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ARTICLE TYPE

A ratiometric fluorescent probe for fast and sensitive detection of peroxynitrite: boronate ester as the receptor to initiate cascade reaction

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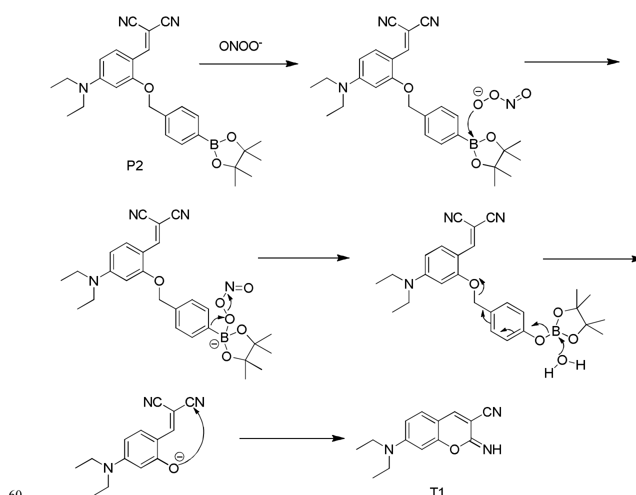
A ratiometric fluorescent probe for the detection of peroxynitrite ($ONOO^-$) was developed based on boronate receptor and intramolecular cyclization. It features fast speed, good selectivity and high sensitivity with a detection limit of 35 nM. The potential in bio-imaging was exemplified in Hela cells.

Peroxynitrite ($ONOO^-$) is an important kind of reactive oxygen species (ROS) which is generated by the reaction of nitric oxide (NO) and superoxide ($O_2^{\cdot-}$).[1] The peroxynitrite anion is relatively stable compared to the acid form (ONOOH) which can decay to nitrate quickly. $ONOO^-$ exists in protonated equilibrium with ONOOH (pKa 6.8)[2] whose half-life is about 1 s at pH 7.40[3]. Abnormality in cellular $ONOO^-$ level is found to be associated with various diseases such as rheumatoid arthritis, acute and chronic inflammatory processes, septic shock, ischemic reperfusion injury, atherosclerosis, stroke, multiple sclerosis, cancer, and so on.[4] Therefore, the development of reliable $ONOO^-$ detection is very significant.

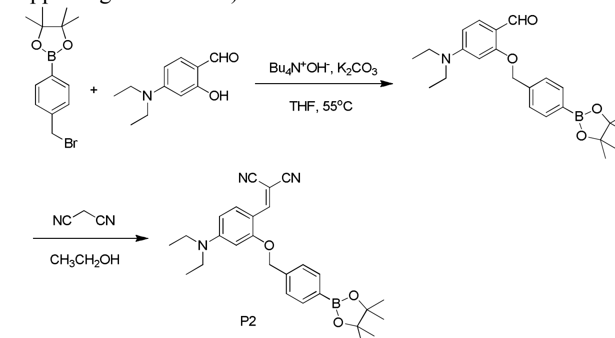
The fluorescence methods have received increasing attention due to its sensitivity, selectivity and versatility for bio-imaging applications[5]. To date, several probes have been developed for the detection of $ONOO^-$ *in vitro*[6]. Some probes are based on the reaction between $ONOO^-$ and an anisole-derived ketone via a dioxirane intermediate which always display a "turn-on" switch[6b, 6d, 6h]. After the addition of $ONOO^-$, the ketone would react with $ONOO^-$ to obtain a dioxirane that would oxidize the phenyl ring to afford dienone product and release the fluorophore. Some probes are designed by using the reducibility of selenium (Se)[6c, 7] and tellurium (Te)[6g]. However, the oxidation of Se in the probe sometimes required at least 600 s to reach a plateau[6c] and the detection limit was not very satisfying. Other reactions such as nitration[8] and utilization of two linked cyanine dyes[9] can also be applied to detect $ONOO^-$. Nevertheless these probes have complex and low-yield synthetic procedures. Most reported probes of $ONOO^-$ have a simple "turn on"[6b, c] or "turn off"[7] signal which sometimes can cause false positive, therefore a ratiometric fluorescent probe is promising since it can provide two-channel fluorescence detection.

$ONOO^-$ could react with arylboronic acids and esters to form the corresponding phenols which is much faster than that of hypochlorous acid (ClO^-) and hydrogen peroxide (H_2O_2)[10]. In addition, the mechanism of the formation of the π-extended iminocoumarin[11] has been previously employed by Kyo Han Ahn's group[12] and Youngmi Kim's group[13] for the detection of fluoride, mercury ions and alkaline phosphatase using the tert-butyldimethylsilyl (TBDMS) ether, vinyl ether and phosphate as receptors. Therefore based on cascade reaction, we designed a new fluorescent probe (**P2**, Scheme 1) for the detection of $ONOO^-$. Probe **P2** had a boronate ester as the receptor for $ONOO^-$ and two nitrile groups for the following cyclization. After adding $ONOO^-$, the boronate ester was oxidized by $ONOO^-$ and the resulting phenolic oxygen would attack the nitrile group, giving rise to a cyclized product which could lead to fluorescence enhancement.



Scheme 1 The mechanism for $ONOO^-$ detection.

The probe **P2** was conveniently prepared in a two-step cascade (scheme 2) and fully characterized by NMR and HRMS (see supporting information)



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Scheme 2 The synthesis of the probe **P2**

With the probe in hand, we first investigated the fluorescence properties of the probe in response to different pH. Free probe **P2** displayed a fluorescence band at 580 nm and had a maximum absorption at 440 nm. The probe showed good stability from pH 4 to 10 (see supporting information). Therefore the detection of $ONOO^-$ was performed in the PBS buffer (pH 7.4) with 10% CH_3CN as a co-solvent.

Next its response to $ONOO^-$ was examined by fluorescence titration. Temporal emission tracking of **P2** (20 μM) in the presence of $ONOO^-$ (0-2.5 μM) suggested that the fluorescence could reach a constant value very quickly, in addition even though the concentration of the probe was low, for example 1 μM, the reaction was still fast and could be finished within 10 s (see the kinetic curves in the supporting information). The rate constant[14] for the reaction between the probe and $ONOO^-$ was $(7.65 \pm 0.268) \times 10^3$ $min^{-1}M^{-1}$ (see supporting information). Upon the addition of $ONOO^-$, the fluorescence intensity at 580 nm decreased gradually and the fluorescence intensity at 480 nm increased dramatically (Figure 1). We then proved the mechanism of the reaction between the probe **P2** and $ONOO^-$. **T1** was synthesized and confirmed to be the product according to the HPLC retention time (Figure 2). The probe shows a linear fluorescence enhancement when the concentration of $ONOO^-$ ranges from 0-0.5 μM (Figure 3). The detection limit of the probe was calculated to be 35 nM according to the fluorescence enhancement at 480 nm (S/N=3)[15]. We also calculated the second-order rate constant for the reaction of the probe with H_2O_2 (see supporting information). The rate constant for the reaction between the probe and H_2O_2 is 75.3 ± 3.56 $min^{-1}M^{-1}$.

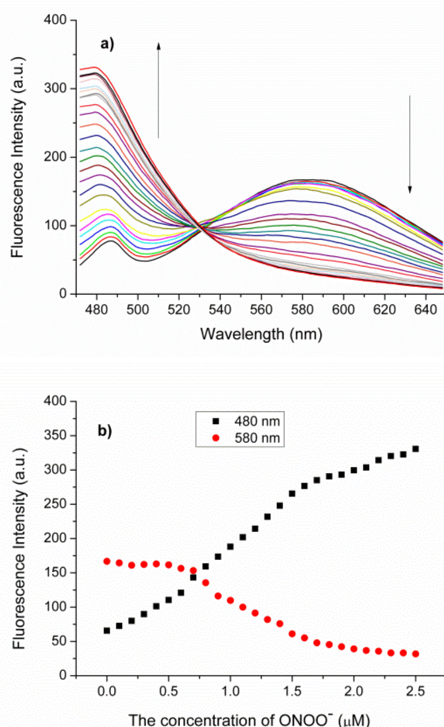

Figure 1. (a) The emission spectra of probe **P2** (20 μM) upon addition of $ONOO^-$ (0-2.5 μM) in PBS buffer solution (0.01M, pH 7.4) with 10% CH_3CN as a co-solvent. (b) Fluorescence responses ($I_{480\ nm}$ and $I_{580\ nm}$) of **P2** (20 μM) to $ONOO^-$ (0-2.5 μM). Excitation wavelength was 440 nm. Slit: 5 nm, 5 nm.

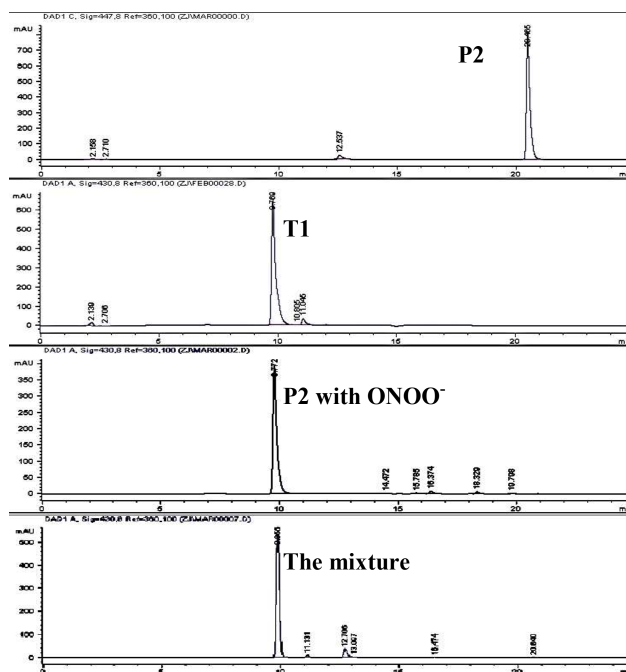

Figure 2. HPLC chromatogram of probe **P2** (100 μM), product **T1** (100 μM), the probe **P2** with $ONOO^-$ (0.5 eq) and the mixture of the reaction system and the product **T1**.

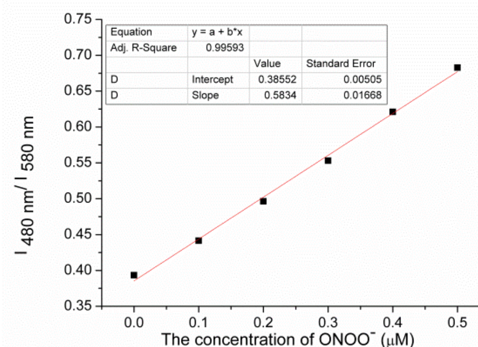

Figure 3. A liner calibration graph of the fluorescence response of the probe **P2** to the concentration of $ONOO^-$ (0-0.5 μM). Excitation wavelength was 440 nm. Slit: 5 nm, 5 nm.

To explore the selectivity and competitiveness of the probe, we examined the fluorescence responses of the probe **P2** to other ROS in PBS buffer (pH 7.4, containing 10% CH_3CN), such as ClO^-, $\cdot OH$, H_2O_2 and $^1O_2$ (Figure 4). Of these species, H_2O_2 induced an obvious fluorescence enhancement only under very high concentrations (larger than 100 μM) after more than half an hour (see supporting information). The reaction between the probe and H_2O_2 was very slow, therefore the discrimination of $ONOO^-$ and H_2O_2 can be achieved by the different rate of the oxidation. We detected the fluorescence in presence of excess peroxo radical species, and we found that only $^1O_2$ would interfere the detection of $ONOO^-$. In addition, ClO^- would slow down the reaction speed between the probe and $ONOO^-$ and it took at least 20 min for the emission at 480 nm to reach a maximum.

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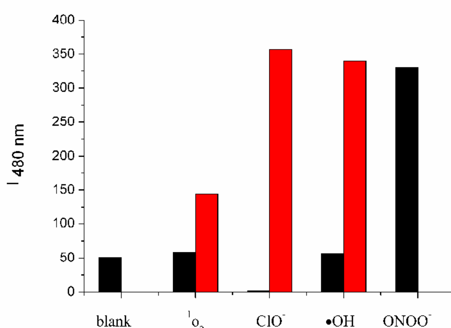

Figure 4. The black columns represented the fluorescence intensity of the probe **P2** (20 μM) in the presence of different ROS including $^1O_2$ (100 μM), ClO^- (100 μM), ·OH (100 μM) and $ONOO^-$ (2.5 μM) in PBS buffer solution (with 10% CH_3CN as a cosolvent). The red columns represented the fluorescence responses of the probe **P2** (20 μM) to $ONOO^-$ (2.5 μM) in the presence of $^1O_2$ (100 μM), ClO^- (100 μM) and ·OH (100 μM). Excitation wavelength was 440 nm. Slit: 5 nm, 5 nm.

At last we applied this probe for bioimaging. RAW264.7 cells were incubated with the probe **P2** (20 μM), lipopolysaccharide (1 μg/mL) and interferon-γ (50 ng/mL) for 4 h, and then phorbol 12-myristate 13-acetate (10 nM) was added. After another 30 min, we could observe obvious fluorescence enhancement. If aminoguanidine (an inhibitor of nitricoxide synthase) and 2,2,6,6-tetramethylpiperidine-N-oxyl (the superoxide scavenger) were added, much weaker fluorescence was detected which indicated that our probe could be used to detect endogenous $ONOO^-$ in the biological systems.

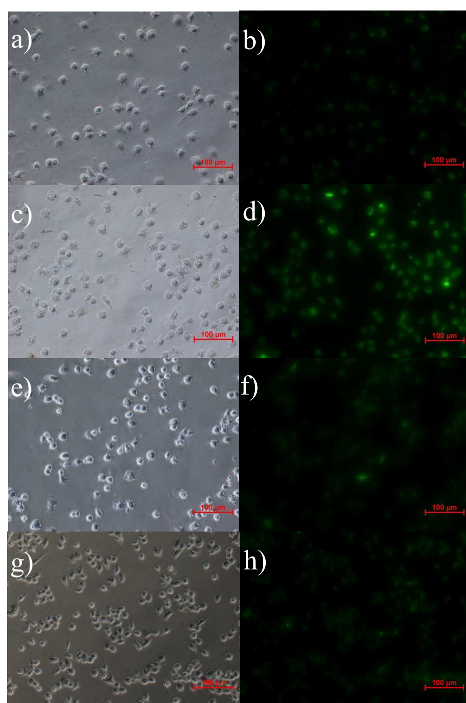

Figure 5. RAW264.7 cells were treated with various stimulants and the probe **P2**. (a) Bright-field image of RAW264.7 cells incubated with **P2** (20 μM) for 4.5 h; (b) Fluorescence image of RAW264.7 cells incubated with **P2** (20 μM) for 4.5 h; (c) Bright-field image of RAW264.7 cells incubated with the probe **P2** (20 μM), lipopolysaccharide (1 μg/mL) and interferon-γ (50 ng/mL) for 4 h, and then phorbol 12-myristate 13-acetate (10 nM) for 30 min; (d) Fluorescence image of RAW264.7 cells incubated with the probe **P2** (20 μM), lipopolysaccharide (1 μg/mL) and interferon-γ (50 ng/mL) for 4 h, and then phorbol 12-myristate 13-acetate (10 nM) for 30 min; (e) Bright-field image of RAW264.7 cells incubated with aminoguanidine (1 mM), the probe **P2** (20 μM), lipopolysaccharide (1 μg/mL) and interferon-γ (50 ng/mL) for 4 h, and then phorbol 12-myristate 13-acetate (10 nM) for 30 min; (f) Fluorescence image of RAW264.7 cells incubated with aminoguanidine (1 mM), the probe **P2** (20 μM), lipopolysaccharide (1 μg/mL) and interferon-γ (50 ng/mL) for 4 h, and then phorbol 12-myristate 13-acetate (10 nM) for 30 min; (g) Bright-field image of RAW264.7 cells incubated with 2,2,6,6-tetramethylpiperidine-N-oxyl (100 μM), the probe **P2** (20 μM), lipopolysaccharide (1 μg/mL) and interferon-γ (50 ng/mL) for 4 h, and then phorbol 12-myristate 13-acetate (10 nM) for 30 min; (h) Fluorescence image of RAW264.7 cells incubated with 2,2,6,6-tetramethylpiperidine-N-oxyl (100 μM), the probe **P2** (20 μM), lipopolysaccharide (1 μg/mL) and interferon-γ (50 ng/mL) for 4 h, and then phorbol 12-myristate 13-acetate (10 nM) for 30 min.

To conclude, we have developed a fluorescent probe connecting boronate ester oxidation and *in situ* cyclization together for the highly sensitive and selective detection of $ONOO^-$. The probe shows a fast fluorescence intensity enhancement toward $ONOO^-$. The detection limit of the probe was calculated to be 35 nM. In addition, the probe can be used to image $ONOO^-$ in living cells which makes it of potential use for the study on $ONOO^-$ related molecular process in the biological systems.

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