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Activatable Photoacoustic Nanoprobes for *In Vivo* Ratiometric Imaging of Peroxynitrite

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While reactive oxygen species (ROS) are chemically reactive mediators that modulate essential functions in living organisms, they play pivotal roles in the acquisition of the hallmarks of cancer.¹ In particular, peroxynitrite (ONOO⁻) is highly reactive and can nitrite tyrosine, cysteine, methionine and tryptophan in different proteins, mutating their biological functions.² In the tumor microenvironment, nitration of T-cell receptor (TCR), CD8 and CC-chemokine ligand 2 (CCL2) can inhibit the binding of CD8⁺ T cells to the peptide major histocompatibility (MHC) class I,³ promote antigen-specific tolerance and impair recruitment of cytotoxic T cells to tumors.⁴ Thus, ONOO⁻ is closely associated with the immunosuppression of tumor,⁵ and real-time *in vivo* imaging of ONOO⁻ in tumor is imperative to understand the underlying mechanism and optimize therapeutic interventions. Although molecular probes have been developed for ONOO⁻ sensing,⁶ they generally rely on fluorescence as the signal readout and few can be excited in the deep-tissue-penetrating near-infrared (NIR) region.⁷ Thereby, current ONOO⁻-sensing probes share the drawbacks of autofluorescence background and limited light penetration depth,⁸ making them less competent for *in vivo* imaging.

In comparison with fluorescence imaging, photoacoustic (PA) imaging exceeds the optical diffusion limit by detection of phonon instead of photon after light excitation, providing deeper tissue penetration (up to ~5 cm) for *in vivo* imaging.⁹ Despite the application of many materials in PA imaging including carbon nanotubes,¹⁰ carbon dots,¹¹ gold nanoparticles,¹² golden carbon nanostructures,¹³ two-dimensional materials¹⁴ and porphyrins,¹⁵ they simply work as the accumulation probes and their signal-to-noise ratios result from the concentration gradient between disease site and normal tissue.¹⁶ In contrast, activatable PA probes undergo intrinsic signal evolution upon detecting molecular targets or events (from “off” to “on” state) and thus have the advantages of low noise background and probe concentration independence.¹⁷ However, activatable PA probes have been far less developed,¹⁸ not to mention activatable probes for ONOO⁻ sensing.

The key challenge to develop activatable PA probes lies in the creation of substantial and specific absorption changes in NIR region upon activation by ONOO⁻, because PA signals are closely correlated with absorption. We recently reported the design of activatable PA probes based on NIR-absorbing semiconducting polymer nanoparticles (SPNs).¹⁹ To generate ratiometric PA signals, a semiconducting polymer (SP) and a NIR dye were utilized as the internal standard with unchanged signals and the sensing component with target-decreased signals, respectively. Although such design has been validated to be flexible for the detection of different targets such as ROS and pH, two optical components must be carefully selected and paired in terms of NIR absorption, complicating the design and partially limit its applicability. In contrast, utilization of a single optically active component that not only changes but also increase its absorption at specific NIR wavelengths upon activation is more straightforward, and thus desired for PA imaging of ONOO⁻.

In this study, we report the design and synthesis of organic semiconducting nanoprobe (OSNs) doped with bulky borane for real-time ratiometric PA imaging of ONOO⁻ in the tumor of living mice. The nanoprobe is composed of a boronate-caged boron-dipyrromethene dye

(BBD) as the primary sensing component that shifts its NIR absorption and thus PA signals in the presence of ONOO^- . However, BBD itself is not qualified for *in vivo* imaging of ONOO^- , because (i) it is not water-soluble; (ii) boronate is not specific to ONOO^- because it can be activated by both ONOO^- and hydrogen peroxide (H_2O_2);²⁰ (iii) its deboronated phenoxide product is pH sensitive, and the formation of conjugate acid at acidic pH tumor environment can compromise the signals. To overcome these limitations, optically inactive but chemically reactive bulky boranes are utilized as the secondary component for the nanoparticles. Such a doping approach consequently transforms BBD into the water-soluble nanoprobe that has high ONOO^- selectivity and increased pH inertness.

Figure 1. Synthesis and characterization of OSNs. (a) Synthesis of BBD. i) $\text{CH}_3\text{COONH}_4$, *n*-BuOH, reflux, 12 h; ii) $\text{BF}_3 \cdot \text{Et}_2\text{O}$, *N,N*-diisopropylethylamine (DIPEA), CH_2Cl_2 , r.t., 12 h; iii) DIPEA, acetonitrile, reflux, 8 h. (b) Chemical structures of the bulky boranes (TPB and TPFB) and the amphiphilic polymer (PEG-*b*-PPG-*b*-PEG). (c) Schematic illustration for the

preparation of OSNs *via* nanoprecipitation (d) Representative TEM image of OSN-B1. (e) DLS profile of OSN-B1. (f) Average hydrodynamic diameters of OSNs in PBS (pH = 7.4).

The primary sensing component (BBD) was synthesized from 3-(3-chloro-4-hydroxyphenyl)-5,5-difluoro-1,7,9-triphenyl-5H-dipyrrolo[1,2-c:2',1'-f][1,3,5,2] triazaborinin-4-ium-5-uide (**3**) by caging the oxygen atom with a methylphenylboronic acid pinacol ester group (**Figure 1a**). The bulky borane doped nanoprobes and the control nanoprobe were prepared *via* nanoprecipitation (**Figures 1b&1c**) with the assistant of an amphiphilic triblock copolymer (PEG-*b*-PPG-*b*-PEG). In addition to BBD as the sensing component, OSN-B1 and OSN-B2 respectively comprised triphenylborane (TPB) and tris(pentafluorophenyl)borane (TPFB) as the secondary component. The molar ratio of the bulky borane to BBD within the nanoprobes was determined to be ~4 (**Figure S1**, Supporting Information). The OSNs possessed similar average hydrodynamic diameters of ~20 nm (**Figures 1e&1f**, and **Figure S2**, Supporting Information) and a typical spherical morphology (**Figure 1d** and **Figure S2d&S2e**, Supporting Information) as shown by dynamic light scattering (DLS) and transmission electron microscopy (TEM), respectively. The size of the OSNs remained nearly the same even after storage in PBS buffer for 30 days and in serum for 7 days (**Figure S2c&S2f**, Supporting Information), showing their excellent aqueous stability.

To study the doping effect on the sensing properties, we measured and compared the absorption spectra of OSNs and BBD itself before and after addition of ROS at the physiological conditions. In the absence of ROS, the absorption spectra of all the probes were nearly the same, showing a maximum peak at 675 nm (**Figure 2a**). Upon addition of ONOO⁻, the absorption of OSNs and BBD at 675 nm gradually decreased, which was accompanied by the appearance of a new peak at 745 nm (**Figure S3**). At the saturation point, the ratios of the absorption at 745 nm to that at 675 nm (Ab_{745}/Ab_{675}) for the OSNs were ~1.7, which was 8-fold higher than that at the initiate state (0.21). Such ratiometric absorption responses were caused by the rapid oxidative cleavage of the borate ester moiety of BBD by ONOO⁻, which

generated the anionic phenoxide product with the characteristic maximum absorption at 745 nm (**Figure 3a** and **Scheme S1**, Supporting Information). The ONOO^- induced oxidation of BBD was confirmed by HPLC analysis (**Figure S4**, Supporting Information), which was also widely reported.^{20a} In contrast, upon addition of H_2O_2 , only BBD and OSN0 increased the ratiometric absorption signals ($\text{Ab}_{745}/\text{Ab}_{675}$) by 2.2 fold (**Figure 2b**), whereas OSN-B1 and OSN-B2 remained nearly the same. Moreover, other ROS including $\text{O}_2^{\bullet-}$, OCl^- , $^1\text{O}_2$, $\bullet\text{OH}$ and $\bullet\text{ON}$ could not induce any absorption change for of all the probes (**Figure 2c** and **Figure S5**, Supporting Information). Thereby, the bulky borane doped OSNs could only be activated by ONOO^- , changing their solution colors from seagreen to dull-yellow (**Figure 2d**). These data proved that the doping of bulky boranes made the OSNs inert to H_2O_2 and subsequently increased ONOO^- specificity.

Figure 2. Absorption responses towards ROS. (a) Absorption spectra of OSNs and BBD (1 $\mu\text{g}/\text{mL}$) after addition of ONOO^- (15 μM) in 1 $\times$ PBS (pH = 7.4). (b) Absorption spectra of OSNs and BBD (1 $\mu\text{g}/\text{mL}$) after addition of H_2O_2 (15 μM) in 1 $\times$ PBS (pH = 7.4). (c) The ratiometric absorption signals ($\text{Ab}_{745}/\text{Ab}_{675}$) of OSNs and BBD (1 $\mu\text{g}/\text{mL}$) in the presence of various ROS 1 $\times$ PBS (pH = 7.4). Control represents assay buffer only. (d) Photographic images of the BBD, OSN0 and OSNs solutions (5 $\mu\text{g}/\text{mL}$) towards of ONOO^- and H_2O_2 .

The specificity of the bulky borane doped OSNs toward ONOO^- should be attributed to the presence of bulky boranes because the nondoped OSN still had the response toward H_2O_2 just as BBD did. Accordingly, the bulky borane molecules were expected to interfere the reaction between BBD and ROS within the nanoparticles. To confirm this assumption, the model reactions were conducted between TPB and ONOO^- or H_2O_2 and mass spectroscopy was used to monitor the products (**Figure S6**, Supporting Information). The results revealed that TPB was reactive to ONOO^- but not H_2O_2 (**Figure 3b**), probably due to the weaker oxidative capability of H_2O_2 as compared with ONOO^- .³ In the presence of ONOO^- , nucleophilic attack first happened, converting TPB into Ph_2BOPh (**Figure S6**, Supporting Information). Then, Ph_2BOPh was further hydrolyzed, yielding Ph_2BOH and PhOH as the final products. Because the bulky borane was in large excess inside the nanoparticles, ROS had to react with the bulky borane first when attacking the nanoparticles (Step 1 in **Figure 3c**). Only after consumption of the bulky borane molecules inside the nanoparticles, the reaction between ROS and BBD could occur (Step 2 in **Figure 3c**). Thereby, the bulky borane served as the ONOO^- -degradable but H_2O_2 -inert shield for BBD within the nanoprobe. In such a way, the reaction between BBD and H_2O_2 was inhibited for OSN-B1&OSN-B2, leading to their specific ratiometric absorption responses toward ONOO^- .

Figure 3. Schematic illustration of reaction mechanisms of BBD (a), bulky borane (TPB) (b), and the bulky borane reinforced nanoprobe (OSN-B1) (c) with ROS (ONOO^- and H_2O_2).

To determine the activation speed of the nanoprobe, the reaction kinetics of OSNs and BBD with ONOO^- or H_2O_2 were studied by time-course absorption measurements (**Figures 4a&4b**). The apparent rate constants (k') of OSN-B1 ($4.96 \times 10^{-3} \text{ s}^{-1}$) and OSN-B2 ($3.92 \times 10^{-3} \text{ s}^{-1}$) were smaller than that of BBD ($5.13 \times 10^{-3} \text{ s}^{-1}$), which was caused by the interference of the bulky borane as illustrated as Step 1 in **Figure 3c**. In addition, due to the extra strong electron withdrawing fluorine substituents of TPFB relative to TPB, Step 1 reaction was slower in OSN-B2 as compared to that OSN-B1, resulting in the further delayed response towards ONOO^- . The reaction between BBD itself and H_2O_2 occurred at $k' = 3.12 \times 10^{-3} \text{ s}^{-1}$ that was slower than that with ONOO^- . This was due to high oxidation capacity of ONOO^- , which was widely observed for other boronated dyes.²⁰ In contrast, no activation was observed for both OSN-B1 and OSN-B2 by H_2O_2 even after incubation for 24 h (**Figure S7**, Supporting Information), confirming that the presence of bulky borane effectively inhibited the reaction between BBD and H_2O_2 . These data showed that OSN-B1 possessed high specificity toward

ONOO⁻ and relatively faster activation as compared with OSN-B2, suggesting OSN-B1 as the best candidate among these probes for *in vivo* imaging.

Because of increased metabolism of glucose and poor perfusion, solid tumors have an acidic microenvironment (pH ~6.8).²¹ To test the feasibility of probe activation in tumor, the absorption responses of OSNs and BBD towards ONOO⁻ were measured at pH ranging from 6.0 to 9.0. All the probes exhibited reduced ratiometric absorption signals (Ab_{745}/Ab_{675}) especially when pH decreased from 7.4 to 6.0 (**Figure 4c** and **Figure S8**, Supporting Information). This was attributed to the protonation of the phenoxide product to its conjugate acid (**3**) at acidic pH as **3** had $pK_a = 7.01$ (**Figure 3a**). Because the conjugate acid (**3**) had the absorption maximum at 675 nm that was the same as BBD, the ratiometric signals were reversed towards the initial unactivated state. However, the decrease rates were slowed down for the doped OSNs. At pH = 6.8, a strong peak at 745 nm was detected for the doped OSNs, which was very weak for OSN0 and nearly invisible for BBD (**Figure 4d**). This observation indicated that the bulky boranes provided a pH-buffering nanoenvironment in the nanoparticles, probably owing to the Lewis acid nature of TPB and TPFB. Thereby, the ratiometric absorption signals of OSN-B1 and OSN-B2 were ~0.85, which were 4-fold higher as compared to their initiate states (0.21). Thereby, the pH-buffering effect of the bulky borane made the doped OSN feasible for PA imaging of ONOO⁻ at acidic tumor microenvironment.

Figure 4. Activation kinetics and pH effect. (a) Ab_{745}/Ab_{675} of the probes (1 $\mu g/mL$) as a function of time in the presence of $ONOO^-$ (15 μM) in 1×PBS (pH = 7.4). (b) Ab_{745}/Ab_{675} of the probes (1 $\mu g/mL$) as a function of time in the presence of H_2O_2 (15 μM) in 1×PBS (pH = 7.4). (c) The $ONOO^-$ activated Ab_{745}/Ab_{675} of the probes (1 $\mu g/mL$) as a function of pH in 1×PBS. (d) Absorption spectra of OSNs and BBD (1 $\mu g/mL$) after addition of $ONOO^-$ (15 μM) at pH = 6.8.

The PA properties of BBD and OSNs were measured. Similar PA spectral profiles with the maximum peak at 680 nm were observed for all the probes (**Figure 5a**), confirmed that the doping of bulky borane had neglectable effect on the PA brightness of BBD. Considering that OSN-B1 had the high $ONOO^-$ selectivity and relatively faster kinetics as compared with others, OSN-B1 was used for PA imaging. In the presence of $ONOO^-$, the PA signal at 750 nm (PA_{750}) significantly increased while the signal at 680 nm (PA_{680}) remained nearly the same (**Figures 5b&5f**). This was consistent with the absorption spectral changes upon addition of $ONOO^-$, which had the isosbestic point near 680 nm (**Figure 2a and Figure S3**, Supporting Information). In contrast, in the presence of other ROS including H_2O_2 , $O_2^{\cdot-}$, OCI^- , 1O_2 , $\cdot OH$ and $\cdot ON$, the PA spectra remained nearly the same (**Figures 5b&5c**). Thereby, the ratiometric PA signals (PA_{750}/PA_{680}) of OSN-B1 increased by ~ 7 -fold after activation by $ONOO^-$, while remaining unchanged for other ROS (**Figure 3c**). Such specific ratiometric responses of OSN-

B1 could be easily distinguished in the PA images when the pseudo green and red were used to respectively assign PA₆₈₀ and PA₇₅₀ (Figure 5d&5e).

Figure 5. *In vitro* ratiometric PA imaging of ROS. (a) PA spectra of OSNs and BBD in 1×PBS (pH = 7.4). (b) PA spectra of OSN-B1 before and after addition of ONOO⁻ or H₂O₂ in 1×PBS (pH = 7.4). (c) The ratiometric PA signals (PA₇₅₀/PA₆₈₀) in the presence of various ROS (15 μM) in 1×PBS (pH = 7.4). (d) PA images of OSN-B1 before and after addition of ONOO⁻ or H₂O₂ 1×PBS (pH = 7.4). (e) Schematic illustration of PA responses of OSN-B1 towards ONOO⁻ and other ROS. (f) PA₇₅₀/PA₆₈₀ of OSN-B1 as a function of the concentration of ONOO⁻ in 1×PBS (pH = 7.4). The error bars represent standard deviations of three separate measurements.

Linear correlation between the ratiometric PA signals and the concentration of ONOO⁻ was found with a limit of detection of ~100 nM (Figure 5f), which was consistent with the ratiometric absorption signals (Figure S9, Supporting Information). According to the previous studies, the physiological steady-state concentration of ONOO⁻ is estimated to be at the nanomolar to low micromolar level, and the basal production rate is at 0.1~1 μM min⁻¹.²² In the pathological regions such as inflammatory microenvironment, the aberrant production rate could be as high as 50~100 μM min⁻¹.⁵ Thereby, the dynamic signal range and sensitivity of OSN-B1 should be ideal to detect ONOO⁻ at pathologically relevant concentrations. Moreover, no obvious cytotoxicity was detected for OSN-B1 against the cancer cells including HeLa and 4T1 as well as the normal cells (mouse fibroblasts cells) (Figure S10, Supporting Information). These data thus confirmed the suitability of OSN-B1 for *in vivo* PA imaging of ONOO⁻.

The capability of OSN-B1 for *in vivo* PA imaging of ONOO⁻ was tested for the subcutaneous 4T1 xenograft tumor of living mice with and without pre-treatment of *N*-acetyl-L-cysteine (NAC). NAC, an antioxidant drug, was used to scavenge ROS and thus reduce the ROS level in tumor.²³ After systemic administration of OSN-B1 (250 μ L, 100 μ g/mL) into the living mice through tail vein, the PA signals were simultaneously monitored at 680 and 750 nm, as illustrated by pseudo green and red colors, respectively (**Figure 6a**). The injected dosage of TPB into the living mice was 1.6 mg/Kg, which was far less than the median lethal dose (LD₅₀, 196 mg/Kg) of TPB.²⁴ Moreover, no weight loss and abnormal behaviors were observed for mice after injection of the nanoprobe. Thus, the nanoprobe should be safe for such *in vivo* imaging applications. To minimize the tissue interference, the PA intensity increments (Δ PA) were used for imaging analysis, defined as the PA intensity after injection of OSN-B1 subtracted by the intrinsic PA intensity of tumor before injection of OSN-B1. The PA increment at 680 nm (Δ PA₆₈₀) for both NAC-treated and untreated mice substantially increased after injection of OSN-B1 (**Figure 6a**), which should be attributed to the enhanced permeability and retention (EPR) effect of OSN-B1. This was confirmed *ex vivo* data showing that OSN-B1 has a relatively high tumor accumulation (**Figure S11**, Supporting Information), consistent with other similar organic nanoparticles.²⁵ In contrast to Δ PA₆₈₀, the PA intensity increment at 750 nm (Δ PA₇₅₀) increased less; moreover, Δ PA₇₅₀ increased more for the untreated mice as compared to NAC-treated mice (**Figure 6a**). This indicated the partial activation of OSN-B1 by ONOO⁻ in the tumor microenvironment. Due to such a difference in the signal evolution, the *in vivo* ratiometric PA signals (Δ PA₇₅₀/ Δ PA₆₈₀) behaved differently over time for NAC-treated and untreated mice: Δ PA₇₅₀/ Δ PA₆₈₀ gradually increased and reached its maximum at 4 h post-injection of OSN-B1 for the untreated mice, whereas Δ PA₇₅₀/ Δ PA₆₈₀ slightly increased for NAC-treated mice over 24 h (**Figure 6b**). At $t = 4$ h, Δ PA₇₅₀/ Δ PA₆₈₀ for the untreated mice is 2.6-fold higher than that for the NAC-treated mice, implying that OSN-B1 was activated more in the untreated mice.

Figure 6. *In vivo* ratiometric PA imaging of ONOO^- . (a) PA images of subcutaneous 4T1 xenograft tumor of NAC-pretreated and untreated living mice ($n=3$) after intravenous administration of OSN-B1 through tail vein injection ($25 \mu\text{g}$ per mouse). The representative PA MIP images with axial view are demonstrated at the post-injection time of 0 and 4 h. (b) The ratiometric PA signals ($\Delta PA_{750}/\Delta PA_{680}$) as a function of post-injection time of OSN-B1 in NAC-pretreated or untreated living mice ($n = 3$). (c) The real-time PA spectra of tumor in living mice after intravenous administration of OSN-B1 at 4 h ($p < 0.05$, $n = 3$).

The real-time *in vivo* PA spectra of tumors at $t = 4$ h showed a strong peak at 750 nm only for the untreated mice (**Figure 6c**); moreover, the spectral profile resembled the *in vitro* spectrum of activated OSN-B1 (**Figure 5b**). The spectral information confirmed that substantial activation of OSN-B1 occurred in the tumor for the untreated mice but not significantly for NAC-treated mice. This difference was caused by the fact that NAC effectively scavenged ONOO^- and reduced the concentration of ONOO^- in tumor. These data thus not only validated the selective activation of the nanoprobe by ONOO^- in tumor but also highlighted its ability to monitor the variation of ONOO^- levels after drug treatment in real time.

In summary, we have proposed a bulky borane doping approach to develop activatable PA nanoprobe for selective ratiometric imaging of ONOO^- . The nanoprobe (OSN-B1&OSN-B2) were composed of two ROS-reactive components: a boronated NIR dye (BBD) that changed its NIR absorption nonspecifically upon reaction with both ONOO^- and H_2O_2 , and a hydrophobic optically-inactive bulky borane (TPB or TPFB) that was selectively reactive to ONOO^- over H_2O_2 . Because the bulky borane was in large excess relative to the boronated NIR dye inside the nanoparticles, the bulky borane acted as a ONOO^- -degradable but H_2O_2 -inert shield to effectively inhibit the reaction between BBD and H_2O_2 . The Lewis acid nature of bulky borane also provided a pH-buffering nanoenvironment for BBD to resist the protonation of the deboronated product from the phenoxide form to its conjugate acid, a conversion process that compromised the ratiometric signals. Thereby, the presence of bulky borane enhanced both ONOO^- selectivity and pH inertness of the nanoprobe (OSN-B1&OSN-B2), permitting effective probe activation by ONOO^- even at tumor-relevant acidic pH (6.8). In contrast, BBD itself and the nondoped nanoprobe (OSN0) failed to do so. Furthermore, the optimum nanoprobe (OSN-B1) possessed an ideal detection window and relatively fast activation kinetics that matched the pathological production of ONOO^- . The advantages of OSN-B1 allowed for ratiometric PA imaging of ONOO^- in the tumor of living mice through systemic administration and also permitted real-time monitoring of ONOO^- along with the drug treatment. By virtue of their good biodistribution, our OSN-based nanoprobe should have the potential to image ONOO^- in other diseases such as chronic inflammation, cardiac ischemia, and neurodegenerative diseases.

To the best of our knowledge, our study not only provides the first activatable PA probe for *in vivo* imaging of a specific ROS after systematic administration, but also reveals for the first time that the reactivity of boronated dye towards ROS can be interfered by the presence of borane molecules. The doping enhanced sensing performance highlights the opportunities provided by intraparticle engineering for the design of activatable PA probes.

Supporting Information

Supporting Information is available online from the Wiley Online Library.

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