Far-Red/Near-Infrared Conjugated Polymer Nanoparticles for Long-Term In Situ Monitoring of Liver Tumor Growth

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The design and synthesis is reported for a fluorescent conjugated polymer (CP), poly{4,4,9,9-tetrakis(4-(octyloxy)phenyl)-4,9-dihydro-indaceno[1,2-b:5,6-b’]dithiophene)-alt-co-[4,7-di(thiophen-2-yl)-2,1,3-benzothiadiazole]} (PIDT-DBT), with absorption and emission profiles fallen within far-red/near infrared (FR/NIR) region and further demonstrate its application in long-term in vitro cell tracing and in vivo imaging of liver tumor growth. PIDT-DBT-Tat nanoparticles (NPs) have an absorption maximum at \( \approx 600 \) nm with an emission maximum at \( \approx 720 \) nm in water. In vitro cell tracing studies reveal that PIDT-DBT-Tat NPs can trace HepG2 liver cancer cells over 8 d. In vivo imaging results indicate that PIDT-DBT-Tat NPs can monitor liver tumor growth for more than 27 d in a real-time manner. Both in vitro and in vivo studies demonstrate that PIDT-DBT-Tat NPs are superior to commercial Qtracker 705 as fluorescent probes. This study demonstrates for the first time the feasibility for long-term in vivo imaging of tumor growth by utilizing CP-based fluorescent probes, which will encourage the development of NIR fluorescent CPs for in vivo bioimaging.

1. Introduction

Cancer is one of the major causes of mortality in the world and the incidence of cancer continues to increase. Effective real-time monitoring of tumor growth is critical to the success of cancer therapy and improvement of patient outcomes.[1–3] However, evaluating tumor growth typically relies on the gross examination of tumor size through physical examination.[4] This approach severely limits the use of clinically relevant orthotopic tumor models and gives little insight in the tumor processes being affected by the treatment. Therefore, development of techniques with ability to noninvasively image tumor growth processes would be beneficial in the evaluation of potential anti-tumor strategies. In vivo imaging technologies have emerged as indispensable tools in cancer research, clinical trials and medical practice, due to their abilities to visualize the location of tumors and assess their biological processes.[5] Among in vivo studies, long-term in situ imaging of tumors is of vital significance in precise diagnosis of cancer, image-guided surgery, and monitoring of the treatment process.[6] Despite the various available bioimaging techniques, such as magnetic resonance imaging,[7] positron emission tomography,[8] single photon emission computing tomography,[9] radiography,[10] and ultrasound,[11] fluorescence imaging with easier maneuverability is an unique nonionizing technique in providing high sensitivity and spatiotemporal resolution.[12,13]

To efficiently realize long-term in situ in vivo fluorescence imaging of tumors, bright and stable near infrared (NIR) (650–900 nm) fluorescent probes are preferentially employed to accumulate within the site of interest through passive (such as enhanced permeability and retention effect) or active (binding to a receptor on the cell surface) targeting strategies.[14,15] Among them, organic fluorophores have been historically applied in vivo applications due to their commercial availability and easy functionalization.[16] However, their use for long-term or real-time in vivo imaging is hampered by their intrinsic drawbacks such as small Stokes shifts and poor photostability.[17] As compared to discrete organic fluorophores, fluorescent nanoparticles (NPs) have advantages in prolonged intracellular retention,[18] which is beneficial for long-term studies. To date, several types of fluorescent NPs (noble metal NPs,[19] upconversion NPs,[20] and hybrid NPs[21]) have been employed for highly sensitive optical imaging of cancer at both cellular and animal levels. However, inorganic nanoparticles face the difficulties in biodegradability and some also show obvious toxicity.[22–24] Fluorescent NPs with great biocompatibility and stable fluorescence in biological environments are preferential for long-term in vivo imaging applications.

Fluorescent conjugated polymers (CPs) based NPs have recently received great attention in bioimaging applications.[25–27] They have been used for targeted in vitro/in vivo cellular imaging, intracellular biomolecule imaging, and in vivo small molecule imaging due to their high brightness, good photostability, low cytotoxicity as well as readily tailored optical properties. Despite of their success in bioimaging, CP NPs have not been used for real-time monitoring of tumor growth, largely due to the lack of highly emissive CPs with suitable absorption and emission profiles. The currently available CP
based far-red (FR)/NIR fluorescent NPs are mainly synthesized through chemical incorporation of narrow-band-gap moieties into CP backbones or physical blending of CP and FR/NIR fluorescent acceptors (e.g., organic dyes, quantum dots (QDs), and CPs). In these cases, the polymer absorption is strongly dependent on the donor component and the short wavelength is not suitable for in vivo bioimaging. Very recently, we have successfully shifted the absorption maxima of highly emissive FR/NIR fluorescent CP NPs to ≈488 and ≈530 nm, by simultaneous incorporation of two narrow-band-gap moieties into CP backbone to realize efficient intra- and intermolecular energy transfer.

As part of our continuous efforts in developing bright FR/NIR CP NPs, in this contribution, we report the design and synthesis of poly[4,4,9,9-tetrakis(4-(octyloxy)phenyl)-4,9-dihydro-s-indaceno[1,2-b:5,6-b′]dithiophene]-alt-co-[4,7-di(thiophen-2-yl)-2,1,3-benzothiadiazole] (PIDT-DBT). The polymer design takes into consideration that alternating strong electron-rich (IDT) and electron-deficient (DBT) units could lead to a strong intramolecular charge transfer band at long wavelength. In addition, the four 4-(octyloxy)phenyl substitutions could minimize π stacking between conjugated backbones to favor high fluorescence in NPs. We further fabricated the CP NPs with cell penetrating peptide, human immunodeficiency virus type 1 (HIV-1) trans-activating transcriptional activator (Tat), on the surface (PIDT-DBT-Tat) and demonstrated their application in long-term in vivo monitoring of liver tumor growth. In vitro cell tracing studies revealed that PIDT-DBT-Tat NPs can trace HepG2 liver cancer cells over 8 d. The NP-labeled cells were further transplanted into the nude mice liver to monitor the liver tumor growth, which showed that the fluorescent signals can be clearly detected for 27 d. Both in vitro and in vivo studies reveal that PIDT-DBT-Tat NPs are superior to commercial Qtracker 705 as fluorescent probes. This study demonstrates for the first time the feasibility of using CP NPs for real-time long-term in vivo monitoring of tumor growth.

### 2. Results and Discussion

#### 2.1. Synthesis and Characterization

Scheme 1a shows the chemical structure of PIDT-DBT. The synthetic route is shown in Scheme S1, Supporting Information. In brief, 2,5-dibromo-p-xylene was oxidized using KMnO$_4$ as an oxidant to give dibromoterephthalic acid 1, which was treated in absolute ethanol under acidic condition to form diethyl 2,5-dibromoterephalate 2. Stille coupling reaction between 2 and 2-tributylstannyl thiophene was performed to afford diethyl 2,5-di(thien-2-yl)terephthalate 3, which was first reacted with 4-octyloxyphenyllithium salt at −78 °C, and then treated under acidic condition to afford 4,4,9,9-tetrakis(4-(octyloxy)phenyl)-4,9-dihydro-s-indaceno[1,2-b:5,6-b′]dithiophene 4. 2,7-Dibromo-4,4,9,9-tetrakis(4-octyloxyphenyl)-4,9-dihydro-s-indaceno[1,2-b:5,6-b′]dithiophene 5 was obtained through bromination of 4. 4,7-Bis(5-(trimethylstannyl)thiophen-2-yl)-2,1,3-benzothiadiazole 6 was synthesized according to previous report. Stille polymerization between 5 and 6 was carried out to give PIDT-DBT with careful purification. The chemical structures of all the intermediates and PIDT-DBT were verified by nuclear magnetic resonance (NMR) spectra. The number average molecular weight and polydispersity index of PIDT-DBT are 18 700 and 1.50, respectively, determined by gel permeation chromatography (GPC) using tetrahydrofuran (THF) as eluent and polystyrene as standard. PIDT-DBT has good solubility in common organic solvents, e.g., toluene, dichloromethane, and tetrahydrofuran.
2.2. Fabrication and Characterization of NPs

Scheme 1b shows the schematic illustration of the fabrication of PIDT-DBT-Tat NPs. The NPs were fabricated by a modified nanoprecipitation method.[31] More specifically, 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG$_{2000}$) and its maleimide modified derivative DSPE-PEG$_{2000}$-Mal were chosen as matrix. Upon addition of a well dissolved DSPE-PEG$_{2000}$/DSPE-PEG$_{2000}$-Mal/PIDT-DBT (1/1/1) mixture in THF solution into tenfold Milli-Q water under continuous ultrasonication, the hydrophobic PIDT-DBT and DSPE components are likely to be embedded into the particle core while the hydrophilic PEG and PEG-Mal segments are extended outside into the aqueous environments to render the NPs with abundant maleimide groups on the surface. The PIDT-DBT-Mal NPs were then conjugated with cell penetrating peptide HIV-1 Tat (RKKRRQRRRC) to afford PIDT-DBT-Tat NPs via click coupling reaction between the maleimide groups on NP surface and thiol groups of peptides at the C-terminus. The obtained nanoparticles were kept in refrigerator at 4 °C, and no obvious suspensions were observed for 2 months.

The volume average hydrodynamic diameters of PIDT-DBT-Tat NPs were studied by laser light scattering (LLS), revealing that PIDT-DBT-Tat NPs have a volume average hydrodynamic diameter of $\approx$56 nm with a narrow size distribution (Scheme 1c). The size and morphology of PIDT-DBT-Tat NPs were further studied by transmission electron microscopy (TEM). As shown in Scheme 1d, the NPs are clearly distinguished with a spherical shape, which have a mean diameter of $\approx$49 nm. The slightly smaller size relative to that revealed by LLS is due to shrinkage of polymeric NPs in dry state.[32] In addition, the hydrodynamic diameter of PIDT-DBT-Tat NPs does not show any obvious change even after incubation at 37 °C in phosphate-buffered saline (PBS, pH = 7.4) solution for 14 d, suggesting that PIDT-DBT-Tat NPs have excellent colloidal stability (Figure S1, Supporting Information). Scheme 1e shows the UV–vis absorption and photoluminescence (PL) spectra of PIDT-DBT-Tat NPs in water. The NPs have an absorption peak at $\approx$600 nm with an emission maximum at $\approx$720 nm, which gives rise to a remarkably large Stoke shift of $\approx$120 nm, endowing the NPs with great potentials to minimize background interference for in vivo bio-imaging application. The absorption spectrum also has a long tail extending over 750 nm, allowing excitation in the FR/NIR region, which is a great advantage for in vivo imaging.

2.3. In Vitro Cell Tracing

To evaluate the performance of PIDT-DBT-Tat NPs as a fluorescent probe, we first studied their in vitro cell tracing ability. HepG2 liver cancer cells were chosen as a model cell line and Qtracker 705 Labeling Kit was used as the benchmark due to its matched emission maximum with PIDT-DBT-Tat NPs. For in vitro cell tracing studies, the HepG2 cancer cells were first incubated with $2 \times 10^{-9}$m PIDT-DBT-Tat NPs and Qtracker 705 at 37 °C for 24 h. The labeled cells were then subcultured in the absence of the probe for designated days and the fluorescence profiles were studied using flow cytometry by counting 10,000 events ($\lambda_{ex} = 561$ nm, 710/50 nm bandpass filter). As shown in Figure 1a, the efficient labeling rate of HepG2 cells upon incubation with PIDT-DBT-Tat NPs remains $\approx$100% till day 2 as compared to the untreated cells. The labeling rate drops to 97.3% and 78.1% after continuous culture for 3 and 4 d, respectively. After 5 d, 53.1% of the cells are still efficiently labeled, while the labeling rate is 23.5% at day 7. On the contrary, only 75.9% and 43.4% of Qtracker 705-treated cells are labeled, while the labeling rate further drops to 18.1% and 4.6% at day 4 and day 6 (Figure 1b), respectively. Compared to Qtracker 705, these results suggest that PIDT-DBT-Tat NPs are more suitable for long-term cell tracing studies with higher fluorescence intensity and longer tracing period.

The efficient labeling of HepG2 cells after incubation with PIDT-DBT-Tat NPs was further confirmed by confocal laser scanning microscopy (CLSM). As shown in Figure 2a, nearly all the cells in suspension show intense fluorescence after incubation with $2 \times 10^{-9}$m PIDT-DBT-Tat NPs for 24 h (day 0 sample for flow cytometry studies). On the contrary, upon incubation with $2 \times 10^{-9}$m Qtracker 705 (Figure 2b), some cells show very dim fluorescence signal and the average intensity is weaker as compared to that incubated with PIDT-DBT-Tat NPs. The 3D
color-coded projection of PIDT-DBT-Tat NP-labeled cells was also obtained, revealing that the NPs are mainly distributed in cytoplasm (Figure S2, Supporting Information). In addition, we also studied the confocal imaging of cells incubated with $2 \times 10^{-9}$ m of PIDT-DBT NPs (without Tat) under the same experimental conditions. As shown in Figure S3, Supporting Information, only very weak fluorescence intensity can be detected from the PIDT-DBT NP-treated cells (Figure S3b, Supporting Information) while much stronger fluorescence signal from the PIDT-DBT-Tat NP-treated cells was observed, suggesting the much lower internalization efficiency of the non-functionalized NPs. The results indicate that the conjugation of Tat peptide on NP surface is essential to enhance the internalization of NPs into living cells. Moreover, the cytotoxicity of PIDT-DBT-Tat NPs was evaluated using methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay. As shown in Figure S4, Supporting Information, the metabolic viabilities of HepG2 and NIH/3T3 cells show negligible difference and remain above 90% after incubation with 2, 4, and $8 \times 10^{-9}$ m PIDT-DBT-Tat NPs for 48 h. The viability of NIH/3T3 cells after incubation with 72 h still remains above 85% (Figure S5, Supporting Information), suggesting that the NPs are of low cytotoxicity during the test period, which is beneficial to long-term in vivo studies.

2.4. In Vivo Monitoring of Liver Tumor Growth

The ability of PIDT-DBT-Tat NPs for real-time in vivo monitoring of liver tumor growth was subsequently evaluated. Surgical operation was conducted at the abdomen of Balb/c nude mice and $4 \times 10^{9}$ of HepG2 cells labeled with $2 \times 10^{-9}$ m PIDT-DBT-Tat NPs or Qtracker 705 were directly injected into the parenchymal cells of the left lobe of liver (two groups, $n = 3$ for each group). Meanwhile, a nude mouse that underwent the same surgical operation without injection of labeled HepG2 cells was used as a control. After injection and suture, the in vivo fluorescence images of these mice were recorded using an IVIS Spectrum Imaging System. As shown in Figure 3, the suture at surgical site shows intense fluorescence upon excitation at 640 nm. After 6 d, the suture was totally absorbed and the cut closed up with no background fluorescence detectable from the surgical site. As shown in Figure 3, the mice injected with PIDT-DBT-Tat NP-labeled HepG2 cells shows intense fluorescence at the surgical site at day 6, due to the high labeling efficiency and efficient NIR emission of PIDT-DBT-Tat NPs. The fluorescence signal remains detectable even after 27 d. On the contrary, no fluorescence can be detected from the mice injected with Qtracker 705-labeled cells at day 6 under the same experimental setup. These results suggest that PIDT-DBT-Tat NPs can serve as a long-term in vivo tumor tracing probe with advantages over commercial Qtracker 705 in real-time fluorescence imaging.

Upon 42 d postinjection of PIDT-DBT-Tat NP-labeled HepG2 cells, one mouse was sacrificed to collect the liver tissue. It can be clearly seen that the transplanted cancer cells have grown into solid tumors (Figure S6, Supporting Information) and spread all over the whole liver to emit intense fluorescent signal (Figure 4a). On the contrary, the liver tissues from mouse without HepG2 cell transplantation show no fluorescent signal under the same experimental conditions. These results indicate the ability of PIDT-DBT-Tat NPs for tracing tumor growth during cancer cell proliferation and invasion without detectable interference in the process. The whole tumor was then mounted and imaged upon excitation at 590 nm using one-photon excited fluorescence microscope. The efficient penetration depth of fluorescence from PIDT-DBT-Tat NPs in tumor tissues was studied by taking images layer-by-layer at a 3 µm interval. The 3D color-coded projection of deep tissue image reveals that the fluorescent signal from the NP-labeled cells can be detected at 230 µm depth in the liver tumor upon excitation at 590 nm, proving the ability of the NIR emissive PIDT-DBT-Tat NPs in deep tissue imaging.
The ex vivo fluorescence imaging of organs (liver, heart, intestine, spleen, kidney, and lung) at day 1 and day 7 after cancer cell transplantation was also performed as shown in Figure S7, Supporting Information. It shows that no fluorescence could be detected from other organs except liver, suggesting the majority of PIDT-DBT-Tat NPs labeled cancer cells remain in the liver and negligible amount of them migrate to other organs during the test period. As the cells are directly injected to a particular site on liver (parenchymal cells) during the experiments, they prefer homing in the organ but not migrating to other organs.

3. Conclusion

To conclude, we report the design and synthesis of a fluorescent conjugated polymer PIDT-DBT with absorption and emission profiles fallen within FR/NIR region. We further demonstrated the preparation of their NIR emissive NPs for long-term in vitro cell tracing and in vivo imaging of liver tumor growth. PIDT-DBT-Tat NPs show an absorption maximum at ≈600 nm and an emission maximum at ≈720 nm in water, which is the first CP used for in vivo cancer cell tracking. Our studies have shown that PIDT-DBT-Tat NPs are superior to Qtracker 705 as fluorescent probes. In vivo imaging results indicate that PIDT-DBT-Tat NPs can monitor liver tumor growth for more than 27 d in a real-time manner. This study demonstrates for the first time the feasibility of utilizing CP-based fluorescent probes for long-term in vivo imaging of tumor growth, which will encourage the development of NIR fluorescent CPs for in vivo bioimaging.

4. Experimental Section

Materials: 1,4-Dibromo-2,5-dimethylbenzene, potassium permanganate (KMnO₄), tert-butyl alcohol, 2-(tributylstannyl)thiophene, bis(triphenylphosphine)palladium(II) dichloride (Pd(PPh₃)₂Cl₂), tetrakis(triphenylphosphine)palladium(0) (Pd(PPh₃)₄), n-butyl lithium (n-BuLi), N-bromosuccinimide (NBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich and used as received. Cell penetrating peptide HIV-1 Tat was purchased from Genicbio, China. Dulbecco’s modified eagle’s medium (DMEM) was a commercial product of National University Medical Institutes (Singapore). Toluene used for polymerization was pretreated with sulfuric acid followed by distillation. All other chemicals were used as received, unless otherwise stated.

Characterization: Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 500 NMR spectrometer (500 MHz for ¹H, referenced to tetramethylsilane (TMS) at δ = 0.00 ppm and 125 MHz for ¹³C, referenced to CDCl₃ at 77.0 ppm). Gel permeation chromatography (GPC) analysis was carried out with Waters 996 photodiode detector and Phenogel GPC columns, using polystyrenes as the standard and THF as the eluent at a flow rate of 1.0 mL min⁻¹ at 35 °C. Laser light scattering (LLS) measurements were performed using Brookhaven instruments corporation (BIC) 90 plus with λ = 655 nm, and the particle diameters were calculated by ZetaPlus Particle Sizing Software Version 3.93. Transmission electron microscopy (TEM) studies were performed on a JEOL JEM-2010 electron microscope with an accelerating voltage of 200 kV. UV–vis spectra were collected on a Shimadzu UV-1700 spectrometer. Photoluminescence (PL) spectra were measured on a Perkin Elmer LS55 equipped with a xenon lamp excitation source and a Hamamatsu (Japan) 928PMT, using 90° angle detection for solution samples. All UV–vis and PL spectra were collected at 24 ± 1 °C. Fluorescence quantum yield was measured using rhodamine 6G in methanol (95%) as the standard. The absorbance of the solutions was kept below 0.1 to avoid internal filter effect. Confocal laser-scanning microscopy (CLSM) images were collected on a Zeiss LSM 410 (jena, Germany) CLSM with imaging soft (Fluoview FV1000). MilliQ water (18.2 MQ) was used for all the experiments.

2.5-Dibromoterephthalic Acid (1): To a suspension of 2,5-dibromo-p-xylene (10.0 g, 37.8 × 10⁻³ M) and cethyl (15 g) in a mixture of water/tert-BuOH (100/100 mL) was added KmnO₄ (29.9 g, 189.4 × 10⁻³ M) in portions within 30 min. The mixture was stirred at 100 °C for 36 h. After cooling to 70 °C, ethanol (40 mL) was added slowly to remove unreacted KmnO₄. The reaction mixture was filtered off and the filtrate was concentrated to ≈20 mL under reduced pressure. The residue was acidified by addition of concentrated HCl (10 mL). The precipitate was collected by filtration and washed with cold ethanol to give 1 as a white crystal (11.2 g, yield: 91%). The product was directly used in the next step without further purification.

Diethyl 2,5-Dibromoterephthalate (2): To a solution of 2,5-dibromoterephthalic acid (10.0 g, 30.8 × 10⁻³ M) in absolute ethanol (50 mL) was added concentrated H₂SO₄ (15 mL). The mixture was refluxed overnight before being cooled to room temperature. The precipitate was collected by filtration and washed with cold ethanol to give 2 as a white crystal (10.9 g, yield: 93%). ¹H NMR (600 MHz, CDCl₃, ppm) δ 8.02 (s, 2 H), 4.41 (q, J = 7.2 Hz, 4 H), 1.41 (t, J = 7.2 Hz, 6 H). ¹³C NMR (150 MHz, CDCl₃, ppm) δ 160.20, 136.40, 135.69, 120.05, 62.33, 14.13.

Diethyl 2,5-Di(thien-2-yl)terephthalate (3): To a solution of diethyl 2,5-dibromoterephthalate (4.3 g, 11.3 × 10⁻³ M) and Pd(PPh₃)₂Cl₂ (397.1 mg, 565.7 × 10⁻⁶ M) in anhydrous THF (15 mL) was added 2-(tributylstannyl)thiophene (10.8 mL, 33.9 × 10⁻³ M). The reaction mixture was stirred at 85 °C under argon atmosphere for 15 h before being cooled to room temperature. The mixture was treated with potassium fluoride aqueous solution (10 wt%, 50 mL) for 2 h. The product was extracted with dichloromethane three times and the combined organic phase was washed with water three times and then dried over MgSO₄. After filtration, the solvent was removed under reduced pressure. The residue was purified by silica gel chromatography (hexane/ethyl acetate = 9/1) to afford 3 as a white solid (3.9 g, yield: 90%). ¹H NMR (600 MHz, CDCl₃, ppm) δ 7.81 (s, 2 H), 7.39 (dd, J = 4.8, 1.2 Hz, 2 H), 7.08 (m, 4 H), 4.21 (q, J = 7.2 Hz, 4 H), 1.15 (t, J = 7.2 Hz, 6 H). ¹³C NMR (150 MHz, CDCl₃, ppm) δ 167.65, 140.46, 134.05, 133.41, 131.84, 127.30, 126.94, 126.43, 61.62, 13.78.
4, 9, 9-Tetakis(4-octyloxyphenyl)-4, 9-dihydro-s-indaceno[1, 2-b: 5, 6-b'] dithiophene (1): A solution of 1-bromo-4,4-oxybenzene (9.975 g, 35.0 \times 10^{-3} \text{ mol}) in anhydrous THF (70 mL) was treated with n-Buli (21.87 mL, 1.6 M in hexane, 35 \times 10^{-3} \text{ mol}) at -78 °C for 1 h. Then a solution of diethyl 2,5-dichloro-4-thiophen-2-yl)terephthalate (1.93 g, 5.0 \times 10^{-3} \text{ mol}) in anhydrous THF (10 mL) was added slowly into the reaction mixture. After the addition was completed, the reaction mixture was stirred at -78 °C for 1 h and then kept at room temperature for 12 h. The solution was quenched with water (20 mL). After solvent removal, the residue was subsequently extracted with dichloromethane (100 mL \times 3), washed with water (100 mL \times 3) and dried over MgSO\(_4\). The organic layer was collected into a round-bottom flask and the mixture was frozen, evacuated, and thawed three times to further remove air. After the mixture was kept at 100 °C overnight in a fume hood to evaporate THF, the CP NPs were further sonicated for 60 s using a microtip probe sonicator at 12 W output (XL2000, Misonix Incorporated, NY). The mixture was stirred vigorously overnight in a sonication bath and the mixture was frozen, evacuated, and thawed three times to further remove air. After the mixture was kept at 100 °C overnight, the reaction was stopped and cooled down to room temperature. The mixture was stirred for 3 h in the presence of light at room temperature and then the solvent was removed under reduced pressure. The residue was subsequently dissolved in dichloromethane (200 mL), washed with water (3 \times 100 mL) and dried over MgSO\(_4\). After solvent removal, the residue was purified by silica gel chromatography (hexane: dichloromethane = 7:3) to afford 5 as a white solid (3.23 g, yield: 78%). \(^1\)H NMR (600 MHz, CDCl\(_3\), ppm) \(\delta\): 7.39 (s, 2 H), 7.24 (d, \(J\) = 4.8 Hz, 2 H), 7.15 (d, \(J\) = 9 Hz, 8 H), 6.97 (d, \(J\) = 4.8 Hz, 2 H), 3.90 (t, \(J\) = 7.2 Hz, 8 H), 1.44 (m, 8 H), 1.32 (m, 32 H), 0.90 (t, \(J\) = 7.2 Hz, 8 H), 1.50 ppm. \(^{13}\)C NMR (125 MHz, CDCl\(_3\), ppm) \(\delta\): 157.95, 156.14, 155.61, 153.35, 141.57, 136.30, 132.68, 117.23, 114.31, 67.89, 61.88, 31.79, 29.33, 29.28, 29.21, 26.05, 22.63, 14.07 ppm.

Poly[4, 4, 9, 9-tetakis(4-octyloxy) phenyl]-4, 9-dihydro-s-indaceno[1, 2-b: 5, 6-b']dithiophene (2) and Poly[4, 4, 9, 9-tetakis(4-octyloxy) phenyl]-4, 9-dihydro-s-indaceno[1, 2-b: 5, 6-b']dithiophene (6) were synthesized by the same procedure as described above. After purification by column chromatography (hexane: dichloromethane = 7:3) to afford 6 as a white solid (0.1241 mg, 0.1 \times 10^{-3} \text{ mol}) in anhydrous THF (70 mL) was treated with n-Buli (21.87 mL, 1.6 M in hexane, 35 \times 10^{-3} \text{ mol}) at -78 °C for 1 h. Then a solution of diethyl 2,5-dithien-2-yl)terephthalate (1.93 g, 5.0 \times 10^{-3} \text{ mol}) in anhydrous THF (10 mL) was added slowly into the reaction mixture. After the addition was completed, the reaction mixture was stirred at -78 °C for 1 h and then kept at room temperature for 12 h. The solution was quenched with water (20 mL). After solvent removal, the residue was subsequently extracted with dichloromethane (100 mL \times 3), washed with water (100 mL \times 3) and dried over MgSO\(_4\). The organic layer was collected into a round-bottom flask and the mixture was frozen, evacuated, and thawed three times to further remove air. After the mixture was kept at 100 °C overnight in a fume hood to evaporate THF, the CP NPs were further sonicated for 60 s using a microtip probe sonicator at 12 W output (XL2000, Misonix Incorporated, NY). The mixture was stirred vigorously overnight in a sonication bath and the mixture was frozen, evacuated, and thawed three times to further remove air. After the mixture was kept at 100 °C overnight, 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 redissolved in dichloromethane (200 mL), washed with water three times, and dried over MgSO\(_4\). After solvent removal, PIDT-DBT (97.9 mg, yield: 71%) was obtained as a black solid by precipitation in methanol. \(^1\)H NMR (500 MHz, CDCl\(_3\), ppm) \(\delta\): 7.95 (br, 7.76 (br), 7.31 (br), 7.14 (br), 7.12 (br), 6.72 (br), 3.86 (br), 1.69 (br), 1.43 (br), 1.35 (br), 1.22 (m, 0.80 (br).

The suspension of PIDT-DBT-Tat NPs: A THF solution (1 mL) containing PIDT-DBT (1 mg) and a mixture of DSPE-PEG\(_{2000}\) (1 mg) and DSPE-PEG\(_{2000}\)Mal (1 mg) was mixed with water (10 mL), followed by sonication for 5 min using a microtip probe sonicator at 12 W output (XL2000, Misonix Incorporated, NY). The mixture was stirred vigorously overnight in a sonication bath and the mixture was frozen, evacuated, and thawed three times to further remove air. After the mixture was kept at -78 °C for 1 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 redissolved in dichloromethane (200 mL), washed with water three times, and dried over MgSO\(_4\). After solvent removal, PIDT-DBT (97.9 mg, yield: 71%) was obtained as a black solid by precipitation in methanol. \(^1\)H NMR (500 MHz, CDCl\(_3\), ppm) \(\delta\): 7.95 (br, 7.76 (br), 7.31 (br), 7.14 (br), 7.12 (br), 6.72 (br), 3.86 (br), 1.69 (br), 1.43 (br), 1.35 (br), 1.22 (m, 0.80 (br).

Synthesis of PIDT-DBT-Tat NPs: A THF solution (1 mL) containing PIDT-DBT (1 mg) and a mixture of DSPE-PEG\(_{2000}\) (1 mg) and DSPE-PEG\(_{2000}\)Mal (1 mg) was mixed with water (10 mL), followed by sonication for 5 min using a microtip probe sonicator at 12 W output (XL2000, Misonix Incorporated, NY). The mixture was mixed vigorously overnight in a sonication bath and the mixture was frozen, evacuated, and thawed three times to further remove air. After the mixture was kept at -78 °C for 1 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 redissolved in dichloromethane (200 mL), washed with water three times, and dried over MgSO\(_4\). After solvent removal, PIDT-DBT (97.9 mg, yield: 71%) was obtained as a black solid by precipitation in methanol. \(^1\)H NMR (500 MHz, CDCl\(_3\), ppm) \(\delta\): 7.95 (br, 7.76 (br), 7.31 (br), 7.14 (br), 7.12 (br), 6.72 (br), 3.86 (br), 1.69 (br), 1.43 (br), 1.35 (br), 1.22 (m, 0.80 (br).
Ex Vivo One-Photon Excited Fluorescence Imaging: In the ex vivo tumor imaging experiment, the mice were euthanized with CO₂ and the livers were collected at 42 d post injection and fixed in 4% paraformaldehyde for one-photon excited 3D fluorescence imaging. The tumor was resected and fixed in 4% paraformaldehyde for 2 h and imaged using Leica TCS SP5X. Confocal images of consecutive layers with approximately 3 µm interval were recorded to generate 3D colored projection to demonstrate the penetration depth of PIDT-DPT-Tat NPs in the tumor. The confocal images were taken upon excitation at 590 nm with a 600–800 nm bandpass filter.

Biodistribution Studies: The mice injected with PIDT-DPT-Tat NP-labeled HepG2 cells were euthanized with CO₂ and the organs (liver, heart, intestine, spleen, kidneys, and lung) were harvested at 1 and 7 d postsurgery, respectively. The collected organs were then imaged using an IVIS Spectrum Imaging System (Xenogen Co., Alameda, CA, USA). The fluorescence images were recorded with 1 s exposure using a filter 720/20 nm upon excitation at 640 nm. The autofluorescence was removed using the software of IVIS Spectrum Imaging System.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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