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Self-Assembled Semiconducting Polymer Nanoparticles for Ultrasensitive Near-Infrared Afterglow Imaging of Metastatic Tumors

Chen Xie, Xu Zhen, Qingqing Miao, Yan Lyu, and Kanyu Pu*

Detection of metastatic tumor tissues is crucial for cancer therapy; however, fluorescence agents that allow to do share the disadvantage of low signal-to-background ratio due to tissue autofluorescence. The development of amphiphilic poly(*p*-phenylenevinylene) derivatives that can self-assemble into the nanoagent (SPPVN) in biological solutions and emit near-infrared afterglow luminescence after cessation of light irradiation for ultrasensitive imaging of metastatic tumors in living mice is herein reported. As compared with the counterpart nanoparticle (PPVP) prepared from the hydrophobic PPV derivative, SPPVN has smaller size, higher energy transfer efficiency, and brighter afterglow luminescence. Moreover, due to the higher PEG density of SPPVN relative to PPVP poly(ethylene glycol), SPPVN has a better accumulation in tumor. Such a high sensitivity and ideal biodistribution allow SPPVN to rapidly detect xenograft tumors with the size as small as 1 mm³ and tiny peritoneal metastatic tumors that are almost invisible to naked eye, which is not possible for PPVP. Moreover, the oxygen-sensitive afterglow makes SPPVN potentially useful for in vivo imaging of oxygen levels. By virtue of enzymatic biodegradability and ideal in vivo clearance, these organic agents can serve as a platform for the construction of advanced afterglow imaging tools.

Detection of metastatic tumor tissues is crucial for cancer therapy, because metastasis is one of the major causes of death from cancer, and its associated complications result in over 90% mortalities.^[1] Optical imaging provides a convenient way to delineate tumor tissues and has been exploited even in clinics for guiding surgery of cancer.^[2] Several kinds of optical imaging agents such as small molecule probes,^[3,4] quantum dots,^[5] graphene oxide,^[6] inorganic nanoparticles,^[7,8] and macromolecular nanoprobe^[9,10] have been developed for detection of tumor metastases. However, these agents rely on fluorescence imaging that requires real-time light excitation, and thus their signals are inevitably mixed with tissue autofluorescence. Thus, fluorescence optical agents generally have low signal-to-background ratios (SBRs) and thus limited sensitivity for detection of metastatic tumor tissues.^[11]

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An emerging paradigm for low-background optical imaging consists in the development of luminescent agents that can emit luminescence after cessation of light excitation, often termed as persistent or afterglow luminescence.^[12] So far, only a few kinds of inorganic nanoparticles have been reported to maintain sufficient afterglow luminescence under biologically related conditions.^[13–15] These persistent luminescent nanoparticles often contain rare-earth metals or heavy metals (such as europium, praseodymium, and chromium) as the energy trap to delay luminescence.^[16–18] Although those metal ions are well known to be toxic, no cytotoxicity and acute in vivo toxicity have been reported for these inorganic nanoparticles, probably because of their low amount in the total particle dosage (100 µg per mouse).^[19] So far, inorganic persistent luminescent nanoparticles have been applied for tumor imaging,^[16] vascular imaging,^[20] in vivo cell tracking,^[17] and in

vitro cell targeting.^[21] However, existing persistent luminescent nanoparticles are not biodegradable, posing a biocompatibility concern for long-term biological applications.^[22]

Semiconducting polymer nanoparticles (SPNs) have evolved into an alternative category of optical agents for molecular imaging and phototherapy.^[23] Dependent on the structures of semiconducting polymers, molecular electronic transition within SPNs can be varied for fluorescence,^[24–26] chemiluminescent,^[27] and photoacoustic imaging^[28,29] as well as for photodynamic^[30,31] and photothermal therapy.^[32,33] By virtue of such versatility, we and others have demonstrated their applications in tumor imaging,^[34,35] evolution of drug-induced toxicity,^[36] monitoring physiological indexes and biomarkers in diseases sites,^[37] and even controlling protein-ion channels^[38] and gene expression.^[39] Recently, we accidentally discovered that poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylenevinylene] (MEH-PPV) could emit light after removal of light excitation.^[40,41] Such light emission can be considered as one type of afterglow luminescence. However, because MEH-PPV is highly hydrophobic, an amphiphilic polymer was required to coprecipitate with it to form water-soluble nanoparticles.^[41] Thus, the resulted nanoparticles were binary micelles with a relatively large size, which could compromise biodistribution and result in unfavorable accumulation in tumor.

We herein report the design and synthesis of water-soluble amphiphilic poly(*p*-phenylenevinylene) (PPV) derivatives for in vivo afterglow luminescence imaging of metastatic tumors in living mice. These polymers have a hydrophobic PPV backbone and hydrophilic poly(ethylene glycol) (PEG) brushes, and thus are able to self-assemble into small nanoparticles in aqueous solution. Such a structure eventually results in preferred accumulation in the tumor of living mice, allowing for ultrasensitive in vivo imaging. In the following, we first describe the synthesis and characterization of the amphiphilic PPV derivatives. Then, we compare their physical and optical properties with our previously reported nanoparticles prepared from coprecipitation of hydrophobic MEH-PPV and an amphiphilic triblock copolymer (PEG-*b*-PPG-*b*-PEG). Finally, we show the advantages of the amphiphilic PPV derivatives in terms of afterglow imaging of ultrasmall and metastatic tumors in living mice.

To endow PPV with good water solubility, the PPVs were designed to have PEG as grafting chains (Scheme 1). First, 4-methoxyphenol was, respectively, reacted with 1,10-dibromodecane and 2-ethylhexyl bromide to give compounds 2 and 3. Compounds 2 and 3 were then treated with paraformaldehyde and HBr to give monomers 4 and 5, respectively. Monomer 4 was then polymerized in the presence of potassium *tert*-butoxide to give the PPV polymer (PPV-Br1) with the bromide groups on the side chains. To synthesize PPV with lower PEG grafting density, monomers 4 and 5 were copolymerized and the molar ratio of monomer 4 to 5 was 8 to 1 to give PPV-BrL. Then, these bromide PPVs were reacted with sodium azide to substitute bromide with azide, affording PPV-N₃1 and PPV-N₃L. The conversion of bromide into azide was confirmed by proton nuclear magnetic resonance (¹H NMR) spectra (Figures S1 and S2, Supporting Information), which showed the shift of the resonance peak of -CH₂Br (3.40 ppm for PPV-Br1, 3.39 ppm for PPV-BrL) into the peak of -CH₂N₃ (3.24 ppm for PPV-N₃1, 3.23 ppm for PPV-N₃L) for both PPV-N₃1 and PPV-N₃L. Finally, PPV-N₃1 and PPV-N₃L were, respectively, reacted with propargyl end-capped PEG (*M*_n = 2000) (PEG-alkyne) through copper(I)-catalyzed alkyne-azide cycloaddition (CuAAC) reaction to give the PEG grafted PPV polymers: PPV-PEG1 and

PPV-PEGL. Their structures were confirmed by ¹H NMR. Taking the ¹H NMR of PPV-PEGL as the example, the peaks of PPV segments (7.54, 7.19, 5.34, 4.67, 4.14–3.79, 1.45–1.11, and 1.09–0.66 ppm) and the peak of the repeating unit of PEG (3.65 ppm) were detected (Figure S3, Supporting Information). Gel permeation chromatography (GPC) showed that both PPV-PEG1 and PPV-PEGL had a molecular weight higher than their corresponding precursors (PPV-Br1 and PPV-BrL), confirming the grafting of PEG onto the PPVs backbone (Table S1, Supporting Information).

Because of the amphiphilicity, PPV-PEG1 could be directly dissolved in phosphate buffer solution (PBS) and self-assemble into small nanoparticles. The optical properties and afterglow mechanism of PPVs were studied (Figures S4 and S5, Supporting Information). PPV-PEG1 exhibited strong afterglow luminescence and its spectrum was almost identical to the fluorescence spectrum with the emission maximum at 580 nm (Figure S4c, Supporting Information). The underlying mechanism of afterglow luminescence (Figure S4f, Supporting Information) was the same as our reported nanoparticles: singlet oxygen (¹O₂) generated from PPVs upon light irradiation can react with the vinyne bond to form dioxetane units,^[42] which can generate photons upon degradation.^[27,36,41] To amplify and redshift afterglow luminescence to NIR region, a ¹O₂ sensitizer, silicon 2,3-naphthalocyanine bis(triethylsilyloxy) (NCBS), was doped into PPV-PEG1 (Figure S6, Supporting Information). However, fluorescence spectra and DLS results indicated the poor fluorescence resonance energy transfer (FRET) from PPV segments to NCBS and low encapsulation efficiency, which should be attributed to the high grafting density of PEG for PPV-PEG1.

To better encapsulate NCBS, PPV-PEGL with a lower PEG grafting density relative to PPV-PEG1 was used to prepare the NCBS-doped nanoparticles (Figure 1a). DLS results, UV and fluorescence spectra showed the successful encapsulation of NCBS and efficient FRET from PPV segments to NCBS (Figure S7a–c, Supporting Information). The afterglow spectra profiles of NCBS-doped nanoparticles were similar to that of the fluorescence spectra (Figure S7d, Supporting

Scheme 1. Synthetic routes of PPV-PEG1 and PPV-PEGL. Reagents and conditions: (i) 1,10-dibromodecane, sodium methoxide, ethanol, refluxing, 2 h; (ii) 2-ethylhexyl bromide, sodium methoxide, ethanol, refluxing, 2 h; (iii) paraformaldehyde, HBr (33 wt% in acetic acid), acetic acid, 70 °C, 4 h; (iv) potassium *tert*-butoxide, tetrahydrofuran (THF), 25 °C, overnight; (v) sodium azide, THF/*N,N*-dimethylformamide (DMF), 40 °C, overnight; (vi) CuBr, *N,N,N',N'',N'''*-pentamethyldiethylenetriamine (PMDETA), PEG-alkyne, THF, 25 °C, 48 h.

Figure 1. Property comparison of SPPVN with PPVP. Schematic illustration of the preparation of a) SPPVN and b) PPVP. c) DLS of SPPVN and PPVP in 1× PBS buffer (pH = 7.4). Inset: representative TEM images of SPPVN and PPVP. The scale bars represent 100 nm. d) Absorption and e) fluorescence spectra of SPPVN and PPVP in 1×PBS buffer (pH = 7.4). f) Afterglow luminescence spectra of SPPVN and PPVP under same mass concentration (130 $\mu\text{g mL}^{-1}$). SPPVN and PPVP solutions were pre-irradiated for 1 min by 514 nm light before the collection of afterglow luminescence signal. The laser power used in the experiments was 1 W cm^{-2} . The error bars represent the standard deviations of three separate measurements.

Information). In particularly, SPPVN (2 w/w% NCBS) had the highest afterglow luminescence intensity among all nanoparticles (Figure S7e, Supporting Information). The afterglow luminescence of SPPVN was long-lasting with a half-life of 4.8 min in 1× PBS buffer at room temperature (Figure S7f, Supporting Information). Moreover, the afterglow intensity of SPPVN induced by pre-irradiation at 808 nm was eight times higher than that induced by pre-irradiation 514 nm, because more $^1\text{O}_2$ (2.6-fold) could be generated from NCBS upon irradiation at 808 nm (Figure S8, Supporting Information). The similar amplified afterglow phenomenon was detected when another photosensitizer, meso-tetraphenylporphyrin (TPP), was used (Figure S9, Supporting Information). As compared with 2% TPP doped nanoparticles (SPPVT), SPPVN had longer emission wavelength (780 vs 660 nm) and higher energy transfer efficiency (51 vs 37%), as well as higher enhancement in the afterglow intensities (20 vs 1.8-fold) than nondoped nanoparticles. Thus, SPPVN with 2 w/w% NCBS was chosen for further studies.

The properties of SPPVN were compared with the previously reported counterpart PPVP, which was prepared via nanoprecipitation of MEH-PPV and NCBS (2 w/w% relative to MEH-PPV) in the presence of an amphiphilic triblock copolymer (PEG-*b*-PPG-*b*-PEG) (Figure 1b). SPPVN had a much smaller hydrodynamic size than that of PPVP (24 vs 34 nm) (Figure 1c).

SPPVN and PPVP had similar UV absorption spectra with two maximum peaks at ≈ 500 and 775 nm, corresponding to the absorption of PPV and NCBS, respectively (Figure 1d). Both SPPVN and PPVP had much stronger fluorescence of NCBS at 775 nm than that of PPV segments at 590 nm; however, the ratio of emission intensity at 780 nm to that of 590 nm for SPPVN was 2.1-fold higher than PPVP, indicating the higher FRET efficiency for SPPVN than PPVP (51 vs 24%) (Figure 1e). The afterglow luminescence spectra of SPPVN and PPVP were similar to their fluorescence spectra, but the afterglow intensity of SPPVN was 1.3-fold higher than that of PPVP under same mass concentration (Figure 1f). Such an improvement in FRET and enhancement in afterglow should be attributed to the closer contact between the PPV segments and NCBS within SPPVN than that in PPVP.

After confirming the excellent physiological stability (Figure S10a, Supporting Information) and cytocompatibility (Figure S10b, Supporting Information), SPPVN was evaluated for in vivo imaging experiments. The tissue-penetration ability of afterglow imaging was compared with NIR fluorescence imaging by detection of the SPPVN solution under a living mouse with the depth of 1.6 cm (Figure 2a). The fluorescence signal was acquired at 780 nm under the excitation at 710 nm, while the afterglow luminescence signal was acquired under bioluminescence mode in IVIS living imaging system after

Figure 2. In vivo tissue-penetration study and lymph node imaging. a) Schematic illustration of fluorescence and afterglow imaging through a living mouse. SPPVN solution was placed under the mouse with the depth of 1.6 cm. b) Fluorescence and afterglow luminescence images of SPPVN solution through a living mouse. The fluorescence image was acquired at 780 nm with excitation at 710 nm. Before the collection of afterglow luminescence images, SPPVN solution ($130 \mu\text{g mL}^{-1}$, $50 \mu\text{L}$) was pre-irradiated at 514 or 808 nm (1 W cm^{-2}) for 1 min. c) SBRs of fluorescence and afterglow luminescence imaging in Figure 4b. d) Schematic illustration of lymph node imaging. e) Fluorescence and afterglow luminescence images of a lymph node in the living mouse at 60 min after intradermal injection of SPPVN ($450 \mu\text{g mL}^{-1}$, $50 \mu\text{L}$) into the forepaw of mouse. Afterglow luminescence images were acquired for 30 s after laser irradiation at 808 nm (0.3 W cm^{-2}) for 1 min. f) SBRs of fluorescence and afterglow luminescence imaging of lymph node in Figure 4e. g) Schematic illustration of hypoxia and normoxia imaging in a living mouse. SPPVN was purged N_2 to remove oxygen before injection. h) Fluorescence and afterglow luminescence images of tumor and skin after local injection of SPPVN ($130 \mu\text{g mL}^{-1}$, $50 \mu\text{L}$). i) Fluorescence and afterglow intensities of tumor and skin after local injection of SPPVN ($130 \mu\text{g mL}^{-1}$, $50 \mu\text{L}$). Error bars represent standard deviations of three separate measurements ($n = 3$). n.s.: not significant, **statistically significant difference ($p < 0.01$, $n = 3$).

pre-irradiation at 514 or 808 nm for 1 min. Because of strong tissue autofluorescence ($2.06 \times 10^7 \pm 1.90 \times 10^6 \text{ p s}^{-1} \text{ cm}^{-2} \text{ sr}^{-1}$), the fluorescence signal from the SPPVN solution was barely distinguishable from the background signal (Figure 2b). In contrast, the background signal of afterglow imaging was very low ($2900 \pm 420 \text{ p s}^{-1} \text{ cm}^{-2} \text{ sr}^{-1}$) due to the elimination of real-time light excitation. Thus, the signal was clearly detectable under both pre-irradiation at 514 and 808 nm. The SBR for afterglow imaging induced by pre-irradiation at 808 nm was 404 ± 63 (Figure 2c), which was ≈ 5.0 and ≈ 304 -fold higher than the afterglow imaging induced by pre-irradiation at 514 nm (80.4 ± 12.5) and NIR fluorescence imaging (1.3 ± 0.2), respectively. These results demonstrated the significantly higher penetration depth and imaging sensitivity of afterglow imaging relative to NIR fluorescence imaging.

The afterglow luminescence of SPPVN decayed slightly faster in vivo as compared with that of in vitro (Figure S11a vs S7f, Supporting Information), because of the higher environment temperature in living mice. Nevertheless, the afterglow of SPPVN could be repeatedly induced by pre-irradiation at 808 nm in living mice for at least six times without obvious decrease in the afterglow intensity (Figure S11b, Supporting Information), demonstrating its ability for long-term in vivo imaging. In comparison with existing inorganic nanoparticles with the emission maxima up to 716 and the half-life up to 200 s,^[12–15] SPPVN has both longer NIR emission (780 nm) and half-life (288 s). Moreover, in vivo afterglow intensity of subcutaneously injected SPPVN ($1.36 \times 10^5 \text{ p s}^{-1} \text{ cm}^{-2} \text{ sr}^{-1} \mu\text{g}^{-1} \text{ mL}^{-1}$) is 27.2-fold higher than that of inorganic persistent nanoparticles such as $\text{ZnGa}_2\text{O}_4\text{:Cr}^{3+}$ nanoparticles (Figure S11c, Supporting

Information). As a result, the SBR for subcutaneously injected SPPVN at $130 \mu\text{g mL}^{-1}$ (4170 ± 179) can be 15.2-fold higher than $\text{ZnGa}_2\text{O}_4:\text{Cr}^{3+}$ nanoparticles (275) even when tested at higher concentration (2 mg mL^{-1}).^[14]

The ability of SPPVN for lymph node imaging was tested after forepaw injection in living mice (Figure 2d). NIR fluorescence and afterglow luminescence images were acquired at $t = 60 \text{ min}$ post-injection. The axillary lymph node was delineated with both afterglow and fluorescence imaging. However, only afterglow imaging showed a low background image of lymph node (Figure 2e). Imaging quantification revealed that the SBR of afterglow imaging (622 ± 104) was 41-fold higher than that of fluorescence imaging (15 ± 3) (Figure 2f). Thus, afterglow imaging using SPPVN could allow mapping the lymph node with a much higher contrast than NIR fluorescence imaging.

Because afterglow was sensitive to oxygen (Figure S4e, Supporting Information), the ability of SPPVN to differentiate hypoxia and normoxia was tested in vivo. The SPPVN nanoparticle solutions were first purged with N_2 to remove residual oxygen, and then the solutions were locally injected into tumor

or under skin (Figure 2g). The fluorescence intensities of locally injected tumor and skin were almost the same (Figure 2h). In contrast, the afterglow intensity of locally injected skin was 2.4-fold higher than that of tumor (Figure 2h,i). This phenomenon was attributed to the hypoxia environment of tumor, which had a low oxygen level that reduced the afterglow intensity of SPPVN. Therefore, these results demonstrated that the oxygen-sensitive afterglow of SPPVN could be used to monitor hypoxia and normoxia.

The afterglow luminescence of SPPVN was applied for in vivo tumor imaging in comparison with PPVP. Both SPPVN and PPVP were exposed to air before injection. To test the ability of early detection, the imaging experiments were conducted for the living mice bearing the xenograft tumor with the size of only $\approx 5 \text{ mm}^3$. Fluorescence and afterglow signals were longitudinally acquired after systemic administration of nanoparticles through tail vein injection. For both SPPVN and PPVP, the afterglow and fluorescence signals gradually increased and reached saturation at 24 h post-injection, but the afterglow imaging images had higher SBR than fluorescence images at each time point (Figures 3a–c). At 24 h, the

Figure 3. In vivo tumor imaging. Fluorescence and afterglow luminescence images of living mice at different time points after intravenous injection of a) SPPVN or b) PPVP ($450 \mu\text{g mL}^{-1}$, $200 \mu\text{L}$). c) SBRs for fluorescence and afterglow imaging of tumor in living mice treated with SPPVN or PPVP as a function of post-injection time. d) Ex vivo fluorescence quantification of major organs from mice at 48 h post-injection of SPPVN or PPVP. e) Ex vivo fluorescence images of major organs of mice at 48 h post-injection of SPPVN or PPVP. The afterglow luminescence images were acquired for 30 s after laser irradiation at 808 nm (0.3 W cm^{-2}) for 1 min. The fluorescence images were acquired at 780 nm with the excitation of 710 nm . Error bars represent standard deviations of three separate measurements ($n = 3$). *Statistically significant difference ($p < 0.05$, $n = 3$).

Figure 4. In vivo peritoneal metastatic tumor imaging. a) Schematic illustration of establishment of metastatic 4T1 tumor model and the imaging procedure. b) Afterglow luminescence intensities of lower quadrant region for SPPVN or PPVP-injected mice as a function of post-injection time. The injection dosage for SPPVN and PPVP is $450 \mu\text{g mL}^{-1}$, $200 \mu\text{L}$. *Statistically significant difference at $t = 1.5$ h ($p < 0.01$, $n = 3$). c) Fluorescence and afterglow luminescence images of mice after skin resection to expose the abdominal cavity at 1.5 h post-injection of SPPVN or PPVP. The tumor regions are marked by white circles. d) Afterglow intensities of background and the tumor regions of SPPVN and PPVP-injected mice marked in (c). n.s.: not significant, **statistically significant difference ($p < 0.01$, $n = 3$). e) H&E stained slices and confocal images of slices of peritoneal metastatic tumor obtained from SPPVN or PPVP-injected mice. The tumor regions are marked by white frames. For the fluorescence images, SPPVN and PPVP nanoparticles were indicated as red color. The nuclei were stained by 4',6-diamidino-2-phenylindole (DAPI) indicated as blue color. Error bars represent standard deviations of three separate measurements ($n = 3$).

SBR in the afterglow imaging of SPPVN-injected mice was 306 ± 20 , 11-fold higher than that in the NIR fluorescence imaging (28 ± 1) (Figure 3c). Because of such high SBR, the tumor was detected with afterglow imaging as early as 40 min post-injection of SPPVN; in contrast, it could only be detected with fluorescence imaging at 4 h post-injection (Figure 3a). For PPVP-injected mice, the tumor could only be visualized at $t = 4$ and 8 h post-injection for afterglow imaging and fluorescence imaging, respectively (Figure 3b). At $t = 40$ min post-injection of nanoparticles, the SBR of afterglow imaging for SPPVN-injected mice was 40 ± 3 , ≈ 22 -fold higher than that of NIR fluorescence imaging (1.8 ± 0.2), and ≈ 2.5 -fold higher than afterglow imaging of PPVP (15.7 ± 1.5) (Figure 3c). As a result of the high sensitivity of SPPVN, its afterglow allowed to detect the tumor with the size as small as $\approx 1 \text{ mm}^3$ at 4 h post-injection with the SBR of 63 ± 10 , which was only possible at 24 h post-injection for NIR fluorescence imaging (Figure S12, Supporting Information). Note that the real-time fluorescence and afterglow luminescence signals from livers for SPPVN-injected tumor-bearing mice were almost the same at each time point for different tumor sizes (5 and 1 mm^3) (Figure S13, Supporting Information). This confirmed that the tumor size did not affect the nanoparticle biodistribution. Moreover, the tumor size that SPPVN detected (1 mm^3) was much smaller than other reported NIR fluorescence imaging probes ($50\text{--}500 \text{ mm}^3$).^[43,44]

To find out the origin of the faster delineation of tumor for SPPVN relative to PPVP, ex vivo biodistribution and in vitro multicellular tumor spheroids (MCTS) uptake were studied. In contrast to PPVP with the highest uptake in liver, SPPVN had the highest uptake in tumor, which was 1.4-fold higher than that in liver (Figure 3d,e and Figure S14, Supporting Information). Such ideal biodistribution of SPPVN was similar to our previous reports on PEGylated semiconducting polymers,^[45,46] which could be attributed to the more stable nondissociable nanostructure of SPPVN as compared with the binary micellar structure of PPVP. The PEG density of nanoparticles for SPPVN was calculated ≈ 1.7 -fold higher than that of PPVP (0.30 vs 0.18 nm^{-2}), which should be another reason for the better biodistribution of SPPVN according to the reported literature.^[47] Furthermore, MCTS uptake studies showed that SPPVN had both higher uptake (1.8 times) and deeper penetration than PPVP after the same incubation time (Figure S15, Supporting Information), probably due to its smaller size relative to PPVP (24 vs 34 nm). Thus, these data implied that in addition to higher afterglow of SPPVN relative to PPVP, the better biodistribution and deeper penetration capabilities of SPPVN relative to PPVP should be responsible for faster detection of tumor in living mice after systemic administration.

To test the ability of SPPVN over PPVP in detection of metastatic tumor tissues, the imaging experiments were conducted on the peritoneal metastatic 4T1 tumor bearing mice.

4T1 cancer cells (2×10^5) were intraperitoneally injected into mice to establish the metastatic tumor model. The mice were randomly divided into two groups and treated with SPPVN or PPVP through tail vein only 3 d after the injection of 4T1 cells (Figure 4a). Fluorescence and afterglow images were acquired after systemic administration of SPPVN or PPVP through tail vein injection. The afterglow signals in the lower quadrant region (excluding liver) gradually increased for SPPVN- but not PPVP-injected mice, indicating the obviously stronger accumulation of SPPVN in tumor tissues (Figure 4b). At $t = 1.5$ h post-injection, skin and peritoneum of mice were resected and the lower quadrant regions of the mice were imaged. For both SPPVN and PPVP-injected mice, only autofluorescence can be detected in the lower quadrant regions. In contrast, strong afterglow spots can be detected on the intestines for SPPVN-injected mice but not for PPVP-injected mice (Figure 4c). Quantification of afterglow signals showed that the afterglow intensity of tumor region for SPPVN-injected mice was 6.1-fold higher than the background signal, while the signal of PPVP-injected mice had no significant statistical difference from the background signal (Figure 4d). Formation of tiny and naked eye barely detectable metastatic tumors on the surface of intestine was confirmed by histological examinations (Figure 4e and Figure S16, Supporting Information). Moreover, the fluorescence imaging of tumor slices indicated that SPPVN had a higher accumulation in the tumor site than PPVP (Figure 4e). These results indicated the faster detection

of tiny peritoneal metastatic tumor tissues for SPPVN relative to PPVP.

The biodegradability of PPV-PEGL was studied in solution and in cell under in vivo mimetic conditions (Figure 5a). Myeloperoxidase (MPO) and H_2O_2 that could be generated in immune cells^[48] were used to mimic the physiological condition, and the concentration of MPO used ($40 \mu\text{g mL}^{-1}$) was in the range of the physiological concentration ($35.4\text{--}82.6 \mu\text{g mL}^{-1}$) calculated according to previous literature.^[49] Incubation of PPV-PEGL with MPO and H_2O_2 led to the gradual decreased and blue-shifted absorption (Figure 5b). This confirmed the enzymatic oxidation of double bonds and the degradation of PPV-PEGL into small segments. Then, PPV-PEGL was incubated with lipopolysaccharides (LPS) activated macrophage RAW264.7 cells for different times to study the intracellular biodegradability. The fluorescence of PPV-PEGL in cells gradually decreased with the increased incubation time (Figure 5c). At 12 h, the fluorescence was 2.3-fold lower than that of the cells without stimulation. These data demonstrated that PPV-PEGL was degradable in biologically relevant conditions.

To study the in vivo clearance of SPPVN, SPPVN was systemic administration into living mice through tail vein, and the fluorescence images at different time points were recorded. After i.v. injection, SPPVN was gradually accumulated into liver and the highest accumulation in liver was found at 1 d post-injection (Figure 5d and Figure S17, Supporting Information). Then, the fluorescence signal in liver gradually decreased and the signal

Figure 5. Biodegradability and clearance studies. a) Schematic illustration of the degradation of PPV-PEGL in the presence of myeloperoxidase (MPO) and H_2O_2 . b) Absorption spectra of PPV-PEGL solutions ($10 \mu\text{g mL}^{-1}$) after treatment with MPO ($40 \mu\text{g mL}^{-1}$) and H_2O_2 (100×10^{-6} M) for different time. c) Confocal fluorescence images of macrophages incubated with PPV-PEGL ($30 \mu\text{g mL}^{-1}$) and stimulated with LPS for different time. The macrophages were costained with Hoechst 33342. d) Quantification of fluorescence intensities of liver in living mice injected with SPPVN ($450 \mu\text{g mL}^{-1}$, $200 \mu\text{L}$) as a function of time. The fluorescence signals were acquired at 780 nm with the excitation of 710 nm. Error bars represent standard deviation of three separate measurements ($n = 3$).

was almost undetectable after 20 d post-injection. Such results indicated that SPPVN could be degraded in liver and cleared out through hepatobiliary excretion within 20 d in living mice.

In summary, we have developed organic and biodegradable NIR afterglow nanoagents based on the amphiphilic semiconducting polymer (PPV-PEGs) and revealed their afterglow mechanism. In addition, by doping PPV-PEGs with photosensitizer can not only redshift the afterglow luminescence spectra but also enhance the afterglow intensity because of the promoted $^1\text{O}_2$ generation under light irradiation. With the optimal doping amount of 2%, the NCBS-doped PPV-PEGL (SPPVN) shows the NIR afterglow with the emission maximum at 780 nm, which is 20-fold brighter than the nondoped PPV-PEGL. The bright and long-lasting NIR afterglow of SPPVN permits deep-tissue imaging through a living mouse with the tissue-penetration depth of 1.6 cm. It also allows detecting lymph nodes and tumor with the SBRs of 602 ± 104 and 40 ± 3 , respectively, that are more than 41 and 22 times higher than NIR fluorescence imaging. In addition, the oxygen-sensitive afterglow of SPPVN makes it promise for imaging of oxygen levels in living mice.

As compared with its counterpart MEH-PPV-based nanoparticles (PPVP), SPPVN has smaller hydrodynamic size (24 vs 34 nm), higher FRET efficiency (51 vs 24%), and brighter afterglow luminescence (1.3-fold). Because of the smaller size and higher PEG density on the nanoparticle surface, SPPVN has higher accumulation in tumor than PPVP. Thus, superior to PPVP, the afterglow of SPPVN is able to delineate the xenograft tumor at the post-injection time as short as 40 min and detect the tumor with the size as small as 1 mm^3 , as well as tiny peritoneal metastatic tumor tissues that are invisible to naked eye. Moreover, SPPVN has ideal enzymatic biodegradability and in vivo clearance. In view of their facile chemical modification, these polymeric afterglow agents can serve as a platform for the construction of ultrasensitive imaging probes for in vivo deep-tissue imaging.

Supporting Information

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

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

lymph node imaging, optical imaging, semiconducting polymer nanoparticles, tumor imaging

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