Reaction-Based Semiconducting Polymer Nanoprobes for Photoacoustic Imaging of Protein Sulfenic Acids

Yan Lyu,† Xu Zhen,† Yansong Miao,‡,† and Kanyi Pu*,†

†School of Chemical and Biomedical Engineering, Nanyang Technological University, Singapore 637457
‡School of Biological Sciences, Nanyang Technological University, Singapore 637551

Supporting Information

ABSTRACT: Protein sulfenic acids play a key role in oxidative signal transduction of many biological and pathological processes; however, current chemical tools rely on visible fluorescence signals, limiting their utility to in vitro assays. We herein report reaction-based semiconducting polymer nanoprobes (rSPNs) with near-infrared absorption for in vivo photoacoustic (PA) imaging of protein sulfenic acids. rSPNs comprise an optically active semiconducting polymer as the core shielded with inert silica and poly(ethylene glycol) corona. The sulfenic acid reactive group (1,3-cyclohexanedione) is efficiently conjugated to the surface of nanoparticles via click chemistry. Such a nanostructure enables the specific recognition reaction between rSPNs and protein sulfenic acids without compromising the fluorescence and PA properties. In addition to in vitro tracking of the production of protein sulfenic acids in cancer cells under oxidative stress, rSPNs permit real-time PA and fluorescence imaging of protein sulfenic acids in tumors of living mice. This study thus not only demonstrates the first reaction-based PA probes with submolecular level recognition ability but also highlights the opportunities provided by hybrid nanoparticles for advanced molecular imaging.

KEYWORDS: organic nanoparticles, photoacoustic imaging, reaction-based probes, sulfenic acids

Development of photoacoustic (PA) imaging agents has attracted significant attention because PA imaging offers higher penetration depth as compared with fluorescence imaging.1,2 Many PA agents including small molecular dyes,3,4 gold nanorods,5 carbon nanotubes,6 two-dimensional materials,7 and porphysomes8 have been developed. However, their signal-to-noise ratios are usually achieved via an enhanced permeability and retention (EPR) effect or lock-and-key molecular recognition between the recognition groups (such as small-molecule ligand, peptide, and antibody) and the overexpressed receptors in disease site.9,10 In contrast, the reaction-based PA probes, which undergo the specific chemical reaction with the target of interest in situ and subsequently enhance signal intensity in disease site, are seldom developed.11−13 However, the reaction-based probes have been proven effective for optical imaging because they are able to identify the specific variant with subtle differences from its analogues.14−16 Such capability provides spatial and temporal information on the submolecular level to track the dynamic biological and pathological events inside living organisms. Thus, the development of reaction-based PA probes is highly desired to further facilitate application of PA imaging in biology and medicine.

Semiconducting polymer nanoparticles (SPNs) assembled from optically active semiconducting polymers (SPs) have emerged as optical nanomaterials for molecular imaging.17−21 By virtue of their excellent optical properties, SPNs have been applied as fluorescent agents for cell tracking,22 tumor imaging,23 ultrafast hemodynamic imaging,24 drug-induced hepatotoxicity evaluation,25 and brain imaging.26 Recently, we revealed that SPNs could efficiently convert light into heat, allowing for photothermal and PA imaging applications.27 We further developed SPNs into smart activatable PA probes for in vivo imaging of reactive oxygen species (ROS)28−30 and pH.31 In addition to their completely organic and biologically inert ingredients,32,33 SPNs possess higher photostability34 and higher PA brightness28 as compared with gold nanorods and carbon nanotubes, respectively. Thus, the merits make SPNs a

Received: September 2, 2016
Accepted: December 20, 2016
Published: December 20, 2016

DOI: 10.1021/acsnano.6b05949
ACS Nano XXXX, XXX, XXX−XXX
versatile platform for the construction of reaction-based PA probes for in vivo molecular imaging.

In this study, we report reaction-based SPN probes (rSPNs) for in vivo PA imaging of protein sulfenic acids. Protein sulfenic acids, as the reversible oxide of cysteine in proteins, are recognized as a center post-translational modification when proteins encounter oxidative stress.35 The vital role of protein sulfenic acids in oxidative signal transduction places them at the forefront of redox biology, relating to many pathological conditions such as cancer,37 cardiac dysfunction,38 ischemia/reperfusion injury, and neurodegenerative diseases.39 However, current chemical probes for the detection of protein sulfenic acids rely on visible fluorescence signals, limiting their utility to in vitro assays.35,40 In contrast, the reaction-based probes (rSPNs) designed here have near-infrared (NIR) fluorescence and PA signals, allowing for real-time imaging of protein sulfenic acids in both living cells and small animals. This nanoprobe comprises 1,3-cyclohexanedione groups on the particle surface that enable specific reaction and covalent binding to the sulfenic acid sites of oxidized proteins.41,42 To minimize the perturbation of such reaction on the fluorescence and PA properties, the optically active SP core is shielded with silica and a poly(ethylene glycol) (PEG) corona. Current chemical probes for the detection of protein sulfenic acids rely on visible fluorescence signals, limiting their utility to in vitro assays. However, the reaction-based probes (rSPNs) designed here have near-infrared (NIR) fluorescence and PA signals, allowing for real-time imaging of protein sulfenic acids in both living cells and small animals.

RESULTS AND DISCUSSION

Two SPs, poly[3-(5-(9-hexyl-9-octyl-9H-fluoren-2-yl)-thiophene-2-yl)-2,5-bis(2-hexyldecyl)-6-(thiophene-2-yl)-pyrrolo[3,4-c]pyrrole-1,4(2H,SH)-dione] (DPPF, SP1) and poly[2,6-(4,4-bis(2-ethylhexyl)-4H-cyclopenta[2,1-b;3,4-b’]-dithiophene)-alt-4,7(2,1,3-benzothiadiazole)] (PCPDTBT, SP2) (Figure 1a), were employed to synthesize the surface-modifiable SPNs (Figure 1b). The THF mixture of SP and an amphiphilic triblock copolymer (PEG-b-PPG-b-PEG) at a mass ratio of 1:100 was first evaporated to yield a thin film. After reconstitution in the HCl solution under ultrasonication, the silicate condensation of tetraethyl orthosilicate (TEOS) efficiently occurred, leading to the formation of a thin silica shell for SPNs. The presence of a silica element in the nanoparticles was detected by energy-dispersive X-ray spectroscopy (EDX), which showed the peak of the silica element (∼1100 cm⁻¹) for the Si–O–Si bond in Fourier transform infrared spectroscopy (FTIR) analysis (Figure S2, Supporting Information).43 The surface structure of SPNs was constructed by adding silane-PEG molecules as the terminators to the reaction. Utilization of a silane-PEG-azide/silane-PEG mixture at a weight percentage of 20 w/w% not only conferred SPNs with a PEG passive layer for biocompatibility and aqueous stability but also yielded the azide reaction groups on the nanoparticle surface for postconjugation.

Click chemistry was used to conjugate the sulfenic acid reactive group, 4-(pent-4-yn-1-yl)cyclohexane-1,3-dione (DYn-2), to the surface of SPNs (Figure 1b). The resulting reaction-based SPNs (rSPNs) were translucent and homologous with

Figure 1. Synthesis and characterization of SPNs. (a) Chemical structures of SP1, SP2, PEG-b-PPG-b-PEG, silane-PEG-azide, silane-PEG, and DYn-2 used for preparation of rSPNs. (b) Schematic illustration of the preparation of rSPNs. (c) DLS profile of rSPN2. Inset: photos of SPN and rSPN solutions (15 μg/mL) in 1 x PBS (pH 7.4). (d) Average diameters of SPNs and rSPNs in PBS (pH 7.4). (e) Representative TEM image of rSPN2.
cyan color in PBS solution, the same as their precursors (Figure 1c, inset). The optical properties of SPNs including absorption and fluorescence spectra before and after conjugation with DYn-2 were the same (Figure S3, Supporting Information).

Dynamic light scattering (DLS) showed that all SPNs had similar hydrodynamic diameters of \(\sim 18\) nm (Figures 1c,d and Figure S4, Supporting Information). The spherical morphology of rSPN2 was observed by transmission electron microscopy (TEM) (Figure 1e). Good aqueous stability was proved by the unchanged diameters of SPNs after stored in the dark at room temperature (24 °C) for more than 1 month (Figure S5, Supporting Information). In addition, rSPN2 was stable in serum at room temperature for 24 h at least (Figure S6, Supporting Information). These data confirmed the weak nonspecific interaction of rSPNs with serum proteins, suggesting the suitability of rSPNs for biological application.

To validate the reactivity toward protein sulfenic acids (Figure 2a), the reaction between rSPN1 and bovine serum albumin (BSA) was studied in the absence and presence of hydrogen peroxide (\(H_2O_2\)). Incubation of BSA with \(H_2O_2\) is a common way to generate sulfenic acids in BSA (BSA-SOH) because sulfenic acid formation in proteins is mainly recognized as the direct product of the reaction between cysteine of proteins and \(H_2O_2\). However, sulfenic acids are a transient oxidative state and likely to be further oxidized into hyperoxidized forms, sulfinic acids (−SOOH), or sulfonic acids (−SOOOH) by excessive \(H_2O_2\). Thus, in order to capture this transient form, rSPN1 was mixed with BSA prior to addition of \(H_2O_2\). The agarose gel electrophoresis showed that the rSPN1/BSA-SOH band migrated much less as compared with rSPN1/BSA and rSPN1 (Figure 2b). The DLS data also verified that the diameter of rSPN1/BSA-SOH (56 nm) was 3.3-fold larger than that of rSPN1 itself (17 nm) (Figure 2c). The diameter of rSPN1/BSA (19 nm) remained almost the same with rSPN1. Both the electrophoresis and DLS data proved the occurrence of the specific reaction between rSPN1 and BSA-SOH, giving rise to the increased size of nanoparticles.

The same phenomenon was observed using rSPN2 for incubation with BSA and \(H_2O_2\) but not for the control nanoprobe (SPN2) (Figure S7a,b, Supporting Information). Note that in the excessive oxidative environment (\(H_2O_2\): higher than 100 µM) the formed BSA-SOH can undergo over-oxidation to form −SOOH or −SOOOH, and thus, the reaction is inhibited, resulting in the unchanged size of rSPN2. Moreover, the absorption and fluorescence spectra of rSPN1/BSA-SOH, rSPN1/BSA, and rSPN1 were nearly identical (Figure 2e,f), suggesting that the covalent bonding of BSA-SOH onto the surface of rSPN1 had no impact on the optical properties of the SP core. Such a surface-inert optical feature of rSPNs should benefit from the silica shell that effectively

Figure 2. In vitro validation of the specific reaction between rSPN1 and the sulfenic acids in BSA (BSA-SOH). (a) Illustration of the mechanism of the specific reaction between rSPNs and BSA-SOH. (b) Agarose gel electrophoresis of rSPN1 and rSPN1 in the presence of BSA or BSA-SOH. (c) Average diameters of BSA, rSPN1, and rSPN1 in the presence of BSA or BSA-SOH. (d) Epi-fluorescence of rSPN1 and rSPN1 in the presence of BSA or BSA-SOH. UV–vis absorption (e) and fluorescence (f) spectra of rSPN1 and rSPN1 in the presence of BSA or BSA-SOH. [rSPN1] = 2 µg/mL; 1 × PBS (pH 7.4). Excitation for fluorescence at 530 nm. Emission for epi-fluorescence images at 670 nm.
H2O2 has been proven to be an effective way to simulate acute oxidative stress and produce R-SOH in cells.37,42 To demonstrate the reactivity of SPN2 toward protein sulfenic acids, rSPN2 was used as the PA and fluorescence probe. rSPN2 had an absorption spectrum ranging from 680 to 860 nm and a fluorescence spectrum with the emission maximum at 840 nm, both of which were ideal for in vivo PA and fluorescence imaging (Figures 3a,b). Under pulsed laser irradiation, rSPN2 emitted PA signals with a maximum close to 680 nm, which was consistent with the absorption (Figure 3c) and similar to that of its counterpart SPN2 (Figure S9, Supporting Information). In addition, the good linearity between the PA signal and the emitted PA signals with a maximum close to 680 nm of rSPN2 and SPN2 in the presence of BSA or BSA-SOH (Figure S10, Supporting Information). In contrast, the PA intensities at 680 nm of rSPN2 and SPN2 decreased obviously along with increased concentration of H2O2, which was its counterpart SPN2 (Figure S8a,b, Supporting Information).

To facilitate in vivo imaging of protein sulfenic acids, rSPN2 was used as the PA and fluorescence probe. rSPN2 had an absorption spectrum ranging from 680 to 860 nm and a fluorescence spectrum with the emission maximum at 840 nm, both of which were ideal for in vivo PA and fluorescence imaging (Figures 3a,b). Under pulsed laser irradiation, rSPN2 emitted PA signals with a maximum close to 680 nm, which was consistent with the absorption (Figure 3c) and similar to that of its counterpart SPN2 (Figure S9, Supporting Information). In addition, the good linearity between the PA signal and the fluorescence of SPN2 existed from 15 to 100 μg/mL (R2 = 0.997) (Figure 3d). Statistical analysis using Manners’ colocalization coefficient ranging from 0 (nonoverlapping) to 1 (100% colocalization) revealed that the colocalization coefficients for all cell groups were higher than 0.58 (Figure 4b). This indicated rSPN2 was mainly located in the endo/lysosome of cells. In addition, the fluorescence intensity of rSPN2 increased obviously along with increased concentration of H2O2 (Figure 4a). As compared with the untreated cells, the fluorescence intensities were, respectively, increased by 3.3- and 8.4-fold for the cells treated with 10 and 100 μM H2O2 (Figure 4c). In contrast, the fluorescence intensities of the corresponding nanoparticles without DYn-2 groups on the surface (SPN2) remained nearly the same in the cells regardless of the treatment concentrations of H2O2 (Figure 4c and Figure S11, Supporting Information).

To demonstrate the reactivity of rSPN2 toward protein sulfenic acids in cells, the fluorescence imaging was conducted on cervical cancer cells (HeLa) with and without the treatment of H2O2. H2O2 has been proven to be an effective way to simulate acute oxidative stress and produce R-SOH in cells.37,42 After incubation with rSPN2 for 24 h, the cells were washed and stained with the endo/lysosome indicator and the nuclei indicator, which were, respectively, depicted in pseudo green and blue colors in the fluorescence cell images. The overlaid images showed that the fluorescence from rSPN2 overlapped well with that from the endo/lysosome indicator regardless of H2O2 treatment (Figure 4a). Statistical analysis using Manders’ colocalization coefficient ranging from 0 (nonoverlapping) to 1 (100% colocalization) revealed that the colocalization coefficients for all cell groups were higher than 0.58 (Figure 4b). This indicated rSPN2 was mainly located in the endo/lysosome of cells. In addition, the fluorescence intensity of rSPN2 increased obviously along with increased concentration of H2O2 (Figure 4a). As compared with the untreated cells, the fluorescence intensities were, respectively, increased by 3.3- and 8.4-fold for the cells treated with 10 and 100 μM H2O2 (Figure 4c). In contrast, the fluorescence intensities of the corresponding nanoparticles without DYn-2 groups on the surface (SPN2) remained nearly the same in the cells regardless of the treatment concentrations of H2O2 (Figure 4c).
Oxidative stress is a hallmark of cancer that distinguishes tumor from normal tissues. As the first step of cysteine in protein, it was observed that the sulfenic acid modifications were more prevalent in tumor tissues taken from patients, relative to matched normal tissues. In vivo reactivity of rSPN2 toward protein sulfenic acids was thus investigated using the xenograft HeLa tumor mouse model. After systemic administration of rSPN2 or the control probe (SPN2) into the tumor-bearing living mice through tail vein injection, both PA and fluorescence images were longitudinally recorded and quantified. The PA and fluorescence intensities gradually increased for both nanoparticles over time (Figure 5). At t = 36 h, the PA intensity of tumor for rSPN2-injected mice was 1.3-fold higher than that for the control probe (SPN2), which was 2.2-fold higher than the PA background of tumor. Similarly, the fluorescence intensity of tumor for rSPN2-injected mice was 1.4-fold higher than that for SPN2, which was 74-fold higher than the fluorescence background of tumor. The % ID/g of tumor vs liver showed the consistent trend (Figure S12, Supporting Information). The observed higher signal-to-noise ratio of fluorescence imaging relative to PA imaging should be mainly attributed to the higher sensitivity of fluorescence; moreover, the substantial PA background at 680 nm from hemoglobin was partially responsible. However, PA imaging had higher resolution and thus allowed for delineating the vascular structures of tumors. As shown in the PA images (Figure 5a), the PA signals were evenly distributed throughout the tumor, implying the efficient extravasation of rSPN2 due to their small size (~18 nm).

The real-time in vivo spectra of tumor (Figure 5e) showed that the PA signals at 680 nm for rSPN2 were 1.5-fold higher than that for SPN2. Moreover, the PA spectral profiles resembled that in solution (Figure 3c), further confirming that the increased PA signals came from the accumulation of nanoparticles. Because both PA and fluorescence signals of rSPN2 were not affected by the surface reactions (Figure 3b,e), the stronger signals of rSPN2-injected mice relative to SPN2-injected mice should result from the specific reaction of rSPN2 to sulfenic acids that facilitated nanoparticles accumulation in tumor. The formation of covalent bonds between rSPN2 and protein sulfenic acids in the tumor led to their slower clearance from the tumor as compared with that for the control probe.
after 36 h (Figure 5c,d). Thus, the signal difference between rSPN2- and SPN2-treated mice was further increased to 1.5- and 2-fold at 48 h for PA and fluorescence images, respectively. The ex vivo PA and fluorescence data illustrated that rSPN2 and SPN2 had quite similar dynamic biodistribution in major organs: the highest was liver uptake, followed by spleen, tumor, and other organs (Figure S13, Supporting Information). It was probably because of their similar sizes and surface structures. This ruled out the organ preference caused by the postconjugation for rSPN2. The in vivo and ex vivo data thus validated the effectiveness of rSPN2 in real-time imaging of protein sulfenic acids in the oxidative microenvironment of tumor of living mice.

To verify the specific bonding of rSPN2 to protein sulfenic acids in the oxidative environment of tumor, the mice injected with rSPN2 or SPN2 were sacrificed at 48 h postinjection of nanoparticles and conducted the immunofluorescence imaging. The antisulfenic acid antibody labeled with a green fluorescence dye was used to costain the tumor tissue samples and was able to specifically bind to the conjugates of the active groups (1,3-cyclohexanedione derivatives) and protein sulfenic acids but nearly showed no reactivity toward both proteins and protein sulfenic acids (Figure 6a). Consistent with the in vivo data, the NIR fluorescence for the tumor tissues of rSPN2-treated mice was stronger than that for SPN2-treated mice (Figure 6b vs 6c). In addition, red signals were well overlaid with the green signals from the antisulfenic acid antibody for the tumor tissues.
of rSPN2-treated mice (Figure 6b); in contrast, weak green signals were detected for the tumor tissues of SPN2-treated mice (Figure 6c). These data proved that the specific covalent bonding between rSPN2 and protein sulfenic acids occurred to facilitate the nanoparticle accumulation in tumors.

CONCLUSIONS

In summary, we have developed reaction-based PA nanoprobes (rSPNs) for imaging of protein sulfenic acids using SPNs as the platform. A templated method was used to synthesize rSPNs with the silica/PEG coating for the SP core so as to conjugate the sulfenic acid reactive 1,3-cyclohexanedione group onto the nanoparticle surface. Such a nanoparticle design resulted in the inert optical and PA features for rSPNs, enabling qualitative correlation between the signal enhancement and the extent of the sulfenic-acid-induced reaction of rSPNs. In vitro studies showed that rSPN2 served as an effective fluorescent probe for real-time tracking of the dynamic changes in the concentration of protein sulfenic acids under elevated oxidative stress in cancer cells. By virtue of the strong NIR absorption and fluorescence, rSPN2 permitted real-time PA and fluorescence imaging of protein sulfenic acids in the tumor of living mice. As compared with its nonreactive control probe (SPN2), substantial enhancement in the PA and fluorescence signals of tumors were observed for rSPN2, proving its capability to specifically react and then covalently bind to protein sulfenic acids in the oxidative microenvironment of tumor. With its good biodistribution, the reaction-based SPNs should be promising for imaging of protein sulfenic acids in other diseases such as cardiac dysfunction, ischemia/reperfusion injury, and neurodegenerative diseases.

To the best of our knowledge, rSPNs represent the first reaction-based PA probes that recognize the submolecular variation of biomolecules and subsequently undergo specific chemical reaction with the target sites to form covalent bonds. The structural flexibility of SPNs will open up opportunities to develop various reaction-based PA probes for advanced molecular imaging in biology and medicine.

METHODS

Chemicals. DYn-2 was purchased from Cayman Chemical Co. Silane-PEG molecules were purchased from Nanocs. All chemicals were purchased from Sigma-Aldrich unless otherwise stated.

Materials Characterization. TEM images were obtained on a JEM 1400 transmission electron microscope with an accelerating voltage from 40 to 120 kV. Dynamic light scattering (DLS) was performed on the Malvern Nano-ZS Particle Sizer. UV–vis spectra were recorded on a Shimadzu UV-2450 spectrophotometer. Fluorescence measurements were carried out on a Fluorolog-3 time correlated single photon counting (TCSPC) spectrofluorometer (Horiba Jobin Yvon). Fluorescence images of SPNs solutions were acquired with the IVIS spectrum imaging system. Fluorescence images were conducted using fluorescence microscopy (Leica, Germany).

Preparation of SPNs. SP1 (1 mg) or SP2 (1 mg) was dissolved in THF (1 mL) by bath sonication and filtered through a polyvinylidene fluoride (PVDF) syringe driven filter (0.22 μm) (Millipore) to obtain the solution (1 mg/mL). Then a mixed THF solution containing SP1 or SP2 (500 μL, 1 mg/mL) and PEG-b-PPG-b-PEG (1 mL, 50 mg/mL) was stirred at room temperature for 3 h to reach homogeneity. After evaporation of the THF solvent with the nitrogen flow, the solid thin film was dispersed in hydrochloride solution (1.6 mL, 0.85 M) with ultrasonication to form a homogeneous suspension. Then the silica condensation was barked on by adding TEOs (90 μL) and subsequently stirring for 2 h at room temperature before adding silane-PEG-azole (Mw 5000) / mPEG-silane (Mw 2000) mixture at the weight percentage of 20% w/w % as terminators (50 μL, 2000 mg/mL in ethanol solution). The termination should last 24 h at room temperature with stirring. To remove hydrochloride and ethanol, the product was washed and centrifuged four times at 3500 rpm for 30 min using an ultracentrifuge tube of 50 kDa (Millipore). The aqueous solution was filtered through a poly(ether sulfone) (PES) syringe driven filter (0.22 μm). The SPNs solutions were diluted in 1× PBS buffer (pH 7.4) and the concentrations were determined by UV–vis absorption according to absorption coefficient of SP1 or SP2.

Synthesis of rSPNs. One batch of SPNs prepared with SP (500 μg) possesses an azide group (~2 μmol). The solid powder of CuSO4 (1 mg) and sodium ascorbate (2 mg) were added into a round-bottomed flask first. After exclusion of oxygen, one batch of SPN solution in PBS buffer (10 mM, pH 7.4) and DMF solution of DYn-2 (400 μL, 1 mg/mL) were added into the sealed flask drop by drop using syringe individually. The solution was stirring under N2 for 2 days at room temperature. The resulting rSPNs were dialyzed to exclude the copper ions, which may be toxic to cells.

In Vitro Study of the Reactivity of rSPNs. The reactivity between rSPNs and protein sulfenic acids has been conducted as follows: BSA powder (8 mg) was dissolved in Tris-HCl buffer (1 mL, 50 mM, pH 7.4). To prereduce protein, tris(2-carboxyethyl)phosphine (TCEP) solution (20 μL, 50 mM) was added in the BSA solution and incubated at room temperature for 30 min. Then the reduced BSA was washed with Tris-HCl buffer to remove TCEP using an ultracentrifuge tube of 50 kDa, and the final volume was made to 1 mL. The reaction system of total volume of 100 μL was made up as follows: rSPN1/BSA-SOH was prepared by mixing with rSPN1 (50 μL, 40 μg/mL), prereduced BSA (25 μL), and Tris–HCl buffer (to the final volume of 100 μL) and adding H2O2 (5 μL, 1 mM) at last. The rSPN1/BSA (without H2O2) and rSPN (without BSA and H2O2) solutions were used as negative controls. The reaction systems were incubated at 37°C for 1 h. The resulting reaction solutions were used for further characterization. For DLS measurement, the final reaction system was diluted in the acetate buffer (100 mM, pH 4.7), reaching isoelectric point of BSA to avoid nonspecific absorption first and then measured as usual.

Agarose Gel Electrophoresis. Agarose gels (4%) were prepared using agarose powder purchased from Bio-Rad (Certified Molecular Biology Agarose) and immersed in 1× Tris/boric acid/EDTA (TBE) buffer, containing sodium dodecyl sulfate (0.1%, SDS). Before loading the gel with the reaction solution of rSPN1, SDS (2 μL, 20%) solution was mixed with the final reaction solution (20 μL). All the samples were then loaded into the wells of the gel and electrophoresed on a horizontal electrophoresis system (Mini-Sub Cell GT; Bio-Rad; electrode spacing 10 cm) for 1.5–2 h at 120 V. At last, an IVIS living imaging system was used to image the locations of nanoparticles in the agarose gel with an excitation at 530 nm and the emission at 670 nm.

In Vitro PA Instrumentation. The in vitro PA spectrum was measured by an optical parametric oscillator, OPO (Continuum, Surelite), generating pulses with duration 5 ns and pulse energy 100 mJ/pulse at 10 Hz repetition rate, which was pumped by a Q-switched 532 nm Nd:YAG laser. The output was tunable from wavelength 680 to 860 nm. The SPNs solution (23 μg/mL) was placed inside a low-density polyethylene (LDPE) tube with an inner diameter (i.d.) of 0.59 mm and outer diameter (o.d.) of 0.78 mm. After irradiation with wavelengths ranging from 680–860 nm with 10 nm increment, the PA signals produced in LDPE tube was coupled to the single-element ultrasound transducer, UST (V323-SU/2.25 MHz, 13 mm active area, and 70% nominal bandwidth, Panametrics). Both the tube and UST were immersed in water medium. PA signals at individual wavelength were collected using the UST and subsequently amplified with a gain of 50 dB. The band pass was filtered (1–10 MHz) by a pulse/receiver unit (Olympus-NDT, S072PR), and the output was digitized with a data acquisition card (GaGe, compuscope 4227) operated at 25 MHz. The data were finally stored in the computer. Peak-to-peak voltage of the PA signals for each wavelength were then normalized with laser
power. The normalized signals were plotted as a function of wavelengths to generate the PA spectra.

**Cell Culture.** HeLa cervical adenocarcinoma epithelial cells were purchased from the American Type Culture Collection (ATCC). HeLa cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (GIBCO, Cat. No. 10596010) supplemented with fetal bovine serum (10%) (GIBCO) and penicillin/streptomycin antibiotics (1%, Sangon Biotech Co., Ltd., Cat. No. B540732-0010). The cells were maintained in an atmosphere of 5% CO₂ and 95% humidified air at 37 °C.

**Cytotoxicity Test.** Cells were seeded in 96-well plates (3000 cells in 100 μL medium per well) for 24 h, and then the original medium was replaced by fresh medium containing SPNs solutions (final concentration 5, 10, 25, 50, and 100 μg/mL). Cells were incubated with SPNs for 24 h, followed by the addition of MTT (20 μL, 5 mg/μL) for 3 h. The media was removed and DMSO (200 μL) was added into each well. The plate was gently shaken for 10 min at room temperature to dissolve all formed precipitates. The absorbance of MTT at 570 nm was measured using a SpectraMax M5 Microplate Cuvette Reader. Cell viability was expressed by the ratio of the absorbance of the cells incubated with SPNs solution to that of the cells incubated with untreated culture medium.

**Cell Imaging.** HeLa cells (human epithelial carcinoma cell line; American Type Culture Collection) were cultured as mentioned. After seeded cells reaching 70% confluency, the cells were washed three times using prewarmed PBS buffer (10 mM, pH 7.4) and incubated with medium containing different concentration of H₂O₂ (0, 10, 100 μM) for 5 min at 37 °C. Then the cells were washed 3 times using PBS buffer and medium containing rSPN2 or SPN2 with the final concentration of 12 μg/mL were added. The cells were incubated with nanoparticles in an atmosphere of 5% CO₂ and 95% humidified air at 37 °C for 24 h. Then the cells were stained with pHrodo Red epidermal growth factor (EGF) Conjugate (ThermoFisher) for the endo/lysosome and with Hoescht 33342 (Nucleic Live ReadyProbes Reagent, Thermo Fisher) for the nuclei as protocol. Fluorescence images of the live cells were taken with the wide-field fluorescence microscope Leica DMi8 (Leica, Germany) equipped with scientific CMOS (sCOMS, Hamamatsu, Japan). Excitation/emission wavelengths were 390 ± 22/440 ± 25 nm for Hoescht, 560 ± 25/607 ± 25 nm for endo/lysosome indicator, and 648 ± 20/694 ± 25 nm for the nanoparticles.

**Tumor Mouse Model.** All animal studies were performed in compliance with the Guidelines established by the Institutional Animal Care and Use Committee (IACUC), Sing Health. To establish tumors in eight-week-old BALB/c, ten to 20 million Hela cells were subcutaneously under the armpit with 0.1 mL. Tumors were allowed to grow before imaging experiments.

**In Vivo PA Imaging.** After nude mice bearing Hela tumors were anesthetized using 2% isoflurane in oxygen, rSPN2 or SPN2 (200 μL, 100 μg/mL) (n = 3) was systematically injected through the tail vein using a microsyringe. Endra Nexus128 PA imaging system was used to acquire the PA images at 680 nm after systematically administration of rSPN2 or SPN2. Data were acquired through a continuous model that took 12 s to obtain one data set. Three-dimensional PA images were 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. Real-time PA spectra of tumor were also recorded with an Endra Nexus128 PA imaging system.

**Biodistribution Method.** After 48 h postinjection with rSPN2 or SPN2 (200 μL, 100 μg/mL) (n = 3), the mice were sacrificed by CO₂ asphyxiation. Major organs as indicated were harvested. Major organ parts were collected and weighted at 100 mg for the PA and fluorescence measurements. For ex vivo PA imaging, organs were harvested and acquired immediately with an Endra Nexus128 PA imaging system.

**Histological Studies.** After 48 h postinjection of nanoparticles, the mice treated with rSPN2 or SPN2 (200 μL, 100 μg/mL) (n = 3) were sacrificed. The tumors were fixed in 4% formalin and dehydrated with 30% sucrose solution. Then, cryostat sectioning was conducted for immunofluorescence staining. The sliced tumor samples were blocked with PBS solution (including 3% BSA and 0.1% TritonX-100) and stained with anticycstene sulfinic acid antibody (no. 07-2139, Merck, 1:3000) for 1 h at 37 °C. The slides were then washed three times with PBS and stained by donkey antirabbit IgG (H-L) secondary antibody (no. A21206, Thermo Fisher, 1:1000) for 30 min at 37 °C in the dark. Then the slices were washed with PBS three times and stained with DAPI (D9542, Sigma) following washing with PBS three times. Finally, the slices were mounted with Fluoromount G (Southern Biotech) and used for immunofluorescence analysis.

**Data Analysis.** The in vivo and ex vivo PA signal intensities and real-time PA spectra were measured by region of interest (ROI) analysis using OsiriX software. Fluorescence signal intensities of cells were measured by ROI analysis using ImageJ. Colocalization coefficients of rSPN2 and pHrodo Red EGF Conjugate were calculated using the function of Coloc2 of ImageJ as protocol (http://imagej.net). Fluorescence signal intensities of mice were measured by ROI analysis using Living Image software for IVIS imaging system. Results were expressed as the mean ± SD deviation unless otherwise stated.

**ASSOCIATED CONTENT**

3 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.6b05949.

Supporting Figures S1–13 detailing the characterization of the SPNs as well as in vitro, in vivo and ex vivo data (PDF)

**AUTHOR INFORMATION**

**Corresponding Author**
*E-mail: kypu@ntu.edu.sg.*

**ORCID**
Kanyi Pu: 0000-0002-8064-6009

**Notes**
The authors declare no competing financial interest.

**ACKNOWLEDGMENTS**

This work was supported by two Nanyang Technological University start-up grants (NTU-SUG: M4081627.120 & M4081533.080), Academic Research Fund Tier 1 from Singapore Ministry of Education (RG133/15: M4011559), and Academic Research Fund Tier 2 from Ministry of Education in Singapore (MOE2016-T2-1-098). We thank Dr. Manojit Pramanik for help with the PA measurements.

**REFERENCES**

Photoacoustic Agents for Deep Brain Tumor Imaging in Living Mice. 


NOTE ADDED AFTER ASAP PUBLICATION

This paper published ASAP on 12/23/2016. Images for the Abstract, Figure 1, and Figure 2 were modified to clarify the linking position on the triazole ring, and the revised version was reposted on 12/29/2016.