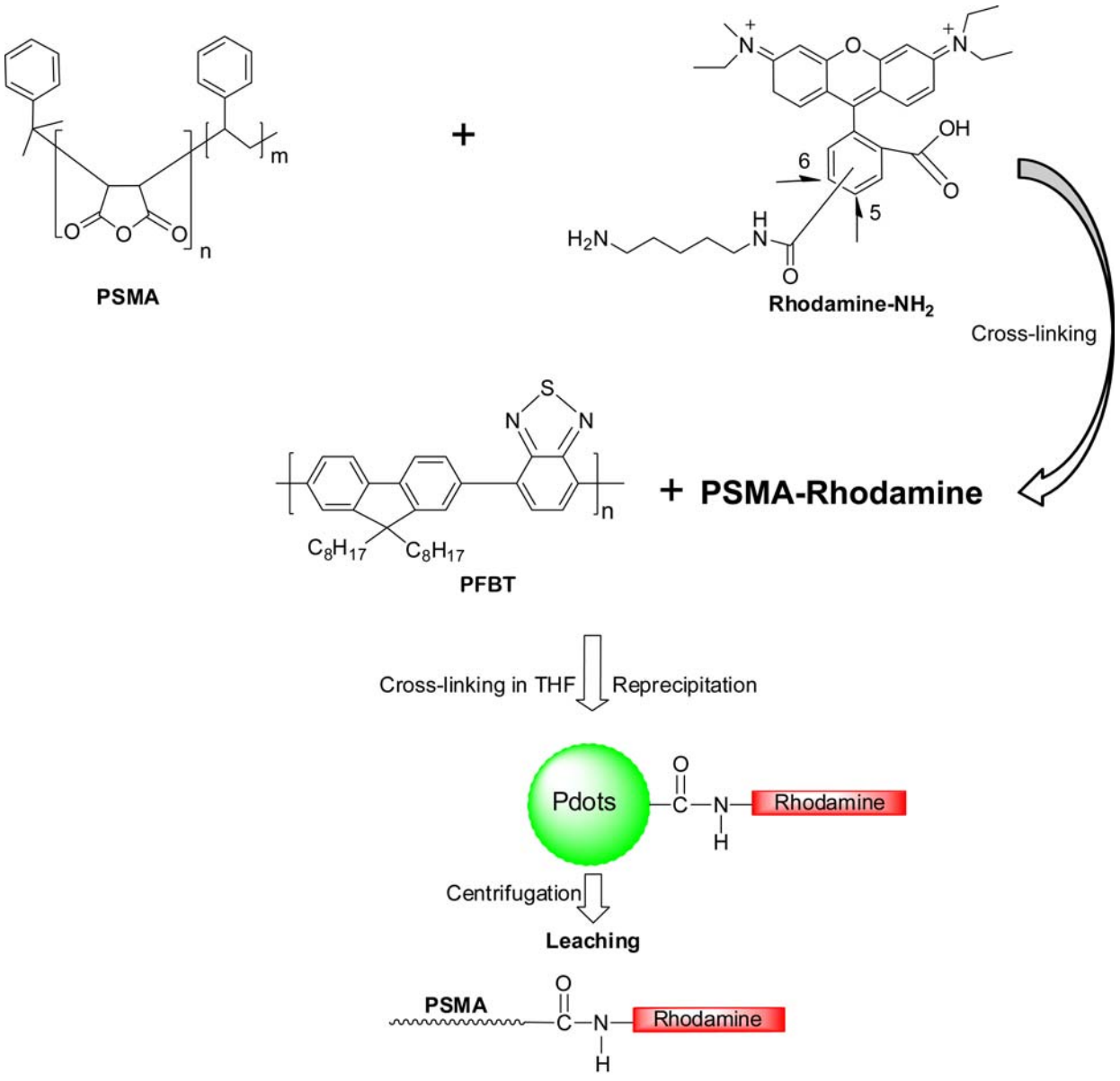
Specific labeling for subcellular imaging. For subcellular microtubule-labeling with cross-linked Pdot-SA conjugates, live HeLa cells were plated on a 22×22-mm glass coverslip and cultured until the density reach 60-70% confluence. The cells were fixed with 4%

paraformaldehyde for 15 minutes and permeabilized with 0.25% Triton-X 100 in PBS for 15 minutes, and blocked with BlockAidTM blocking buffer for 30 mins. The fixed and blocked HeLa cells were incubated sequentially with 5 μg/mL biotinylated monoclonal anti-α-tubulin antibody (Biolegend, San Diego, CA, USA) and 5 μg/mL cross-linked Pdot-SA conjugates for 40 minutes each and followed by two washing seteps after each incubation. For the control, no biotinylated monoclonal anti-α-tubulin antibody was added. The Pdot-tagged cells were then counterstained with Hoechst 34580 and imaged immediately on a fluorescence confocal microscope (Zeiss LSM 510).

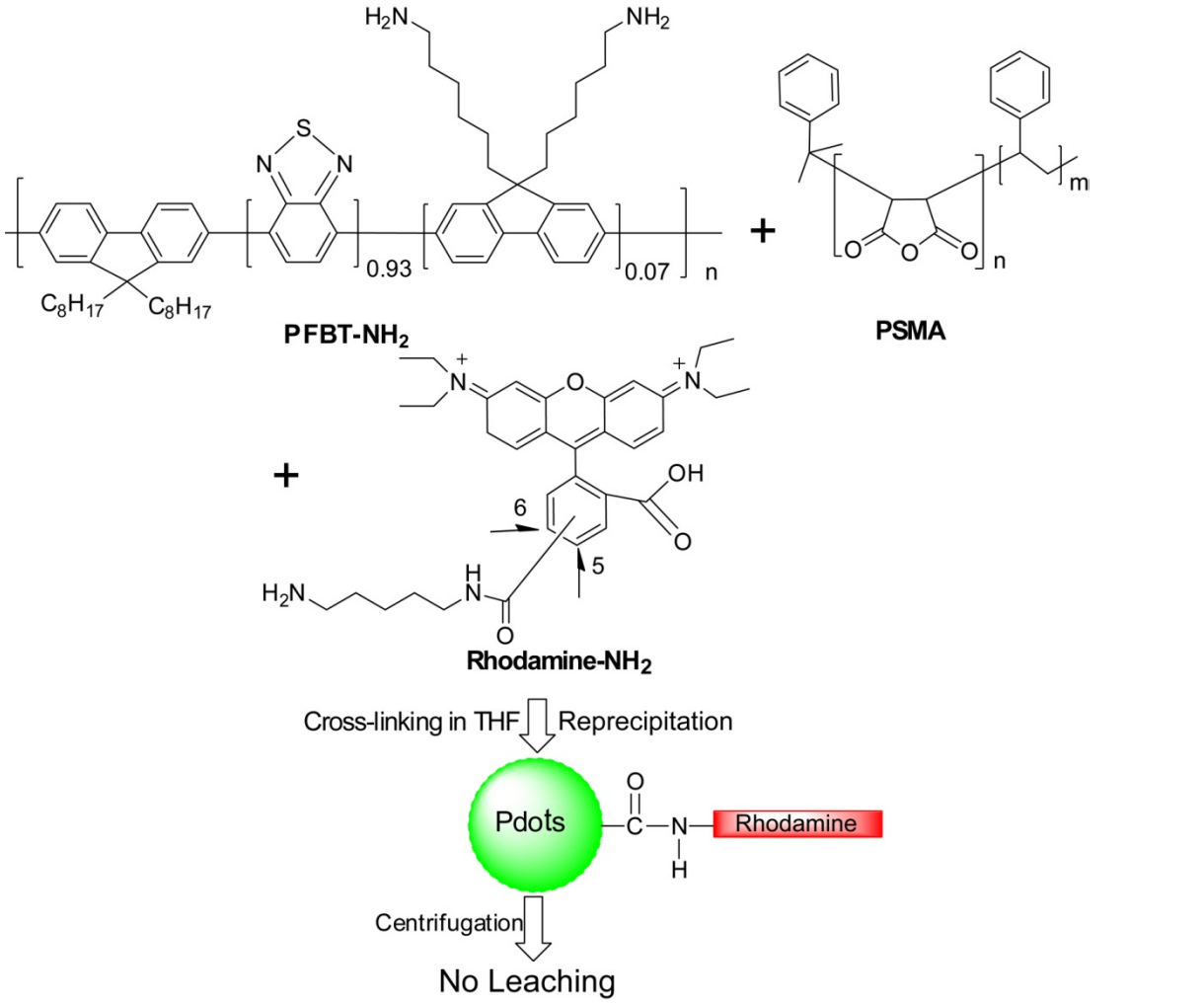
Cellular surface/subcellular imaging. The fluorescence images of MCF-7 cells (for cellular surface imaging) and HeLa cells (for subcellular imaging) were acquired with a fluorescence confocal microscope (Zeiss LSM 510). Blue channel, green channel emission and bright field image were simultaneously obtained. The blue channel was excited by 405 nm diode laser, while the green channel was excited by 488 nm Argon laser. An EC Plan-Neofluar 40×/1.30 Oil DIC and a Plan-Apochromat 63×/1.40 Oil DIC objective lens were utilized for cellular surface imaging and subcellular imaging, respectively.



Scheme S1. Scheme of the dye-leaching method for examining the stability of PSMA functionalized Pdots formed by the co-condensation method.



Scheme S2. Scheme of the dye-leaching method for examining the stability of PSMA functionalized Pdots formed by the co-condensation method: Rhodamine-NH_2 was covalently linked to PSMA first, and then the PSMA-NH-Rhodamine was physically blended with PFBT polymer to form Pdots.



Scheme S3. Scheme of the dye-leaching method for examining the stability of the cross-linked Pdots where PSMA and PFBT-NH_2 were covalently cross-linked together to form stable functionalized Pdots that showed no dye leaching.

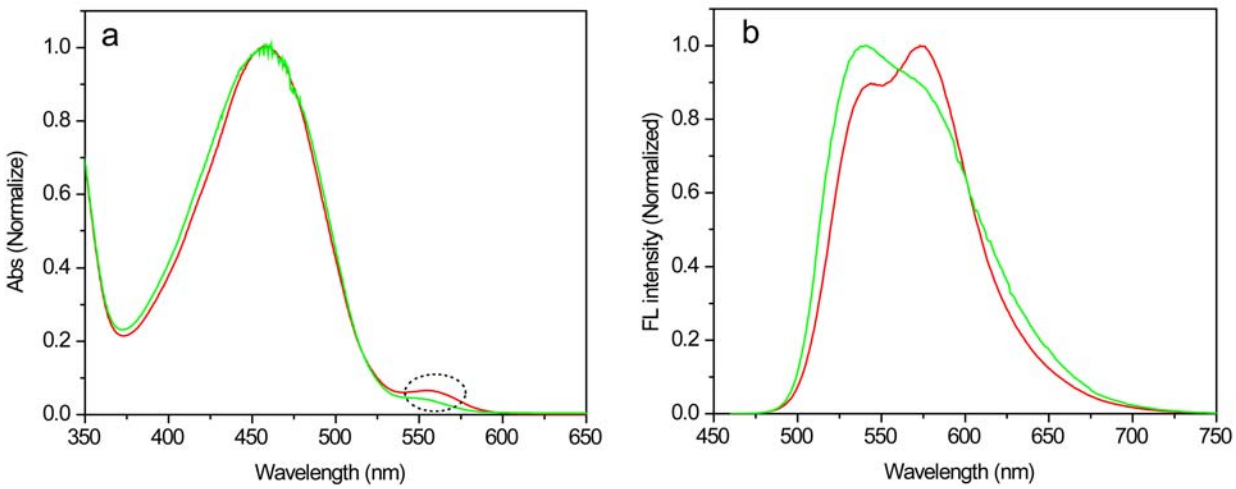


Figure S1. The absorption (a) and fluorescence (b) spectra of Pdot-Rhodamine nanoparticles functionalized by physical-blending and covalent cross-linking methods (shown in Scheme S2). The circled regions indicate the absorption peak of Rhodamine, which clearly showed significant dye leaching in physically blended PSMA Pdots but not in cross-linked Pdots.

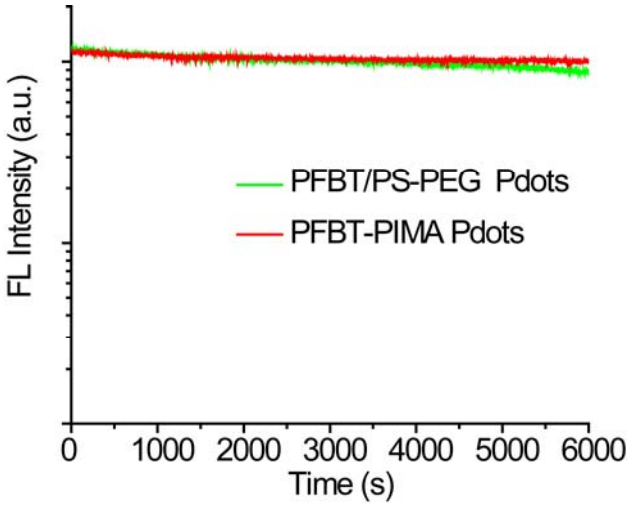


Figure S2. The intensity trajectories of aqueous suspensions of PFBT/PS-PEG Pdots and PFBT-NH-PIMA Pdots.

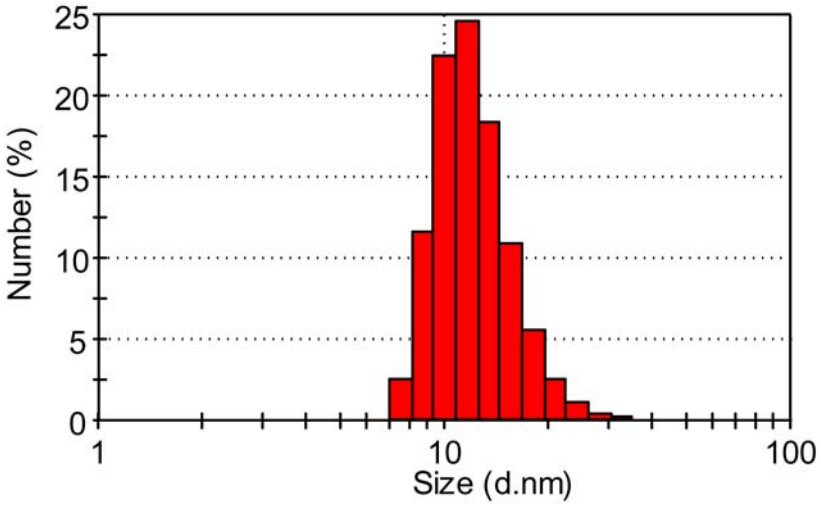


Figure S3. Histogram of the particle size of the PFBT-NH-PSMA Pdots measured by DLS (average diameter ~ 11 nm).

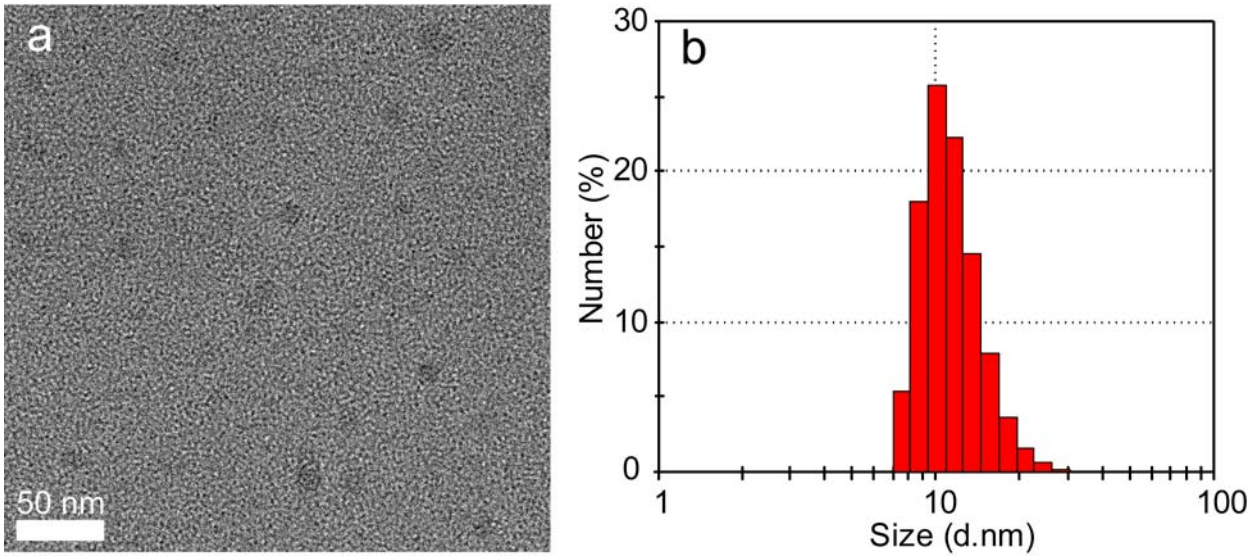


Figure S4. (a) Typical TEM image of cross-linking PFO-NH-PIMA Pdots. Scale bar: 50 nm. (b) Histogram of the particle size of the PFBT-NH-PIMA Pdots measured by DLS (average diameter ~ 10.0 nm).

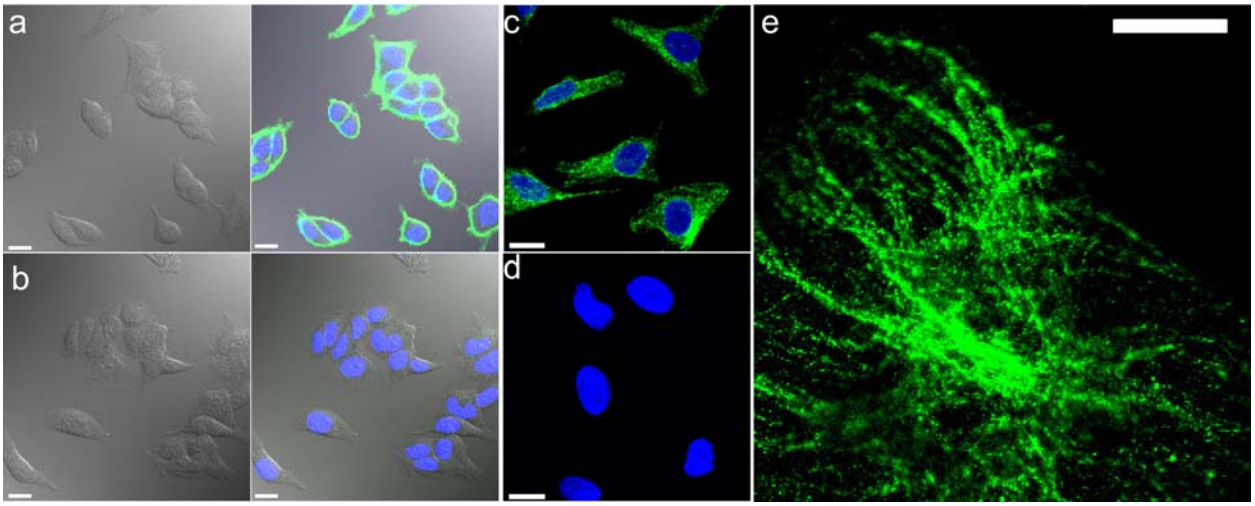


Figure S5. Specific cellular imaging using cross-linked PFBT-NH-PMA Pdots. The blue channel was from the fluorescence of the nucleus stain Hoechst 34580, while the green channel was from emission from PFBT-NH-PIMA Pdots. **(a, b)** Confocal fluorescence microscopy images of cellular surface labeling using Pdot streptavidin. Panel a shows positive labeling and panel b shows negative labeling performed under the same condition but in the absence of biotinylated primary antibody. Images from left to right: Nomarski (DIC) images; combined DIC and fluorescence images. All cells used were MCF-7 breast-cancer cells. **(c, d, e)** Confocal fluorescence microscopy images of microtubules in HeLa cells labeled with Pdot-streptavidin. The combined fluorescence images for the positive labeling (c) and the negative labeling (d) under the same condition but in the absence of biotinylated anti-α-tubulin. The green fluorescence image from Pdot labeled microtubules (e). All the scale bars represent 20 μm.

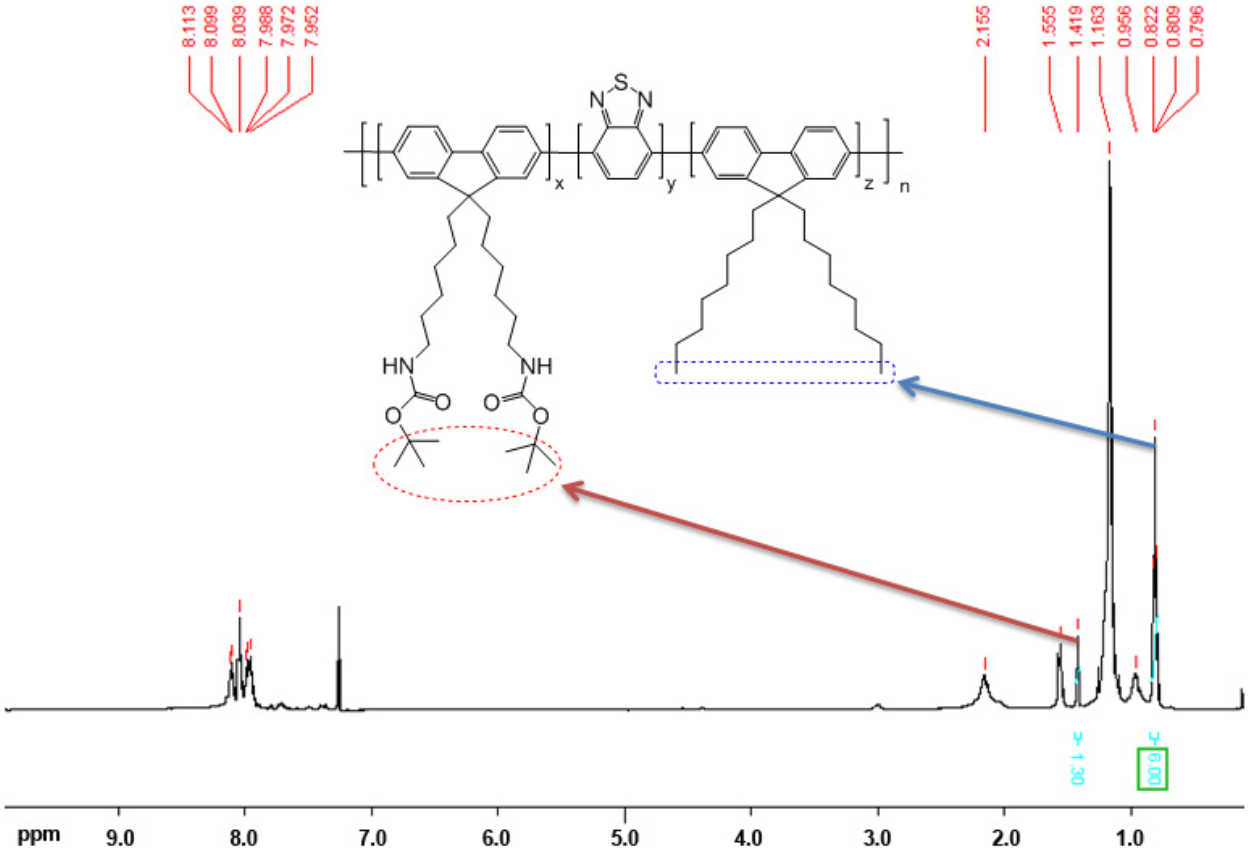


Figure S6. ^{1}H-NMR spectra of PFBT-NH_2 polymer.

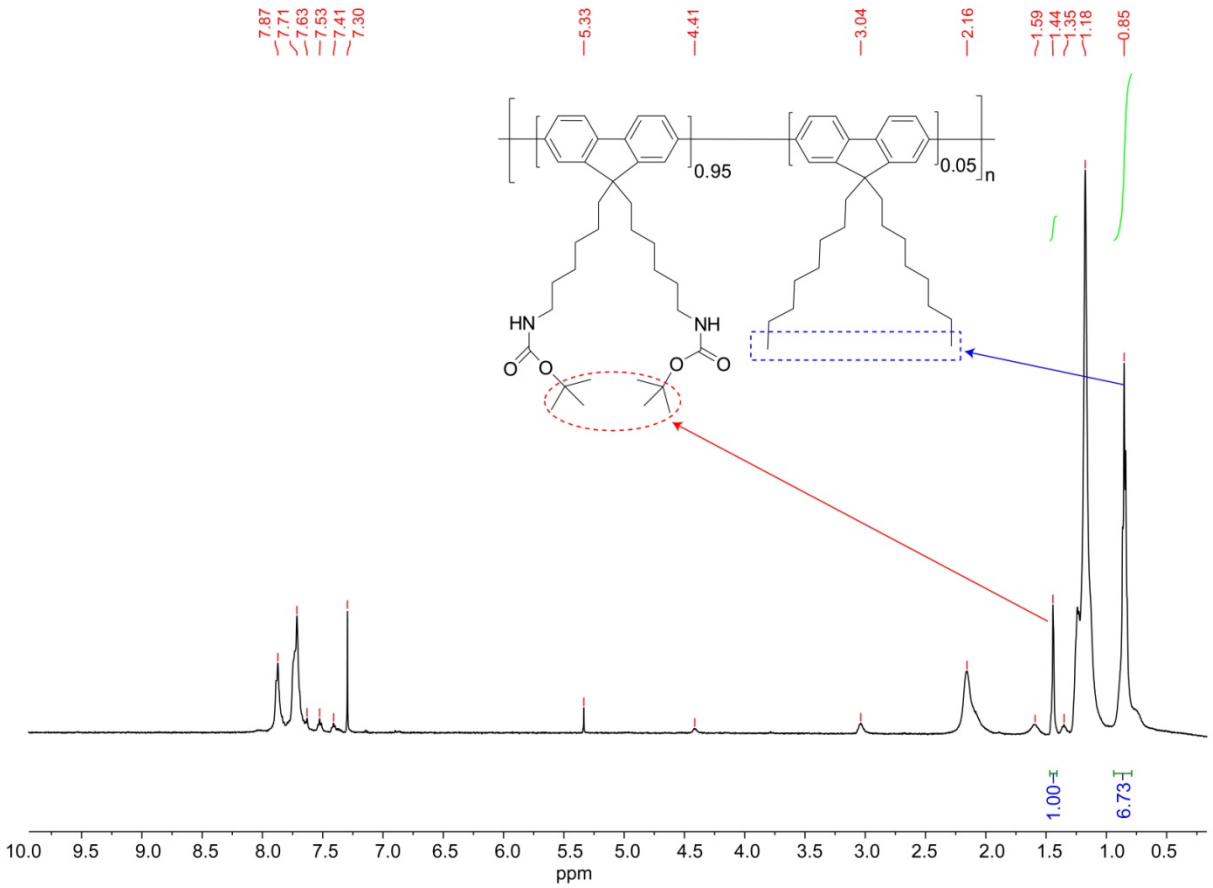


Figure S7. ^{1}H-NMR spectra of PFO-NH_2 polymer.