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2016

Xie, C., Upputuri, P. K., Zhen, X., Pramanik, M., & Pu, K. (2016). Self-quenched semiconducting polymer nanoparticles for amplified in vivo photoacoustic imaging. *Biomaterials*, in press.

<https://hdl.handle.net/10356/84546>

<https://doi.org/10.1016/j.biomaterials.2016.12.004>

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Self-Quenched Semiconducting Polymer Nanoparticles for Amplified In Vivo Photoacoustic Imaging

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Abstract

Development of photoacoustic (PA) imaging agents provides opportunities for advancing PA imaging in fundamental biology and medicine. Despite the promise of semiconducting polymer nanoparticles (SPNs) for PA imaging, the molecular guidelines to enhance their imaging performance are limited. In this study, semiconducting polymers (SPs) with self-quenched fluorescence are synthesized and transformed into SPNs for amplified PA imaging in living mice. The self-quenched process is induced by the incorporation of an electron-deficient structure unit into the backbone of SPs, which in turn promotes the nonradiative decay and enhances the heat generation. Such a simple chemical alteration of SP eventually leads to 1.7-fold PA amplification for the corresponding SPN. By virtue of the targeting capability of cyclic-RGD, the amplified SPN can effectively delineate tumor in living mice and increase the PA intensity of tumor by 4.7-fold after systemic administration. Our study thus provides an effective molecular guideline to amplify the PA brightness of organic imaging agents for *in vivo* PA imaging.

Keywords: Photoacoustic imaging; Semiconducting polymer nanoparticles; Tumor imaging; Contrast agents

Introduction

Development of photoacoustic (PA) imaging agents has become a new hotspot in the field of biomaterials because PA imaging provides deeper tissue penetration and higher spatial resolution as compared with traditional optical imaging techniques [1]. As most biological and pathological processes do not have detectable variations in intrinsic PA contrast, many exogenous agents have been developed for PA imaging, which include near-infrared (NIR) emitting dyes [2–4], fluorescent proteins [5,6], metallic nanoparticles (e.g. gold nanoparticles) [7–11], carbon nanotubes [12–15], 2D graphene analogues [16–18] and porphyrins [19–21]. However, organic dyes, fluorescent proteins, and metallic nanoparticles usually have poor photostability [22,23] unless specific modification or precaution is undertaken; carbon nanotubes and graphene analogues generally possess broad and multi-peak PA spectral profiles [24,25], making their signals less distinctive from the tissue background; and porphyrins could be phototoxic as they are efficient in the production of single oxygen upon light illumination and are usually integrated with photodynamic therapy [26,27]. Thus, alternative PA imaging agents with improved properties are highly demanded to fully explore the potential of PA imaging in fundamental biology and medicine.

Semiconducting polymer nanoparticles (SPNs) have emerged as a new category of optical nanomaterials for molecular imaging [28–29]. By virtue of their unique optical properties, SPNs have been applied for fluorescence imaging such as cell tracking [30], tumor imaging [31], and ultrafast hemodynamic imaging [32] as well as for chemiluminescence imaging of hepatotoxicity [33] and neuroinflammation [34]. In addition, we have recently revealed that SPNs can efficiently convert photon energy into heat, permitting photoacoustic imaging of tumor [35], reactive oxygen species (ROS) [22] and pH [36] as well as photothermal related applications such as photothermal

1 ablation of tumors [37] and real-time activation of neurons [38]. SPNs not only have good
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3 biocompatibility as a result of their completely organic and biologically inert ingredients but also
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5 can be much more photostable than gold nanorods and photoacoustically brighter than carbon
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7 nanotubes [22,23].
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11 To improve the PA brightness of SPNs, we have screened a series of SPs and also engineered
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13 the intraparticle structure of SPNs [35]. Massive screening of SPs have led to better PA agents but it
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15 is a random approach that inevitably requires a huge amount of time and synthetic effort. In
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17 contrast, intraparticle engineering of SPNs is a rational approach that aims to enhance the heat
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19 generation by doping a secondary optically active component into SPNs [37]. However, it not only
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21 faces the challenge of potential leakage of the secondary component from the nanoparticles but also
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23 has the issue of decreased absorption coefficient on a per particle basis. Thereby, simple yet
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25 effective guidelines to improve the PA brightness of SPNs remain to be revealed to advance SPNs
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27 in PA imaging.
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37 We herein report a self-quenched molecular engineering approach to develop SPNs with
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39 amplified PA brightness for targeted *in vivo* imaging of tumor. The molecule design of SPs lies in
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41 the incorporation of an electron-deficient structure unit into the polymer backbone to promote the
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43 nonradiative decay and in turn quench the fluorescence for enhanced heat generation. Such a
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45 self-quenched backbone design of SP thus led to the amplified PA brightness for the corresponding
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47 SPNs. As follows, the synthesis of the self-quenched SPs and their transformation into the
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49 water-soluble SPNs are described first. The optical and PA properties are then discussed to reveal
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51 the underlying mechanisms that governs the amplification of PA brightness. At last, the
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53 proof-of-concept application of the self-quenched SPN in targeted PA imaging of tumor in living
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mice is demonstrated.

Results and Discussion

The self-quenched SPs were designed using poly{3-(5-(9-hexyl-9-octyl-9H-fluoren-2-yl)thiophen-2-yl)-2,5-bis(2-hexyldecyl)-6-(thiophen-2-yl)pyrrolo[3,4-c]pyrrole-1,4(2H,5H)-dione (PDPPF, SP0) as the backbone framework. PDPPF derivatives were found to show excellent light and thermal stability [39], and thus were suitable for laser intensive tasks such as PA imaging. Benzothiadiazole (BT) was used as the electron-deficient structure unit and incorporated in the PDPPF backbone to induced fluorescence quenching. The BT-containing self-quenched SPs and the control SP were synthesized *via* Pd-catalyzed Suzuki polymerization (Figure 1a). Condensation polymerization between Monomers 1 and 2 led to the alternating copolymer (SP0), while random polymerization between Monomers 1, 2 and 3 yielded the self-quenched SPs. According to the doping amount of BT in the backbone, the self-quenched SPs were termed as SP5 and SP10, which are respectively composed of 5% and 10% of BT within the backbone. The characteristic peaks at 8.14 and 7.92 ppm corresponding to the protons on the benzyl ring of BT were identified in the proton nuclear magnetic resonance spectra (^1H NMR), confirming the successful incorporation of BT into the backbone. Moreover, the integral ratios of the proton signals of BT to the proton signals of thiophene (8.92 ppm) further revealed that the actual doping amounts were close to 5% and 10% for SP5 and SP10, respectively. Gel permeation chromatography (GPC) showed that the molecular weights of SPs were within the range of 30000 to 50000 g mol^{-1} (Table S1, Supporting information). All the polymers had good solubility in tetrahydrofuran (THF). This is critical for the subsequent nanoprecipitation process because a well-dissolved THF solution of SP is a prerequisite to form homogeneous nanoparticles with a

narrow dispersity after addition into the poor solvent (water) under sonication.

Figure 1. (a) Synthetic routes of SPs (SP0, SP5 and SP10). Reagents and conditions: i) Palladium-tetrakis(triphenylphosphine) ($\text{Pd}(\text{PPh}_3)_4$), K_2CO_3 , methyltriocetylammmonium chloride, toluene/ H_2O , 100 °C, 24 h. (b) Schematic illustration of the preparation of SPNs *via* nanoprecipitation.

Nanoprecipitation was used to transform the hydrophobic SPs into the water-soluble SPNs. The amphiphilic triblock copolymer (PEG-*b*-PPG-*b*-PEG) was used to co-precipitate with the SP so as to afford the PEG-coating passivated surface for the SPNs. The SPNs solutions were transparent, showing similar cyan color (Figure 2a). Dynamic light scattering (DLS) showed that all the SPNs had the similar hydrodynamic diameters of ~40 nm (Figures 2c&2d), and the transmission electron microscopy (TEM) revealed a typical spherical morphology with the average diameter of ~25 nm (Figure 2b). The slightly smaller size estimated by TEM relative to DLS was probably attributed to

the shrinkage of nanoparticles in the dry state during the preparation of TEM samples. No precipitation and obvious change in size were observed for the SPNs even after 30 days of storage in phosphate buffer solution (PBS, pH = 7.4) or fetal bovine serum (FBS) (Figure 2e). Furthermore, no cytotoxicity of the SPNs was detected by using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Figure 2f). These data demonstrate the excellent aqueous stability and good cytocompatibility of the SPNs, suggesting their suitability for biological application.

Figure 2. Characterizations of SPs. (a) Photograph of the SPs solutions. From left to right: SPN0, SPN5, SPN10. Representative TEM (b) and DLS (c) of SPs: SPN10. (d) Average diameters of SPNs solutions determined by DLS. (e) The hydrodynamic diameters of SPN10 ($25 \mu\text{g mL}^{-1}$) as a function of storage time at room temperature in $1 \times$ PBS (pH = 7.4) and 10% FBS. (f) Cell viability of Hela cells after incubation with SPN10 in various concentrations. The error bars represent the standard deviation of three separate measurements.

To study the effect of doping BT on optical properties of the SPNs, their UV-vis and

photoluminescence (PL) spectra were measured in PBS (pH = 7.4) at the same concentration of 10 $\mu\text{g mL}^{-1}$. All SPNs had a major extinction band with the two vibronic peaks at 616 and 665 nm. With the increase of BT amount, the vibronic extinction peak at ~ 665 nm gradually decreased; meanwhile, a new extinction band ranging from 700 to 800 nm appeared, which was assigned to the BT-containing segments (Figure 3a). Such an extinction change verifies the successful doping of BT into the backbone of the SP. The fluorescence of the BT-free nanoparticle (SPN0) had an emission maximum at 710 nm. With the increase of the BT amount, the emission intensity at 710 nm substantially decreased by 7.3 and 9.4-fold for SPN5 and SPN10, respectively (Figures 3b&3c). The IVIS fluorescence images further illustrated that the fluorescence signals of SPN10 were quenched to the level that is as low as the background noise (Figure 3c). The quenched fluorescence of SPN10 should be attributed to the efficient electron transfer process from the DPPF segments to the DPPBT segments in the highly compact core of nanoparticles. Also, the fact that the extinction of SPN10 showed no intensity decrease under continuous illumination at 680 nm for 60 min confirmed its good photostability, showing the feasibility for long-term optical imaging applications (Figure 3d).

The PA properties of the SPNs were measured and compared at the same concentrations at 680 nm. Among these SPNs, SPN10 had the highest PA brightness at 680 nm (Figures 4a&b), which was respectively 1.14 and 1.70-fold higher than SPN5 and SPN0. In addition, the PA amplitude of SPN10 was 6.62-fold higher than the gold nanorods (GNR) with the absorption maximum at 670 nm (Figure S4, Supporting Information) when comparing at the same mass concentration. The PA spectrum of SPN10 was close to its absorption, both of which showed the BT-related band in the range of 700 to 800 nm (Figure 4c vs Figure 3a). Also, the PA spectral profile of SPN10 was

distinct from that of animal blood, making it easy to be detected from blood. Moreover, the PA amplitudes of SPN10 at 680 nm were then determined at a series of concentrations from 5 to 1000 $\mu\text{g mL}^{-1}$ (Figure 4d). As expected a linear relationship between the PA intensities and the concentrations was observed for SPN10, showing the feasibility for the signal quantification.

Figure 3. UV-vis and Fluorescence properties of SPNs. Normalized extinction (a), fluorescence spectra (b) of SPNs solutions in PBS (pH = 7.4), the concentrations of SPNs were $10 \mu\text{g mL}^{-1}$. (c) Fluorescence intensities and images of IVIS fluorescence of SPNs solutions in PBS at same concentration. (d) UV stability of SPN10 under continuous irradiation for 1 h. The error bars represent the standard deviation of three separate measurements.

To understand the underlying mechanisms that govern the difference in the PA brightness of the SPNs, the fluorescence and PA intensities of the SPNs are normalized at the same concentration and summarized in Figure 4e. It can be noted that the order of the PA intensities for the SPNs is opposite to that for the PL intensities. This phenomenon can be rationalized by considering the

energy dissipation after light excitation. The photon energy absorbed by a chromophore can be dissipated through three major pathways: fluorescence, nonradiative thermal deactivation and long-life species (phosphorescence). Because these SPNs are not phosphorescence, the third pathway can be neglected. Thus, upon light excitation, the fluorescence and thermal deactivation compete with each other to release the energy in the form of photons and heat, respectively. With the incorporation of BT into the SPNs, the nonradiative channels are promoted, leading to fluorescence quenching and thus enhanced thermal deactivation. Because the photoacoustic signals are mainly determined by the heat generation, the enhanced thermal deactivation eventually results in the increased PA brightness for the BT-containing SPNs (SPN5&SPN10) relative to the BT-free SPN (SPN0).

Figure 4. PA properties of SPNs. (a) PA signals of SPNs and GNR at 680 nm at the same concentration (0.23 mg mL^{-1}). (b) Comparison of PA amplitudes of SPNs and GNR under the same concentration (0.23 mg mL^{-1}). The PA amplitudes were calculated as the difference between the maximum and minimum intensities for each sample in Figure 4a. (c) PA spectra of SPN10 (0.5 mg mL^{-1}).

mL⁻¹) in PBS (pH = 7.4) and mouse blood. (d) The PA amplitudes at 680 nm as a function of concentrations of SPN10. Inset: PA images of SPN10 at different concentrations (from left to right: 62.5, 125, 250, 500, 1000 $\mu\text{g mL}^{-1}$). (e) Normalized PA and fluorescence intensities based on the same mass extinction coefficients of SPNs at 680 nm (PBS, pH = 7.4). The error bars represent the standard deviation of five separate measurements.

With the highest PA brightness among the SPNs, SPN10 was further developed into nanoprobe for *in vivo* PA imaging. To endow SPN10 with the capability to target cancer cells, a cyclic-RGD endcapped PEG-*b*-PPG-*b*-PEG (RGD-PEG-*b*-PPG-*b*-PEG-RGD) was designed and synthesized by firstly conjugating *N*-hydroxysuccinimide (NHS) to the ends of PEG-*b*-PPG-*b*-PEG, followed by substituting the NHS with cyclic-RGD (Figure S5, Supporting Information). Nanoprecipitation of SP10 and RGD-PEG-*b*-PPG-*b*-PEG-RGD led to cyclic-RGD conjugated SPN10 (SPN10-RGD) (Figure 5a). No obvious difference in size (~ 40 nm) and zeta-potential (-6 ± 1 and -6 ± 2 mV for SPN10 and SPN10-RGD, respectively) was observed for SPN10 and SPN10-RGD (Figure S7, Supporting Information). Such low zeta-potentials for both SPNs indicated that the stability of nanoparticles were not owing to the electrostatic repulsion but their steric stabilization nature from the dense PEG groups on the particle surface. The targeting capability of SPN10-RGD was validated for *in vivo* imaging of xenograft 4T1 tumor in living mice along with the control SPN10. Before systemic administration of nanoparticles, the tumors showed weak PA signals at 680 nm owing to the relatively low intrinsic absorption of oxyhemoglobin and deoxyhemoglobin in the NIR region under the experimental conditions (Figure 5b). In contrast, the PA signals in tumor areas gradually increased for both SPN10 and SPN10-RGD over time after systemic administration of nanoparticles through tail vein injection (Figure 5c & Figure S8, Supporting Information). In particular, SPN10-RGD treated mice had higher PA signals in tumor area as compared with

SPN10-treated mice at each post-injection time point. This indicated that the presence of cyclic-RGD indeed helped the tumor uptake of nanoparticles, as the $\alpha_v\beta_3$ integrin receptors were overexpressed on the surface of 4T1 cells [40]. At $t = 4$ h post-injection, the PA signals reached the maxima for both SPNs, which were 4.7 and 2.6-fold higher than that of the tumor background for SPN10-RGD and SPN10, respectively. The enhanced PA signals permitted clear visualization of tumors using the SPNs (Figure 5b).

Figure 5. *In vivo* PA imaging of xenograft 4T1 tumors. (a) Synthesis of the targeted SPN (SPN10-RGD). (b) PA images of tumor after systemic administration of SPN10-RGD or SPN10 (30 μ g in 120 μ L) for 0, 4 and 24 h, respectively. The representative PA maximum imaging projection (MIP) images with axial view for SPN10-RGD and SPN10. (c) Quantification of PA intensities at 680 nm of tumor as a function of post-injection time of SPN10-RGD and SPN10. (d) *In vivo* real-time PA spectra extracted from the tumors of living mice after systemic administration

of SPN10-RGD and SPN10 for 4 h. (e) *Ex vivo* PA quantification at 680 nm of major organs of mice 24 h after systemic administration of SPN10-RGD and SPN10 (* $p < 0.01$, $n = 3$).

Real-time *in vivo* PA spectra extracted from the tumors of SPN10-RGD and SPN10-injected mice at $t = 4$ h resembled the PA spectrum in solution (Figure 5d vs Figure 4c). Such a spectral similarity confirmed that the enhancement of PA signals in tumor areas came from the accumulation of the SPNs. Moreover, the PA intensity of tumors at 680 nm for SPN10-RGD treated mice was 1.78-fold higher than that for SPN10 treated mice (Figure 5d), which again showed the advantage of targeted imaging. After 4 h post-injection, both SPN10 and SPN10-RGD treated mice showed a decrease of PA signals in tumor areas, indicating the clearance of nanoparticles from the tumors. However, SPN10-RGD showed a slower clearance rate than SPN10 especially from 8 to 24 h, which was attributed to the active targeting effect from cyclic-RGD. The *ex vivo* biodistribution for SPN10-RGD and SPN10 was acquired at $t = 24$ h post-injection (Figure 5e). Both SPNs had noticeable uptake in the major reticuloendothelial system (RES) organs such as liver and spleen. However, by virtue of the targeting effect, SPN10-RGD showed 1.94-fold higher accumulation in tumor as compared with SPN10, which was consistent with the real-time PA measurement. These data not only demonstrated the effectiveness of SPNs in delineating the tumors of living mice but also highlighted the importance of targeting in the optimization of *in vivo* PA imaging.

Conclusion

We have proposed a self-quenched molecular approach to amplify the PA brightness of SPNs. The straightforward synthetic method allowed for the well-controlled incorporation of the electron-deficient BT unit into the backbone of PDPPF, yielding the BT-containing SPs

(SP5&SP10). The presence of BT had no effect on the size of the SPNs but could effectively promote nonradiative decay and in turn quench the fluorescence of the SPNs. Because the photon energy saved from quenched fluorescence upon light excitation is released in the form of heat, the PA brightness for the BT-containing SPNs could be enhanced by up to 1.6-fold as compared with the control SPN. The proof-of-concept application of the amplified SPN (SPN10) was demonstrated for *in vivo* imaging of tumor. Conjugation of cyclic-RGD to the surface of SPN10 permitted effective targeting to the 4T1 tumors in living mice after systemic administration. Particularly, SPN10-RGD delineated the tumor in a clearer way with the PA intensity that is 1.8-fold higher as compared with the non-targeted nanoparticles. Our study thus not only brings in a new generation of SPNs for PA imaging but also provides a new molecular design guideline to amplify the PA brightness for organic nanoparticles.

Experimental Section

Chemicals. All chemicals were purchased from Sigma-Aldrich unless otherwise stated. 2,2'-(9,9-Dioctyl-9H-fluorene-2,7-diyl)bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolane) (FLR), 3,6-Bis(5-bromothiophen-2-yl)-2,5-bis(2-hexyldecyl)pyrrolo[3,4-c]pyrrole-1,4(2H,5H)-dione (DPP) and 2,1,3-Benzothiadiazole-4,7-bis(boronic acid pinacol ester) (BT) were purchased from Luminescence Technology Corp.. Cyclo (RGDfK) was purchased from i-DNA Biotechnology Pte Ltd.

Characterization. Dynamic Light Scattering (DLS) was determined on a Malvern Nano-ZS Particle Sizer. TEM images were obtained from a JEM 1400 transmission electron microscope with an accelerating voltage from 40 to 120 kV. Nuclear magnetic resonance (NMR) spectra were recorded using a Bruker Avance II 300MHz NMR, CDCl₃ and D₂O were used as the solvents. Gel

permeation chromatography (GPC) results were performed by Shimadzu LC-VP system with polystyrenes as the standard and high purity of THF as the eluent. UV-vis spectra were measured from a Shimadzu UV-2450 spectrophotometer. Fluorescence measurements were carried out on a Fluorolog 3-TCSPEC spectrofluorometer (Horiba Jobin Yvon). Fluorescence images were obtained by IVIS spectrum imaging system.

PA measurement. For *in vitro* measurement, an optical parametric oscillator, OPO (Continuum, Surelite), which pumped by a Q-switched 532 nm Nd:YAG laser was used as an excitation source. OPO can generate tunable laser pulses within 680-920 nm wavelength range with 5 ns pulse duration, 100 mJ/pulse energy at 10 Hz repetition rate. The solution containing samples were placed inside a low-density polyethylene (LDPE) tube with an inner diameter (ID) of 0.59 mm and outer diameter (OD) of 0.78 mm. The sample containing LDPE tube, and the single-element ultrasound transducer, UST (V323-SU/2.25 MHz, 13 mm active area, and 70% nominal bandwidth, Panametrics) were immersed into water medium for coupling of PA signals to UST. The LDPE tube was irradiated with wavelengths ranging from 680 - 920 nm with 10 nm increment. PA signals were collected using the UST and these signals were subsequently amplified using a gain of 50 dB, and band pass filtered (1-10 MHz) by a pulser/receiver unit (Olympus-NDT, 5072PR). Finally, the output signals from the pulser/receiver unit was digitized with a data acquisition card (GaGe, compuscope 4227) operated at 25 MHz and the acquired signals were stored in the computer. Peak-to-peak voltage of the PA signals was then normalized with the laser energy at each wavelength and were plotted against the wavelength to generate the PA spectrum.

General synthetic procedure for SPs. DPP, FLR and BT with different molar ratio for the corresponding SPs were placed into a 50 mL schlenk tube followed by addition of

Palladium-tetrakis(triphenylphosphine) ($\text{Pd}(\text{PPh}_3)_4$) (5 mg) and potassium carbonate (828 mg, 6 mmol). Then a mixture of water (3 mL) and toluene (5 mL) with methyltrioctylammonium chloride (1 mg) were added to the reaction tube, and the reaction vessel was degassed by three-freeze-pump-thaw circles. The mixture was vigorously stirred at 100 °C for 24 h to start the reaction, and then the solvent was removed under pressure. The obtained solid was re-dissolved in excess dichloromethane and washed with brine 3 times. The organic phase was collected and precipitated into excess methanol. The obtained solid was washed several times by methanol and acetone and then dried under vacuum for 24 h to afford the SP polymers. For SP1, SP2 and SP3, the molar ratio of DPP, FLR and BT is 0.5/0.5/0, 0.5/0.45/0.05 and 0.5/0.4/0.1, respectively. SP0: ^1H NMR (300 MHz, CDCl_3 , δ): 8.99, 7.80-7.67, 7.64, 7.56, 4.12, 2.05, 1.48-0.95, 0.91-0.56; SP5: ^1H NMR (300 MHz, CDCl_3 , δ): 8.99, 8.14, 7.92, 7.80-7.67, 7.64, 7.56, 4.12, 2.05, 1.48-0.95, 0.91-0.56; SP10: ^1H NMR (300 MHz, CDCl_3 , δ): 8.99, 8.14, 7.92, 7.80-7.67, 7.64, 7.56, 4.12, 2.05, 1.48-0.95, 0.91-0.56.

Synthesis of carboxyl ended PEG-*b*-PPG-*b*-PEG. PEG-*b*-PPG-*b*-PEG (1 g) was dissolved into 20 mL anhydrous tetrahydrofuran (THF), and to the solution was added sodium hydride (100 mg) under ice bath and stirred for 1 h. Succinic anhydride (100 mg) was then added into the mixture and the reaction was carried out for 24 h at room temperature. The solid was removed to obtain the filtrate and THF was evaporated under reduced pressure. Water was added under vigorous sonication to dissolve the residue. The obtained solution was dialysis against pure water for 2 days to remove the low molecular weight by-products, yielding carboxyl ended PEG-*b*-PPG-*b*-PEG. ^1H NMR (300 MHz, CDCl_3 , δ): 4.24 (t, 4H), 3.64 (m, 800H), 3.55 (m, 130H), 3.40 (m, 65H), 2.60 (m, 8H), 1.15 (m, 195H).

Synthesis of NHS-endcapped PEG-*b*-PPG-*b*-PEG. Carboxyl ended PEG-*b*-PPG-*b*-PEG (40 mg) was dissolved into freshly distilled THF (5 mL) with vigorous stirring. *N*-hydroxysuccinimide (1 mg), 4-dimethylaminopyridine (2 mg) and dicyclohexylcarbodiimide (2 mg) were then added into the solution and the reaction was allowed to stir at room temperature for 48 h. After removing precipitation through filtration, the resulting solution was concentrated and precipitated into excess diethyl ether to obtain NHS-PEG-*b*-PPG-*b*-PEG-NHS.

Synthesis of RGD-endcapped PEG-*b*-PPG-*b*-PEG. NHS-PEG-*b*-PPG-*b*-PEG-NHS (20 mg) was dissolved into anhydrous *N,N*-dimethylformamide (DMF) (2 mL) followed by addition of 4 mg of RGDfK. The solution was stirred at room temperature for 24 h and then dialysis against pure water to remove excess RGDfK. RGD-PEG-*b*-PPG-*b*-PEG-RGD was obtained after lyophilization. ¹H NMR (300 MHz, D₂O, δ): 1.16 (m, PPG, CH₃), 1.56, 1.66, 1.86, 2.68, 2.91, 3.07 (m, RGD), 3.54 (m, PPG, CHCH₂), 3.69 (m, PEG, CH₂), 3.92, 4.26, 4.55, 4.72, 6.86, 7.11 (m, RGD).

Preparation of semiconducting polymer nanoparticles (SPNs). SPNs was prepared by a nanoprecipitation method. Briefly, SP (0.25 mg) and PEG-*b*-PPG-*b*-PEG (20 mg) or RGD-PEG-*b*-PPG-*b*-PEG-RGD (20 mg) were dissolved into 1 mL of THF under sonication. The obtained solution was rapidly injected into a mixture of water (9 mL) and THF (1 mL) solution under vigorous sonication with a sonicator under 110 W for 1 min. The THF in the solution was then removed under a gentle nitrogen flow, and the resulting solution was purified by filtered through a 0.22 μm PVDF syringe driven filter (Millipore). The obtained SPNs solutions were concentrated through ultrafiltration and then stored under 4 °C for further use.

Cell Culture and Cytotoxicity Test. 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) with 10% FBS (fetal bovine serum) (GIBCO) in a humidified environment containing 5% CO₂ and 95% air at 37 °C. Cells were then seeded in 96 well plates (5000 cells in 200 µL per well) and cultured for 24 h, then SPN10 (final concentration 5, 10, 25, 50 and 100 µg/mL) solutions were added into the cell culture medium. Cells were incubated for 24 h and followed by adding MTS (100 µL, 0.1 mg/mL) for another 4 h. The absorbance of MTS was measured by using a microplate reader at 490 nm. Cell viabilities were calculated by the ratio of the absorbance of the cells incubated with SPN10 solution to that of the cells incubated with cell culture medium only.

Tumor Mouse Model. All animal experiments were performed in compliance with the Guidelines established by the Institutional Animal Care and Use Committee (IACUC), SingHealth. To establish tumor models in six-week-old female nu/nu mice, two million 4T1 cells which 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 mm (approximately 10-15 days) before being used for *in vivo* imaging experiments.

***In Vivo* PA imaging of tumor.** Nude mice were anesthetized using 2% isoflurane in oxygen with a catheter was applied to the tail vein. The mice were placed in the Endra Nexus128 PA imaging system, and were scanned to determine the endogenous signal of tumors at 680 nm before systemically administration with SPN10 (30 µg in 120 µL) (n = 3) or SPN10-RGD (30 µg in 120 µL) (n = 3) through catheter. Data was acquired by a continuous model took 12 s to obtain one data set. Real-time PA spectra were recorded with a LAZR instrument (Visualsonics, 2100 High-Resolution Imaging System). For *ex vivo* PA imaging, mice were sacrificed by CO₂

1 asphyxiation, organs were harvested and acquired immediately with Endra Nexus128 PA imaging
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3 system. PA images were reconstructed off-line using data acquired from all 128 transducers at each
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5 view with a back-projection algorithm. The algorithm corrects for pulse-to-pulse variations in the
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7 laser intensity and small changes in the temperature that affect acoustic velocity in the water. The
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9 reconstructed raw data is analyzed using OSiriX software.
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13 **Data analysis.** PA signal intensities were measured by region of interest (ROI) analysis using
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15 OsiriX. Intensities of fluorescence were determined by ROI analysis using IVIS living imaging
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17 system. Results were expressed as the mean $\pm$ SD deviation unless otherwise stated. All statistical
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19 calculations were performed using GraphPad Prism v. 6 (GraphPad Software Inc., CA, USA).
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26 **Acknowledgements**

27
28 This work was supported by Nanyang Technological University start-up grant (NTU-SUG:
29
30 M4081627.120), Academic Research Fund Tier 1 from Singapore Ministry of Education
31
32 (RG133/15: M4011559, and RG31/14: M4011276) and Academic Research Fund Tier 2 from
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34 Ministry of Education in Singapore (ARC2/15: M4020238).
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