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

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

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 (O2−).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) whose half-life is about 1 s at pH 7.40. 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. 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 plateau6c and the detection limit was not very satisfying. Other reactions such as nitration8 and utilization of two linked cyanine dyes9 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”10 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 (ClO2−) and hydrogen peroxide (H2O2)10. In addition, the mechanism of the formation of the π-extended iminocoumarin11 has been previously employed by Kyo Han Ahn’s group12 and Youngmi Kim’s group13 for the detection of fluoride, mercury ions and alkaline phosphatase using the tert-butylidimethylsilyl (TBDM) 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).
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% CH₃CN 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⁻ (0.25 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 for the reaction between the probe and ONOO⁻ was $(7.65±0.268)×10^3$ 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). We also calculated the second-order rate constant for the reaction of the probe with H₂O₂ (see supporting information). The rate constant for the reaction between the probe and H₂O₂ is $75.3±3.56$ min⁻¹ M⁻¹.

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% CH₃CN), such as ClO⁻, OH, H₂O₂ and ¹O₂ (Figure 4). Of these species, H₂O₂ 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 H₂O₂ was very slow, therefore the discrimination of ONOO⁻ and H₂O₂ 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 ¹O₂ 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.

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% CH₃CN as a co-solvent. (b) Fluorescence responses (I₄₈₀ nm and I₅₈₀ 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 linear 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.
Figure 4. The black columns represented the fluorescence intensity of the probe P2 (20 μM) in the presence of different ROS including O2 (100 μM), ClO (100 μM), -OH (100 μM) and ONOO- (2.5 μM) in PBS buffer solution (with 10% CH3CN 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 O2 (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 4.5 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.

We are grateful for the financial support from the State Key Program of National Natural Science of China (21236002), the National Basic Research Program of China (2010CB126100), the National High Technology Research and Development Program of China (2011AA10A207).

Notes and references


