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Nanoparticle Regrowth Enhances Photoacoustic Signals of Semiconducting Macromolecular Probe for In Vivo Imaging

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Smart molecular probes that emit deep-tissue penetrating photoacoustic (PA) signals responsive to the target of interest are imperative to understand disease pathology and develop innovative therapeutics. This study herein reports a self-assembly approach to develop semiconducting macromolecular activatable probe for in vivo imaging of reactive oxygen species (ROS). This probe comprises a near-infrared absorbing phthalocyanine core and four poly(ethylene glycol) (PEG) arms linked by ROS-responsive self-immolative segments. Such an amphiphilic macromolecular structure allows it to undergo an ROS-specific cleavage process to release hydrophilic PEG and enhance the hydrophobicity of the nanosystem. Consequently, the residual phthalocyanine component self-assembles and regrows into large nanoparticles, leading to ROS-enhanced PA signals. The small size of the intact macromolecular probe is beneficial to penetrate into the tumor tissue of living mice, while the ROS-activated regrowth of nanoparticles prolongs the retention along with enhanced PA signals, permitting imaging of ROS during chemotherapy. This study thus capitalizes on stimuli-controlled self-assembly of macromolecules in conjunction with enhanced heat transfer in large nanoparticles for the development of smart molecular probes for PA imaging.

Photoacoustic (PA) imaging is a hybrid imaging modality that detects the conversion of photon energy into acoustic pressure waves, and thus provides deeper tissue penetration relative to traditional optical methods.^[1,2] Because few endogenous chromophores including hemoglobin and melanin can generate PA signals, synthetic exogenous chromophores are often used to enhance the signal to background of PA imaging.^[3–5] To understand the physiology of diseases at molecular level, smart activatable PA probes have been developed to transduce the molecular targets or events of interest into the changes in PA signals.^[6–9] Until now, activatable PA probes based on porphyrins,^[10] semiconducting polymer nanoparticles,^[11–15] and inorganic nanoparticles^[16,17] as the framework have been used for imaging of enzymes, chemical mediators, and physiological indexes in living animals. The probe design mechanism mainly relies on the target-induced change in the probe composition or the chemical structures of reference chromophores.^[18–20] To generate distinct PA signals after activation, this mechanism

requires the dramatic variation in absorption in responses to the changed structures. However, due to the limitation of near-infrared (NIR) chromophores having such properties, other probe design approaches are highly desired to broaden the application of PA imaging in medicine.

Molecular self-assembly has been applied to transform small molecules or nanoparticles into high-order complexes through noncovalent intermolecular interactions for biological applications.^[21] In particular, integration of molecular self-assembly with biological events such as enzymatic reactions and ligand–receptor binding provides a spontaneous yet controlled way to generate on-demand production of large nanostructures with desired properties that are different from its precursor.^[22] Such self-assembly induced change has led to a new generation of fluorescence molecular probes for imaging of enzyme activity such as hydro-

lase,^[23] alkaline phosphatase,^[24] and caspase.^[25,26] Moreover, in situ self-assembly strategy has been revealed to combine the advantages of both small molecules and large nanoparticles for in vivo molecular imaging and therapy.^[25] The small size of molecular probe has deep-tissue penetration and idea biodistribution; while in situ self-assembly of small molecular probes into large nanoparticles leads to increased residence time in the target tissue of interest such as tumor.^[25,27] However, self-assembly molecular probes that change their PA signals after activation are rare and remain to be challenging.

In this study, we report a self-assembly approach to develop semiconducting macromolecular probes with target-enhanced PA signals for in vivo imaging of reactive oxygen species (ROS). ROS is a hallmark of many pathological processes, and thus detection of ROS is imperative to diagnosis and treatment of many life-threatening diseases such as cancer.^[28] However, PA molecular probes capable of monitoring ROS in living animals along with cancer treatment have been less exploited.

To transduce the aberrant ROS level into the cure for self-assembly, an amphiphilic semiconducting macromolecule, zinc-tetra(2-((1-(3-amino-3-oxopropyl)-1H-1,2,3-triazol-4-yl)-methoxy)-2-oxo-1-(4-((4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)benzyl)oxy)phenyl)ethyl methoxy poly(ethylene glycol) succinate)phthalocyanine (PCBP), is designed and synthesized (Figure 1a). PCBP comprises phthalocyanine and poly(ethylene glycol) (PEG) as the hydrophobic core and hydrophilic arms, respectively. These two components are covalently linked by the

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Figure 1. a) Design and mechanism of the self-assembly macromolecular probe (PCBP) for PA imaging of ROS. b) Synthetic routes of PCBP and PCP. Reagents and conditions: (i) sodium sulfide nonahydrate, water, 50 °C, 5 h; (ii) 3-azidopropanoic acid, *N,N*-diisopropylethylamine, *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate, anhydrous *N,N*-dimethylformamide (DMF), 25 °C, 48 h; (iii) mPEG-alkyne, copper(II) sulfate pentahydrate, sodium ascorbate, DMF, 25 °C, 48 h. (iv) mPEG-PBA-alkyne, copper(II) sulfate pentahydrate, sodium ascorbate, DMF, 25 °C, 48 h.

phenylboronic acid pinacol ester groups, well known to be able to be cleaved by ROS including hydrogen peroxide (H_2O_2) and peroxyxynitrite (ONOO^-).^[29] The control probe, zinc-tetra(3-(4-((methoxy poly(ethylene glycol))methyl)-1H-1,2,3-triazol-1-yl)propenamide)phthalocyanine (PCP), without the ROS linker is also synthesized for comparison (Figure 1b). In the presence of ROS including H_2O_2 and ONOO^- , the aryl boronic ester group is oxidized and the boronic pinacol ester is then released. The remaining un-masked phenol undergoes a self-immolative

process, which is a quinone methide rearrangement, cleaving hydrophilic PEG from PCBP and leaving hydrophobic phthalocyanine behind. Due to increased hydrophobic interaction and π - π stacking,^[30] residual phthalocyanine segments self-assemble and regrow into large nanoparticles. According to the previous theoretical and experimental studies, large nanoparticles have increased heat transfer as compared with small nanoparticle and single-molecule state.^[27,31–35] Thus, such an ROS-induced self-assembly of PCBP eventually results

in enhanced PA signals for imaging of ROS both in solution and in the tumor of living mice.

The amphiphilic semiconducting macromolecules (PCBP and PCP) were synthesized by using zinc-tetranitrophthalocyanine (TNPC) as the starting material (Figure 1b). Zinc rather than other metal ions was chosen here, (i) because zinc-phthalocyanine shows almost no fluorescence and thus benefits photoacoustic signals; (ii) zinc is more biocompatible as compared with Cu, Co, and Cr; (iii) zinc-phthalocyanine has a longer absorption wavelength than iron-phthalocyanine.^[36] The nitro groups of TNPC were first reduced by sodium sulfide nonahydrate to give zinc-tetraaminophthalocyanine (TAPC) with four amino groups. Amidation reaction between TAPC and 3-azidopropanoic acid was then conducted to afford azide-modified TAPC, zinc-tetra(3-azidopropanamide)phthalocyanine (TAPC-N₃). The proton nuclear magnetic resonance (¹H NMR) spectrum of TAPC-N₃ showed the disappearance of resonance

peak at 6.24 ppm which assigned to the protons of amino groups of TAPC (Figure S1 vs Figure S2, Supporting Information). Similarly, the Fourier transform infrared spectrum of TAPC-N₃ displayed two new peaks at 1648 and 2101 cm⁻¹, which respectively assigned to the signals of carbonyl and azide groups (Figure S3, Supporting Information). Both results confirmed the conversion of TAPC into TAPC-N₃. Alkyne-end-capped PEGs with (mPEG-PBA-alkyne) or without (mPEG-alkyne) the ROS-responsive boronate moiety were synthesized (Scheme S1, Supporting Information). The correct structures of mPEG-PBA-alkyne, mPEG-alkyne and all the intermediates were confirmed by ¹H NMR and mass spectroscopy. TAPC-N₃ was then conjugated with mPEG-PBA-alkyne or mPEG-alkyne through copper(I)-catalyzed alkyne-azide cycloaddition click reaction to obtain the final products the ROS-responsive probe (PCBP) and the control probe (PCP), respectively. Taking the ¹H NMR of PCBP as an example, the peaks of zinc-phthalocyanine

Figure 2. In vitro characterization of the macromolecular probe (PCBP) along with the control probe (PCP). Representative TEM a) and DLS b) of PCBP. c) Average diameters of PCBP and PCP determined by DLS. d) Average diameters of PCBP as a function of time incubated with PBS (pH = 7.4) and FBS, respectively. e) Cell viability of HeLa cells after incubation with PCBP under various concentrations of phthalocyanine. f) Normalized absorption of PCBP and PCP solutions in PBS (pH = 7.4). g) PA spectra of PCBP and PCP in PBS (pH = 7.4). h) The PA amplitude at 700 nm as a function of concentrations of PCBP. i) PA amplitude of PCBP and GNR at 700 nm under the same mass concentration of the optical components. The error bars represent the standard deviation of three separate measurements.

(9.29, 8.32, and 7.77–7.64 ppm) and the peaks for PEG-PBA part (6.82–6.60, 5.84, 4.97, 4.58, 4.02, 3.74, 3.51, 2.54, and 1.24 ppm) were all in presence (Figure S4, Supporting Information). These results demonstrated the correct chemical structures of the ROS-responsive and control probes.

Both PCBP and PCP could be dissolved in water and form small nanoparticles with a spherical morphology as indicated by transmission electron microscopy (TEM) (Figure 2a). Dynamic light scattering (DLS) showed a relatively narrow size distribution of PCBP with an average hydrodynamic size of 20 ± 2 nm which was similar with that of PCP (18 ± 6 nm) (Figure 2b,c). No precipitation and obvious change in size were detected for PCBP during the storage in phosphate buffer solution (PBS) (pH = 7.4) or fetal bovine serum (FBS) for more than 20 d (Figure 2d). Furthermore, no obvious cytotoxicity of PCBP was observed in 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay (Figure 2e). These results showed the good aqueous stability and cytocompatibility of PCBP for biological applications.

The optical properties of PCBP and PCP were studied under physiological conditions. Both PCBP and PCP had broad absorption peaks ranging from 550 to 850 nm with a similar spectral profile (Figure 2f). In addition, their absorption spectra were different from of TAPc-N₃ in good organic solvent which had a sharp absorption peak in the NIR region with the maximum at 695 nm (Figure S5 in the Supporting Information vs Figure 2f). The blueshifted absorption of PCBP and PCP relative to that of

TAPc-N₃ indicated the formation of H-aggregate in aqueous solution. This was reasonable as they stayed in small nanoparticle state rather than single-molecule state in aqueous solution.

The PA spectra of PCBP and PCP were acquired under the same mass concentration of phthalocyanine ranging from 680 to 900 nm. Similar PA spectra were obtained for PCBP and PCP in terms of both spectral profile and intensity, which were consistent with their absorption spectra (Figure 2g vs Figure 2f). The PA amplitudes of PCBP at 700 nm were then measured at a series of concentrations of phthalocyanine from 12 to 188 $\mu\text{g mL}^{-1}$. A linear relationship between PA amplitude and concentration was observed (Figure 2h). In addition, the PA amplitude of PCBP was 4.2-fold higher than that of gold nanorods (GNR) with the maximum absorption near 700 nm under the same mass concentration of optical components (Figure 2i). These results indicated that PCBP was an ideal candidate for PA imaging with the feasibility for signal quantification.

Changes in the size of the macromolecular probe (PCBP) were then studied upon the addition of different ROS in the PBS buffer (pH = 7.4). The average diameter of PCBP increased gradually with H₂O₂, which reached approximately 350 nm in the presence of H₂O₂ (Figure 3a). This phenomenon was attributed to the ROS-induced cleavage of PEG chains, leading to enhanced hydrophobicity and thus regrowth into large nanoparticles. Similar to H₂O₂, ONOO could react with boronate moiety,^[29] and result in increased particle size for PCBP. In contrast, the size increase was not observed for other ROS including

Figure 3. The size and PA responses of the macromolecular probe (PCBP) towards ROS. a) Average diameters of PCBP and PCP under different concentrations of H₂O₂. Inset: TEM image of PCBP after addition of H₂O₂ (5 mM). b) Average diameter and PA amplitude of PCBP toward different ROS (5 mM) in PBS (pH = 7.4). c) PA amplitudes of PCBP and PCP as a function of H₂O₂ concentration. d) PA amplitudes at 700 nm as a function of average diameters of PCBP. The error bars represent the standard deviation of three separate measurements. The concentrations of phthalocyanine for both PCBP and PCP in the experiments are 50 $\mu\text{g mL}^{-1}$.

hydroxyl radical, singlet oxygen, hypochlorite, and superoxide anion (Figure 3b). Moreover, the size of the control probe (PCP) did not change for all ROS, reflecting that the increased size of PCBP was specifically caused by ROS-induced cleavage.

Change of PA amplitude for PCBP in the presence of different concentrations of H_2O_2 was then measured in solution. Upon addition of H_2O_2 , the PA amplitude of PCBP at 700 nm increased linearly (Figure 3c), validating the feasibility for quantification of H_2O_2 . The limit of detection was determined to be 0.15 mM under the probe concentration of $50 \mu\text{g mL}^{-1}$. Such a detection range is physiologically relevant as the static concentration of H_2O_2 in living organisms ranges from 0.1×10^{-6} to 2×10^{-6} M, and the probe activation can be detected over time as the dynamic accumulation of ROS.^[37,38] At the saturation point, the PA amplitude of PCBP was increased by ≈ 1.4 -fold relative to its original (Figure 3c). Similar effect was observed for ONOO^- (Figure 3b). On the contrary, the PA amplitude of the control probe (PCP) remained almost unchanged for all ROS (Figure S6, Supporting Information). Therefore, these results demonstrated such enhanced PA signals were specific to H_2O_2 and ONOO^- . Moreover, the PA amplitudes of PCBP were proportional to the nanoparticle diameters (Figure 3d). Theoretically, the PA pressure is proportional to the time derivative of the heat flux transferred to the fluid, and we have shown that

coating the organic nanoparticles with a thin silica layer to change the pathway of heat dissipation can enhance PA signals.^[31] Because the absorption spectra of PCBP in the absence and presence of ROS are the same (Figure S7, Supporting Information), such enhanced PA signals for PCBP along with ROS-induced nanoparticle regrowth should be attributed to increased rate of heat transfer within large nanoparticles,^[27,31,32] which was also observed for gold nanorods.^[33]

The self-assembly macromolecular probe (PCBP) was evaluated for in vivo PA imaging of ROS in the subcutaneous 4T1 xenograft tumor model. D,L-Buthionine-(S,R)-sulphoximine (BSO), a drug that reduces the level of glutathione, was used to further elevate the ROS level at tumor site. After systemic administration of PCBP into living mice through tail vein, PA images were recorded at 700 nm. As tumors had weak intrinsic PA signals because of the absorption of hemoglobins in the NIR region, PA amplitude increment at 700 nm (ΔPA_{700} , defined as the PA amplitude of tumor site after probe injection deducted by the background signal of tumor before injection) was utilized in data analysis to minimize the interference of tissue background. ΔPA_{700} for both BSO-pretreated and untreated mice gradually increased after injection of the probe, both of which reached the maximum at 8 h postinjection (Figure 4b,c). This indicated that PCBP efficiently accumulated at the tumor site

Figure 4. PA imaging of ROS variation in the tumor of living mice along with drug treatment using the macromolecular probe (PCBP). a) Illustration of the mechanism for PA imaging of ROS in tumor using PCBP. PCBP first accumulates into tumor through EPR effect and then are activated by ROS to self-assemble and regrow into large nanoparticles, eventually resulting in enhanced PA signals. b) Representative PA maximum intensity projection images of tumors for BSO-pretreated and untreated mice ($n = 3$) after systemic administration of PCBP ($30 \mu\text{g}$ per mouse) through tail vein. c) PA amplitude increment at 700 nm (ΔPA_{700}) as a function of postinjection time of PCBP in BSO-pretreated and untreated mice. d) Ex vivo quantification of PA of major organs from mice with (BSO) or without (control) pretreatment of BSO 30 h after systemic administration of PCBP. *Statistically significant difference ($p < 0.01$, $n = 3$).

for both BSO-pretreated and untreated mice. However, at each time point, ΔPA_{700} for BSO-pretreated mice was higher than that of untreated mice. At 8 h postinjection, ΔPA_{700} for BSO-pretreated mice was 2.0-time higher than that of untreated mice. This proved that the elevated ROS level in the tumor of BSO-pretreated mice effectively activated the self-assembly of PCBP, leading to the formation of large nanoparticles with stronger PA signal at 700 nm (Figure 4a). Moreover, this signal enhancement was larger than the value (1.4-fold) tested in solution, indicating that other factors were relevant. Thus, increased residence time induced by the formation of large nanoparticles at tumor site should partially contribute to such an additional enhancement in the PA signals for BSO-treated mice. This was also witnessed by the fact that ΔPA_{700} at 24 h postinjection was almost identical to that of 8 h postinjection for BSO-pretreated mice, which was substantially decreased for untreated mice (Figure 4c).

The real-time in vivo PA spectral profile extracted from the tumors of BSO-pretreated was similar to that for untreated mice (Figure S8, Supporting Information). However, the absolute PA signal at 700 nm for BSO-pretreated mice was 2.03 times higher than that for untreated mice. Ex vivo biodistribution for BSO-pretreated and untreated mice were determined at 30 h postinjection (Figure 4d). PCBP exhibited noticeable uptake in major reticuloendothelial system organs such as liver and spleen for both BSO-pretreated and untreated mice. However, BSO-pretreated mice showed a higher PA amplitude in tumor relative to that of untreated mice, which again proved that the self-assembly induced the increase in the particle size and led to prolonged retention time in the tumor of living mice.

In summary, we have developed a smart PA probe that can self-assemble and regrow for target-enhanced PA imaging of ROS in living animals. The semiconducting macromolecular probe (PCBP) is composed of three key components: an NIR-absorbing hydrophobic phthalocyanine core, four hydrophilic PEG arms, and an ROS-responsive linker. Such a macromolecular structure allows PCBP to remain stable in aqueous solution but to self-assemble and regrow into large nanoparticles upon ROS-induced cleavage of PEG. This reassembly process is selective to $ONOO^-$ and H_2O_2 , and can lead to enhanced PA signals. In addition, the small size of the macromolecular probe at its intact state (20 nm) is beneficial to penetrate into the tumor tissue of living mice, while the ROS-activated regrowth of nanoparticles prolongs the tissue retention and enhances the PA signals, permitting imaging of ROS during drug treatment.

Our study provides a supramolecular approach that capitalizes on the stimuli-controlled self-assembly of macromolecules in conjunction with the increased heat transfer in large nanoparticles to develop smart molecular probes for PA imaging. In view of the unique structure of phthalocyanine, the macromolecular probe can be functionalized with other metal atoms such as ^{64}Cu radioisotope for positron emission tomography or manganese ion for magnetic resonance imaging. Such a probe design approach can also be generalized for the detection of other physiologically important targets such as enzymes, which only requires the modification of stimuli-responsive linker.

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.

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