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Self-Assembly of Semiconducting Polymer Amphiphiles for In Vivo Photoacoustic Imaging

*Chen Xie, Xu Zhen, Qunli Lei, Ran Ni and Kanyi Pu**

Dr. C. Xie, Dr. X. Zhen, Dr. Q. Lei, Prof. R. Ni, Prof. K. Pu
School of Chemical and Biomedical Engineering, Nanyang Technological University,
Singapore 637457, Singapore
E-mail: kypu@ntu.edu.sg

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Photoacoustic (PA) nanoagents have been developed for molecular imaging at the penetration depth that is higher than traditional optical imaging. Despite the advantages of semiconducting polymer nanoparticles (SPNs) over other inorganic nanoparticles for PA imaging, their synthetic method is generally limited to nanoprecipitation, which is likely to cause the issue of nanoparticle dissociation. We herein report the synthesis of near-infrared (NIR) absorbing semiconducting polymer amphiphiles (SPAs) that can spontaneously self-assemble into homogenous nanoparticles for *in vivo* PA imaging. As compared with their counterpart nanoparticles (SPN1) prepared through nanoprecipitation, SPAs generally have higher fluorescence quantum yields but similar size and PA brightness, making them superior over SPN1. Optical and simulation studies reveal that the PEG grafting density plays a critical role in determining the packing of SP segments inside the core of nanoparticles, consequently affecting the optical properties. The small size and structurally-stable nanostructure in conjunction with a dense PEG shell allow SPAs to passively target the tumor of living mice after systemic administration, permitting both PA and fluorescence imaging of tumor at the signals that are ~1.5-fold higher than that of liver. Our study thus not only provides the first generation of amphiphilic optically-active polymers for PA imaging but also highlight the molecular guidelines for development of organic NIR imaging nanomaterials.

1. Introduction

Photoacoustic (PA) imaging is a hybrid imaging modality that combines optical excitation with ultrasonic detection and thus can provide deeper tissue penetration and higher spatial resolution as compared with traditional optical imaging techniques.^[1] However, apart from very few naturally occurring light absorbers including melanin and hemoglobin,^[2] most tissues do not contain other endogenous molecules to give PA signals. To detect biological or pathological processes of interest, development of exogenous PA agents is thus essential. Till now, many materials have been developed as PA agents, which include near-infrared (NIR) dyes,^[3] fluorescence proteins,^[4] inorganic nanoparticles,^[5] dye-loaded perfluorocarbon nanoparticles,^[6] carbon materials,^[7] two-dimensional materials^[8] and porphysomes.^[9] However, organic dyes, fluorescence proteins and metallic nanoparticles are usually confronted with the issues of poor photostability^[10] unless certain precaution is conducted prior to application. Carbon materials and two-dimensional materials generally have broad PA spectral profile,^[11] making them hard to be differentiated from the tissue background particularly at low concentrations. Porphysomes have high PA brightness but might be phototoxic as they are capable to produce single oxygen upon light illumination.^[12] To fully explore the potential of PA imaging in life science, alternative PA imaging agents with improved properties are highly demanded.

As a new category of photonic nanomaterials, semiconducting polymer nanoparticle (SPNs) derived from semiconducting polymers (SPs) have attracted a great deal of attention due to their excellent optical properties and good biocompatibility.^[13] SPNs have been applied for fluorescence imaging such as cell tracking,^[14] tumor imaging^[15] and ultrafast hemodynamic imaging^[16] as well as for chemiluminescence imaging of drug-induced injury^[17] and neuroinflammation.^[18] Moreover, SPNs generally possess high photothermal conversion efficiency, allowing for photothermal therapy^[19] and real-time activation of neurons under NIR

irradiation.^[20] With such a high ability to convert photon energy into heat, SPNs can act as flexible nanoplatform for *in vivo* PA imaging of tumor,^[21] reactive oxygen species (ROS)^[10] and pH.^[22] Our preliminary studies also reveal that SPNs are more photostable than gold nanorods and acoustically brighter than carbon nanotubes.^[10]

Despite the promise of SPNs for PA imaging, the synthesis of SPNs is limited to encapsulation of SPs into amphiphilic block copolymers through nanoprecipitation.^[18,19] Thus, most existing SPNs in principle are binary micelles that could undergo dissociation.^[23] According to the previous reports, escape of amphiphilic copolymer from the nanoparticles can potentially lead to aggregation during blood circulation;^[24] moreover, the present of proteins or other *in vivo* substances can accelerate the dissociation process due to their interaction with micelles.^[25] Such *in vivo* dissociation can cause the alternation in the optical properties and also poor biodistribution of nanoparticles.^[26] Thereby, other approaches to develop structurally stable SPNs are desired but remains to be revealed.

In this study, we report the synthetic approach towards structurally stable SPNs by grafting hydrophilic poly(ethylene glycol) (PEG) onto the backbone of semiconducting polymers. The result is the NIR-absorbing semiconducting polymer amphiphiles (SPAs) that can spontaneously self-assemble into nanoparticles in aqueous solutions. To study the relationship between the PEG grafting density and the properties of SPAs, four SPAs with different PEG side chain lengths and grafting densities were first designed and synthesized (**Figure 1**). Poly(2,5-bis(2-hexyldecyl)-3,6-di(thiophen-2-yl)-2,5-dihydropyrrolo[3,4-c]pyrrolo-1,4-dione-*alt*-9,9-dioctyl-9H-fluorene) (PDPPF, SP1) was used as the backbone framework, because PDPPF derivatives showed excellent photo- and thermal-stability and were suitable for laser intensive tasks such as PA imaging.^[27] Then, the optical and PA properties of these SPAs were investigated and compared with the SPN (SPN1) prepared *via* nanoprecipitation. It was found that the PEG grafting density played an important role in determining the inner structure of

nanoparticles and thus can affect the optical properties of SPAs. At last, the proof-of-concept application of SPAs for *in vivo* PA and fluorescence imaging was demonstrated in living mice.

Figure 1. Illustration of preparation of SPN1 *via* nanoprecipitation and self-assembly of SPAs (SPA1PEG2, SPA1PEG5, SPA2PEG2 and SPA2PEG5) into nanoparticles.

2. Results and Discussion

SPAs were synthesized *via* the Pd-catalyzed Suzuki polymerization followed by copper(I)-catalyzed alkyne-azide cycloaddition (CAAC) reaction (**Scheme 1**). First, monomer 2 was respectively polymerized with monomer 1 and monomer 3 to yield SP1-Br and SP2-Br with different grafting densities of bromide group. Then, SP1-Br and SP2-Br were reacted with sodium azide to substitute bromide with azide, giving SP1-N₃ and SP2-N₃, respectively. The conversion of bromide into azide was almost complete as detected by the proton nuclear magnetic resonance spectra (¹H NMR) of SP1-N₃ and SP2-N₃ (**Figure S1 and S2, Supporting Information**), showing the shift of the resonance peak of $-\text{CH}_2\text{CH}_2\text{Br}$ (3.37 ppm for SP1-Br, 3.37 and 3.22 ppm for SP2-Br) into that of $-\text{CH}_2\text{CH}_2\text{N}_3$ (3.23 ppm for SP1-N₃, 3.22 and 3.06

ppm for SP2-N₃) for both SP1-N₃ and SP2-N₃. At last, SP1-N₃ and SP2-N₃ were respectively react with methoxy-PEG-alkyne (M_n = 2000 or 5000) through CAAC reaction to give the final products, SPAs. Taking the ¹H NMR of SPA2PEG2 as an example (**Figure S3, Supporting Information**), the peak for repeating unit of PEG (3.6 ppm) and the peaks of SPA backbone (7.90-7.41, 4.17, 1.26 and 1.10 ppm) were in presence. Gel permeation chromatography (GPC) showed that the molecular weights of SPs were all in the range of 30000 to 55000 g mol⁻¹ (Table S1, Supporting information). Due to the grafting of PEG onto the side chains, SPA1PEG5 and SPA2PEG5 had an increased molecular weight as compared with their precursors SPA1PEG2 and SPA2PEG2, respectively. These results demonstrated the successful synthesis of SPAs.

Scheme 1. Synthetic routes of SPAs. Reagents and conditions: i) Palladium-tetrakis(triphenylphosphine) (Pd(PPh₃)₄), K₂CO₃, methyltrioctylammonium chloride, toluene/H₂O, 100 °C, 24 h; ii) NaN₃, tetrahydrofuran (THF)/N,N-dimethylformamide (DMF), 25 °C, overnight; iii) CuSO₄·5H₂O, sodium ascorbate, methoxy-PEG-alkyne, THF/DMF, 25 °C, 48 h.

All SPAs could be directly dissolved in phosphate buffer solution (PBS), showing the similar cyan color (**Figure 2a**). Hydrodynamic diameters of all the nanoparticles were determined by dynamic light scattering (DLS), showing the sizes in the range of 20 to 40 nm (Figure 2c&2d). Using SPA2PEG2 as an example, transmission electron microscopy (TEM) showed a spherical morphology with the diameter of ~25 nm (Figure 2b), which was slightly

smaller as compared with the DLS result (~ 30 nm). The larger sizes of SPA1PEG2 and SPA2PEG2 (more than 30 nm) as compared with SPA1PEG5 and SPA2PEG5 (~ 20 nm) were attributed to the increased hydrophobicity caused by shorter PEG chains of SPA1PEG2 and SPA2PEG2. No precipitation and obvious change in size were observed for SPAs during storage in PBS (pH = 7.4) or fetal bovine serum (FBS) even for 30 days (Figure 2e). Meanwhile, no obvious change in size and morphology was detected for SPAs after irradiation by 680 nm laser for 1 h (**Figure S6, Supporting Information**). Furthermore, no cytotoxicity of SPAs was detected using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Figure 2f). These results showed the excellent aqueous stability and good cytocompatibility of SPAs, indicating their suitability for biological application.

Figure 2. In Vitro Nanoparticle Characterizations. (a) Photograph of the nanoparticle solutions. From left to right: SPN1, SPA1PEG2, SPA1PEG5, SPA2PEG2, SPA2PEG5. Representative TEM (b) and DLS (c) of SPAs: SPA2PEG2. (d) Average diameters of SPN1 and SPAs determined by DLS. (e) DLS data of SPA2PEG2 as a function of time incubated with PBS (pH=7.4) and FBS, respectively. (f) Cell viability of HeLa cells after incubation with SPA2PEG2 solutions in various concentrations. The error bars represent the standard deviation of three separate measurements.

To study the optical properties of SPAs, their absorption and photoluminescence (PL) spectra were determined in PBS (pH = 7.4) and compared with SPN1. SPN1 had two vibronic absorption peaks at 616 and 665 nm (**Figure 3a**). In contrast, the two absorption peaks were respectively blue-shifted to 601 and 640 nm for the SPAs, among which SPA2PEG2 had the weakest absorption peak at 640 nm. The spectral difference was also observed for PL. Among all the nanoparticles, SPA2PEG2 had the strongest PL intensity, which was ~11-fold higher than SPN1 (Figures 3b and 3c). In fact, SPA2PEG2 had the highest quantum yield (4.31%), followed by SPA2PEG5 (3.13%), SPA1PEG2 (2.77%), SPA1PEG5 (2.73%) and SPN1 (1.02%). Furthermore, the fluorescence decay of SPA2PEG2 is different from other nanoparticles (Figure 3d). As widely reported in the previous literatures,^[28] the highly electron-delocalized backbones of SPs result in strong π - π stacking and in turn aggregation when SPs are in solid states or in poor solvents; the aggregation of SPs are generally accompanied by the red-shifted absorption, quenched fluorescence and different fluorescence decay. Thus, the spectral differences for these SP nanoparticles indicated that the SP segments of SPAs were less aggregated as compared with that for SPN1, and SPA2PEG2 had the least aggregated SP segments among all the nanoparticles.

The highest PL quantum yield, in conjunction with the high photostability of SPA2PEG2 as witness by its unchanged fluorescence under continuous illumination at 640 nm for more than 60 min (Figure 3f), revealed that SPA2PEG2 should be the best candidate among these nanoparticles for fluorescence imaging applications. The capability of SPA2PEG2 for cell imaging was studied and compared with SPN1 using confocal laser scanning microscopy (CLSM). Both SPN1 and SPA2PEG2 entered Hela cells as observed by red fluorescence in the cellular cytoplasm. However, SPA2PEG2-treated cells had higher red fluorescence as compared with SPN1-treated cells (Figure 3g). Imaging quantification further revealed that the red fluorescence of SPA2PEG2-treated cells was ~8-fold higher than that of SPN1-treated cells

(Figure 3h). These data showed that the higher fluorescence quantum yield of SPA2PEG2 relative to SPN1 make it a better candidate for fluorescence cell imaging.

Figure 3. UV-vis and fluorescent properties of SPN1 and SPAs. Normalized absorption (a), fluorescence spectra (b) of SPN1 and SPAs solutions in PBS (pH = 7.4). Fluorescent intensities (c) and IVIS fluorescence images (d) of SPN1 and SPAs solutions in PBS with normalized absorption in 640 nm. (e) Fluorescent lifetimes of SPN1 and SPAs solutions in PBS. (f) Fluorescence stability of SPA2PEG2 under continuous irradiation for 1 h. (g) Confocal laser scanning microscopy (CLSM) images of HeLa cells after 24 h incubation with SPN1 and SPA2PEG2. The nuclei were stained by 4',6-diamidino-2-phenylindole (DAPI) indicated as blue color. (h) Comparison of PL intensities of HeLa cells stained with SPN1 and SPA2PEG2. The error bars represent the standard deviation of three separate measurements.

To find out the effect of PEG grafting density on the optical properties of SPAs, the quantum yields of SPAs was correlated with the absorption of triazole at 254 nm. Because triazole groups were generated along with the reaction of PEG grafting, the number of triazole

groups was identical to that of PEG chains. Thus, the absorption of triazole at 254 nm represented the PEG grafting density. As shown in **Figure 4a**, a linear relationship was observed for the quantum yields and the absorption at 254 nm, proving that a high PEG grafting density was beneficial to the fluorescence brightness of SPAs. Theoretical simulation was further conducted to reveal the effect of PEG grafting density on the nanostructures of SPAs. The models of SPA2PEG (high grafting density) and SPA1PEG (low grafting density) were constructed by coarse grained model (Figure 4b). The simulation results illustrated that the SP backbone in the core of SPA2PEG nanoparticle was obviously less aggregated as compared to that for SPA1PEG nanoparticle (Figure 4c). Moreover, the densities of SP core and PEG shell of SPA2PEG within 2 nm from center was respectively lower and higher than those of SPA1PEG (Figure 4d). These differences were attributed to the stronger side-chain hindrance caused by the higher density of PEG grafts within SPA2PEG relative to that of SPA1PEG. The experimental and simulation data clearly confirmed that an increase in the PEG grafting density substantially reduced the aggregation of SP segments within the core of SPAs but increasing the density of PEG shell, ultimately contributing to enhanced fluorescence brightness.

Figure 4. Theoretical simulation of SPAs. (a) Quantum yield of SPAs as a function of Abs at 254 nm of SPAs. (b) The coarse grained model for simulation of SPA2PEG and SPA1PEG. (c) The structures of nanoparticles and hydrophobic SP cores of SPA2PEG and SPA1PEG. (d) The densities of main chains and PEG chains of SPAs as a function of distance from the center of nanoparticle.

Figure 5. PA properties of the nanoparticles. (a) PA spectrum of SPA2PEG2 in PBS (pH = 7.4). (b) PA signals of GNRs, SPN1 and SPAs at 680 nm at the same mass concentration of the optical components. (* $p > 0.05$) (c) The PA amplitudes at 680 nm as a function of concentrations of SPA2PEG2. (d) PA images of SPA2PEG2 at different concentrations. The error bars represent the standard deviation of five separate measurements.

The PA properties of the nanoparticles were then measured and compared at the same optical concentrations at 680 nm. The PA spectrum of SPA2PEG2 was close to its absorption ranging from 680 to 760 nm, both of which showed the maximum intensity at 680 nm (**Figure 5a**). Furthermore, despite the difference in fluorescence, all the nanoparticles showed almost the same PA brightness at 680 nm (**Figure 5b**), suggesting that the PEG density had no impact on the PA properties of the nanoparticles. In addition, when comparing at the same mass

concentration of the optical components, the PA amplitude of SPA2PEG2 was 3.9-fold higher than that of the gold nanorods (GNRs) with the absorption maximum at 670 nm (**Figure S8, Supporting Information**), indicating that SPA2PEG2 was a better candidate for PA imaging. This result reflected that the PA brightness was not only determined by the competition between fluorescence and non-radiative thermal decay, but also affected by the heat dissipation within the nanoparticles.^[29] As SPAs and SPN1 had the different nano-structures, the heat dissipation should vary from each other. The similar PA brightness implied that the two factors (non-radiative thermal decay and heat dissipation) were canceled out in the system. The PA amplitudes and images of SPA2PEG2 at 680 nm were determined at a series of concentrations from 0.5 to 8 mg mL⁻¹ (Figures 5c and 5d). With increasing the concentrations, the PA signals gradually increased as shown by the PA images in Figure 5d. As expected, a linear relationship between the PA intensities and the concentrations was observed for SPA2PEG2, showing the feasibility for the quantification of PA signals.

As the best candidate among all the nanoparticles, SPA2PEG2 was further evaluated for *in vivo* PA and fluorescence imaging of xenograft 4T1 tumor in living mice. Before systemic administration of SPA2PEG2, the tumors showed weak PA signals at 680 nm because of the relatively low intrinsic absorption of oxyhemoglobin and deoxyhemoglobin in the NIR region under the experimental conditions (**Figure 6a**). As oxyhemoglobin and deoxyhemoglobin had very weak autofluorescence, no signal was detected in the fluorescence image before systemic administration (Figure 6b). After systemic administration of SPA2PEG2 through tail vein injection, both the PA and fluorescence signals in tumor areas gradually increased over time (Figure 6d), implying the capability of SPA2PEG2 for passive tumor targeting through the enhanced permeability and retention (EPR) effect. The PA signals in the tumor area reached the maximum at $t = 6$ h post-injection, while the fluorescence signals reached the maximum at $t = 8$ h post-injection (Figure 6d). However, it should be noted that the PA signals in the tumor at $t = 6$ and 8 h post-injection showed no statistical difference ($p > 0.05$). At this time point, the

PA signal was 3-fold higher than that of the tumor background. As a result of high resolution, the maximum intensity projection (MIP) and 3D PA images at 6 h post-injection was able to clearly depict that PA signals came from the areas within and outside the blood vessels in the tumor (Figure 6a). This revealed that SPA2PEG2 was able to passively target the tumor and extravasate the blood vessels probably owing to their relatively small size (~ 35 nm in diameter).

Figure 6. *In vivo* imaging of SPA2PEG2. (a) Representative PA maximum imaging projection (MIP) (upper) and 3D (lower) images of tumor from a systemic administration of a living mouse 0 h, 6 h and 24 h post-injection of SPA2PEG2 ($30 \mu\text{g mouse}^{-1}$). (b) Real-time fluorescence imaging of tumor bearing mouse after systemic administration of SPA2PEG2 ($30 \mu\text{g mouse}^{-1}$) at 0 h, 8 h and 24 h. (c) *Ex vivo* fluorescence image of major organs of mice 24 h after systemic administration of SPA2PEG2. (d) PA and fluorescence intensity in tumor area as a function of post-injection time for SPA2PEG2 intravenous injected mice. (* $p > 0.05$) (e) *In vivo* real-time PA spectra extracted from the tumors in living mice after systemic administration of SPA2PEG2 or saline for 6 h. (f) *Ex vivo* quantification of ΔPA and $\Delta\text{fluorescence}$ of major organs from mice ($n = 3$) 24 h after systemic administration of SPA2PEG2. ΔPA and $\Delta\text{fluorescence}$ were calculated by subtracting the intensities for the organs of saline-treated mice from those of the corresponding organs of SPA-injected mice. The *ex vivo* signals for the

organs of saline-treated mice are shown in **Figure S9, Supporting Information**. Error bars represent standard deviations of three separate measurements ($n=3$).

Real-time *in vivo* PA spectra extracted from the tumors of SPA2PEG2-treated mice at $t = 6$ h resembled the PA spectrum of SPA2PEG2 in solution but differed from that of saline-treated mice (Figure 6e vs Figure 5a). Besides, the PA intensity at 680 nm for the tumor of SPA2PEG2-treated mice was ~ 3 -fold higher than that for saline-treated mice, consistent with the PA data in Figure 6d. Such a spectral similarity verified that the enhancement of PA signals in tumor areas came from the accumulation of SPA2PEG2. The *ex vivo* biodistribution for SPA2PEG2 was acquired by both PA and fluorescence at $t = 24$ h post-injection (Figures 6c&6f). Tumors had the strongest PA and fluorescence signals, which was followed by liver, kidney, spleen, lung, intestine and muscle. Moreover, fluorescence signals of tumor were ~ 1.8 -fold higher than that of liver, indicating preferred tumor accumulation for SPA2PEG2. According to our previous reports,^[19,22] the SPNs prepared by coprecipitation with PEG-*b*-PPG-*b*-PEG often had the highest uptake in liver instead of tumor despite the similar PEG coating. Thereby, the ideal biodistribution of SPA2PEG2 should be attributed to its non-dissociable nanostructure passivated by a dense PEG shell that minimized the reticuloendothelial uptake and promoted the EPR effect.

3. Conclusion

We have designed and synthesized a series of NIR-absorbing SPAs composed of a hydrophobic SP backbone grafted by the hydrophilic PEG chains and applied them for *in vivo* PA imaging. The amphiphilic nature of SPAs allowed them to spontaneously self-assemble into homogenous nanoparticles in biologically-relevant medium with the small diameter of ~ 35 nm. As compared with their counterpart nanoparticle (SPN1) prepared by nanoprecipitation, SPAs generally had higher fluorescence quantum yields but similar PA brightness, showing that SPAs were superior to SPN1 for optical imaging applications. In particular, simulation results revealed that the SPA with a higher PEG grafting density had a looser core with which SP segments were less

aggregated relative to that for the SPA with a lower PEG grafting density. This inner nanoparticulate difference led to the highest fluorescence quantum yield of SPA2PEG2 with the highest PEG grafting density among all the nanoparticles, which was ~11-fold higher than that of SPN1. By virtue of its small size and structurally stable nanostructure, SPA2PEG2 was able to passively target the tumor of living mice after systemic administration through tail vein injection, permitting both PA and fluorescence imaging of tumor. At the optimal time point, the PA signals for the tumor of SPA2PEG2-administered mice was 3-fold higher than that for the background noise of tumor. Moreover, the ultrahigh PEG density and non-dissociable nanostructure of SPA2PEG2 endowed it with reduced reticuloendothelial uptake and promoted EPR effect, leading to the tumor signals that were the highest among all the tested organs (~1.5-fold higher than that of liver).

Our study thus not only provides the first generation of amphiphilic optically-active polymers for PA imaging but also highlight the importance of PEG grafting density in determining their optical and biophysical features for *in vivo* applications. By virtue of the self-assembly nature, our NIR-absorbing SPAs should also suit for encapsulation of drug molecules or sensing components in the hydrophobic domain, permitting other theranostics and molecular imaging applications.

4. Experimental Section

Chemicals: All chemicals were purchased from Sigma-Aldrich unless otherwise mentioned. 2,2'-(9,9-Dioctyl-9H-fluorene-2,7-diyl)bis(4,4,5,5-tetramethyl-1,3,2-dioxaborolane) (FLR), 2,5-bis(6-bromohexyl)-3,6-bis(5-bromothiophen-2-yl)pyrrolo[3,4-c]pyrrole-1,4(2H,5H)-dione (DPP-Br) and 3,6-Bis(5-bromothiophen-2-yl)-2,5-bis(2-hexyldecyl)pyrrolo[3,4-c]pyrrole-1,4(2H,5H)-dione (DPP) were purchased from Luminescence Technology Corp.. Poly(ethylene glycol) methyl ether alkyne (methoxy-PEG-alkyne, $M_n = 2000$ and 5000) were purchased from J&K Scientific Ltd. 2,7-Bis[9,9(-bis(6-bromohexyl)-fluorenyl)-4,4,5,5-tetramethyl-1,3,2]dioxaborolane (FLR-Br) was synthesized according to the previous literature.^[30]

Characterization: DLS was performed on a Malvern Nano-ZS Particle Sizer. TEM images were captured from a JEM 1400 transmission electron microscope with an accelerating voltage from 40 to 120 kV. ^1H NMR spectra were recorded using a Bruker Avance II 300MHz NMR, CDCl_3 was used as the solvent. GPC results were obtained by Shimadzu LC-VP system with polystyrenes as the standard and high purity of THF as the eluent. UV-vis spectra were obtained from a Shimadzu UV-2450 spectrophotometer. Fluorescence measurements were carried out on a Fluorolog 3-TCSPEC spectrofluorometer (Horiba Jobin Yvon). Luminescence lifetime and photostability measurements were achieved on Edinburgh Instruments (F 900). Fluorescence images were obtained by IVIS spectrum imaging system. Quantum yields of SPAs were measured using Indocyanine Green (ICG) as the standard with a known quantum yield of 1% in H_2O .^[27]

PA Instrumentation: An optical parametric oscillator, OPO (Continuum, Surelite), pumped by a Q-switched 532 nm Nd:YAG laser was used as the excitation source. OPO can generate tunable laser pulses within 680-920 nm wavelength range with 5 ns pulse duration, 100 mJ pulse⁻¹ 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 in 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. Respective PA signals were collected using the UST and these signals were subsequently amplified with 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 Procedure for SP1, SP1-Br and SP2-Br: DPP (0.5 mmol), FLR (0.5 mmol), Palladium-tetrakis(triphenylphosphine) ($\text{Pd}(\text{PPh}_3)_4$) (5 mg) and potassium carbonate (828 mg, 6 mmol) were placed in a 50 mL schlenk tube. 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 and then the solvent was removed under reduced pressure. The obtained solid was re-dissolved by excess dichloromethane and washed with water for three times. The organic phase was concentrated and precipitated into excess methanol. The obtained solid was washed three times by methanol and then dried under vacuum for 24 h to afford the SPs. SP1: ^1H NMR (300 MHz, CDCl_3 , δ): 8.99 (br, 2H), 7.80-7.67 (m, 4H), 7.64 (s, 2H), 7.56 (s, 2H), 4.12 (br, 4H), 2.05 (br, 6H), 1.48-0.95 (m, 68H), 0.91-0.56 (m, 22H); SP1-Br: ^1H NMR (300 MHz, CDCl_3 , δ): 9.00 (br, 2H), 7.87-7.53 (m, 8H), 4.21 (br, 4H), 3.44 (br, 4H), 2.07 (br, 4H), 1.90 (br, 8H), 1.40 (br, 2H), 1.26 (br, 4H), 1.22-0.95 (m, 20H), 0.92-0.56 (m, 12H); SP2-Br: ^1H NMR (300 MHz, CDCl_3 , δ): 8.97 (br, 2H), 7.74 (d, 4H), 7.65 (br, 2H), 7.59 (br, 2H), 4.19 (br, 4H), 3.44 (br, 4H), 3.28 (br, 4H), 2.09 (br, 4H), 1.90 (br, 8H), 1.67 (br, 4H), 1.40 (s, 2H), 1.31-1.00 (br, 4H), 1.22-0.95 (m, 10H), 0.92-0.51 (m, 4H).

General Procedure for SP1-N₃ and SP2-N₃: SP1-Br or SP2-Br (10 mg) was dissolved into a mixture of THF (3 mL) and DMF (4 mL). Then sodium azide (2 equiv to bromide group of SP1-Br or SP2-Br) was added into the solution. The reaction was carried out at room temperature overnight. After that the solvent was removed under reduced pressure, an excess of dichloromethane was added to the residue. The resulting solution was washed three times with water and the organic phase was collected. The obtained solution was then concentrated and precipitated into excess methanol to obtain solid which was washed with methanol for three times. The obtained solid was dried under vacuum overnight to obtain SP1-N₃ or SP2-N₃. SP1-

N₃: ¹H NMR (300 MHz, CDCl₃, δ): 9.00 (br, 2H), 7.87-7.53 (m, 8H), 4.21 (br, 4H), 3.30 (br, 4H), 2.07 (br, 4H), 1.90 (br, 8H), 1.40 (br, 2H), 1.26 (br, 4H), 1.22-0.95 (m, 20H), 0.92-0.56 (m, 12H); SP2-N₃: ¹H NMR (300 MHz, CDCl₃, δ): 8.97 (br, 2H), 7.74 (d, 4H), 7.65 (br, 2H), 7.59 (br, 2H), 4.19 (br, 4H), 3.30 (br, 4H), 3.13 (br, 4H), 2.09 (br, 4H), 1.90 (br, 8H), 1.67 (br, 4H), 1.40 (s, 2H), 1.31-1.00 (br, 4H), 1.22-0.95 (m, 10H), 0.92-0.51 (m, 4H).

General Procedure for SPA1PEG and SPA2PEG: SPA1PEG and SPA2PEG polymers were prepared by using CAAC reaction. Briefly, SP1-N₃ or SP2-N₃ (3 mg) was dissolved into a mixture of THF (3 mL) and DMF (4 mL) solution. Copper(II) sulfate pentahydrate (2 equiv to azide group of SP1-N₃ or SP2-N₃), sodium ascorbate (10 equiv to azide group of SP1-N₃ or SP2-N₃) and methoxy-PEG-alkyne (M_n = 2000 or 5000, 2 equiv to azide group of SP1-N₃ or SP2-N₃) were added into previous solution subsequently. The reaction was carried out at room temperature under nitrogen atmosphere for 48 hour. After that the solvents were removed under reduced pressure and the remaining residue was dissolved into water. The resulting solution was dialysis against DI water to remove the salt and excess methoxy-PEG-alkyne in the system. The SPA1PEG or SPA2PEG polymers were obtained after lyophilization. SPA1PEG and SPA2PEG: ¹H NMR (300 MHz, CDCl₃, δ): 7.89-7.43, 4.67, 4.61, 4.40-4.11, 3.88, 3.65, 3.55, 3.38, 1.25, 1.18-0.97, 0.92-0.50.

Preparation of Nanoparticles: SPN1 was prepared by a nanoprecipitation method. Briefly, SP1 (0.25 mg) and PEG-*b*-PPG-*b*-PEG (20 mg) were dissolved into 1 mL of THF. The obtained solution was rapidly injected into a mixture of water (9 mL) and THF (1 mL) solution under continuous sonication with a sonicator under 110 W for 1 min. Then THF in the solution was removed under a gentle nitrogen flow, and the resulting solution was purified by filtration through a 0.22 μm PVDF syringe driven filter (Millipore). The obtained SPN1 solution was concentrated to different concentrations through ultrafiltration and then stored under 4 °C for further use. SPA1PEG2, SPA1PEG5, SPA2PEG2 and SPA2PEG5 nanoparticles were prepared

by directly dissolving the corresponding polymers into DI water under continuous sonication for 5 min. The resulting solutions were stored under 4 °C.

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 which contains 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 SPA2PEG2 (final concentrations: 5, 10, 25, 50 and 100 µg mL⁻¹) 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⁻¹) 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 SPA2PEG2 solution to that of the cells incubated with cell culture medium only.

Cell Imaging: Hela cells were cultured based on the method described above. The cells were respectively incubated with SPN1 and SPA2PEG2 (20 µg mL⁻¹) for 24 h before staining the nuclei with DAPI. Confocal images of the cells were captured by using a LSM510 confocal laser scanning microscopy (Carl Zeiss, Germany) with the excitation wavelength of 640 nm for samples and 405 nm for DAPI.

Coarse Grained Model for SPAPEG Amphiphiles: In order to simulate SPAPEG amphiphiles, a coarse-grained model of the SPAPEG was established (Figure 5a). Red beads (diameter 0.5 nm) were the hydrophobic main chains (N=20), which means every SPA monomer composed by 4 beads. Cyan and blue beads (diameter 0.35 nm) were the hydrophobic linkers and PEG (m=45) respectively. In our model, we assumed that only half of the linkers were attached by PEG chains for both low and high density bottle brushes due to the molecular crowding effects of PEG (PEG reaction rate 50%). Since the experiments observed a similar micelle size for low and high tethering density systems, the number of chain number in the micelle was fixed to be 5 in both cases for the convenience of direct comparison.

Tumor Mouse Model: All animal experiments were performed in compliance with the Guidelines established by the Institutional Animal Care and Use Committee (IACUC), Sing Health. To establish tumor models in six-week-old female nu/nu mice, two million 4T1 cells suspended in 50 mL of 50% v/v mixture of Matrigel in supplemented DMEM (10% fetal bovine serum, 1% pen/strep (100 U mL⁻¹ penicillin and 100 µg mL⁻¹ 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 used for *in vivo* imaging experiments.

In Vivo PA Imaging of Tumor: 4T1 tumor xenografted nude mice were anesthetized using 2% isoflurane in oxygen, and 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 systemic administration with SPA2PEG2 (30 µg in 120 µL) (n=3) or saline (120 µL) (n=3) through catheter. Data was acquired through a continuous model that took 12 s to obtain one data set. For *ex vivo* PA imaging, mice were sacrificed by CO₂ asphyxiation, and organs were embedded in agar phantom and acquired immediately with Endra Nexus128 PA imaging system. Three-dimensional PA image was reconstructed off-line using data acquired from all 128 transducers at each view and a back-projection algorithm. The algorithm corrects for pulse-to-pulse variations in the laser intensity and small changes in the temperature that affect acoustic velocity in the water. The reconstructed raw data was analyzed using OSiriX software.

In Vivo Fluorescent Imaging: 4T1 tumor xenografted nude mice were anesthetized using 2% isoflurane in oxygen, SPA2PEG2 was systematically injected through the tail vein using a microsyringe. Fluorescence animal imaging was performed using IVIS spectrum imaging system. Fluorescence images of the mice were acquired at designated time points after nanoparticle administration. The first and last PA images were recorded 30 min and 24 h post-injection, respectively. For *ex vivo* fluorescence imaging, mice were sacrificed by CO₂ asphyxiation, and the tumor, liver, kidney, spleen, lung, intestine and muscle were harvested

for *ex vivo* fluorescence imaging to estimate the tissue distribution of the SPA2PEG2 nanoparticles.

Data Analysis: PA signal intensities were measured by region of interest (ROI) analysis using OsiriX. Intensities of fluorescence were determined by ROI analysis using IVIS living imaging system. Results are expressed as the mean $\pm$ SD deviation unless otherwise stated. All statistical calculations were performed using GraphPad Prism v. 6 (GraphPad Software Inc., CA, USA).

Supporting Information

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

Acknowledgement

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The table of contents entry should be 50–60 words long:

Near-infrared absorbing semiconducting polymer amphiphiles (SPAs) that can spontaneously self-assemble into homogenous nanoparticles are synthesized. The small size and structurally-stable nanostructure in conjunction with a dense PEG shell allow SPAs to passively target the tumor of living mice after systemic administration, permitting both photoacoustic and fluorescence imaging of tumor.

Keyword: polymer amphiphile, photoacoustic imaging, nanoparticles, self-assembly

C. Xie, X. Zhen, Q. Lei, R. Ni and K. Pu*

Self-Assembly of Semiconducting Polymer Amphiphiles for In Vivo Photoacoustic Imaging