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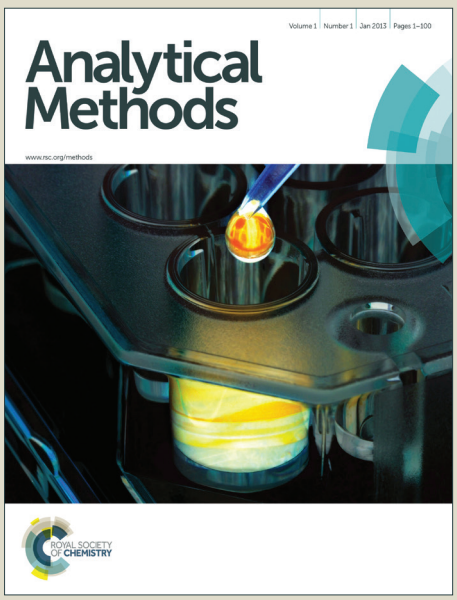

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ARTICLE TYPE

An arylboronate-based fluorescent probe for peroxynitrite with fast response and high Selectivity

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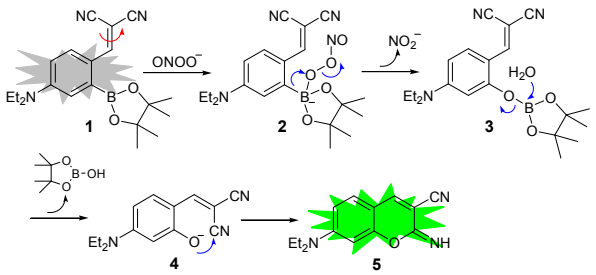
An arylboronate-based fluorescent probe for peroxynitrite with fast fluorescence response and high selectivity was developed based on the peroxynitrite-triggered assembly of aminocoumarin fluorescent dye.

Peroxynitrite ($ONOO^-$), a highly reactive oxidant and an efficient nitrating agent in living systems, is generated through the reaction of nitric oxide (NO) and superoxide ($O_2^{\bullet-}$) under diffusion control.[1] $ONOO^-$ and its secondary metabolites ($NO_2^{\bullet}$, $CO_3^{\bullet-}$, and $^{\bullet}OH$) can react with a wide array of biomolecules, such as proteins, lipids, and nucleic acids, eventually resulting in cell death. As a result, $ONOO^-$ has been implicated in a variety of disease states, including Alzheimer's disease, arthritis, cancer, autoimmune and inflammatory disease, and other disorders.[2] However, evidences also revealed that $ONOO^-$ plays a positive role in either signal transduction via nitrating tyrosine residues[3] or immunogenic response against invading pathogens.[4] Therefore, methods for detection of cellular $ONOO^-$ are of considerable importance for both disease diagnosis and exploration of its various pathophysiology.

Fluorescent probe technique is well suited to meet the need to map the spatial and temporal distribution of biomolecules within living cells due to its sensitivity, visualization, and nondestructive detection.[5] The early stage fluorescent probes for $ONOO^-$ are based on the leuco and nonfluorescent dihydrorhodamine and dihydrofluorescein, which can be converted to fluorescent products upon being oxidized by $ONOO^-$.[6] However, these probes also respond to other reactive oxygen species (ROS) such as $^{\bullet}OH$ and ClO^-, and thus have little selectivity toward $ONOO^-$. Afterwards, a number of small-molecule fluorescent probes with the improved selectivity were exploited based on the $ONOO^-$-triggered specific reactions, including aromatic nitration,[7] formation of dioxirane with activated ketones,[8] oxidation of organoselenium/organotellurium[9] or phenol[10] or 4-hydroxyaniline groups,[11] chemical transformation of boronic acid[12] or boronate[13] to phenol, and other.[14] However, considering the very short lifetime (~10 ms) of $ONOO^-$ in biological systems,[2d] fastly probing the species still remains challenging. As far as we know, there are only few fluorescent probes that could detect $ONOO^-$ within seconds to date.[8b,9b,11]

Based on the reaction of arylboronate with H_2O_2 to produce phenol, arylboronate-containing fluorescent probes have been utilized for detecting and imaging H_2O_2 in living cells.[15] Despite the long response time, the possibility of oxidation by $ONOO^-$ was not tested. In 2009, it was reported that arylboronate could react rapidly with $ONOO^-$ to form stable hydroxy derivatives; moreover, the rate constants (~ 1.6×10^6 M^{-1} s^{-1}) are nearly a million times bigger than that of H_2O_2 (2.2 M^{-1} s^{-1}).[16] The big differences in rate constants could be explained with at least two factors: (1) in aqueous solution, ONOOH has a pK_a value of 6.7, whereas H_2O_2 has a higher value of 11.7; thus, $ONOO^-$ should be a better nucleophile than H_2O_2 in physiological medium; (2) in the reactions of arylboronate with $ONOO^-$ or H_2O_2, it is easier for $ONOO^-$–arylboronate adduct than for H_2O_2–based one, formed by the initial reaction of arylboronate with $ONOO^-$ and H_2O_2 respectively, to undergo the subsequent aryl migration reaction because NO_2^- is a better leaving group than OH^-. Given these, it is very promising for arylboronate-based fluorescent probes to realize the fast response toward $ONOO^-$. In fact, this has been examplified by two such fluorescent probes reported recently, which were able to detect $ONOO^-$ within 10 s and 15 s, respectively.[13a,c] However, just like a double-edged sword, the fast response of the probes is often accomponied by the interference by H_2O_2 especially in lengthened incubation time. Therefore, further efforts are still required for reseatchers to improve the response rate while containg the high selectivity.

Herein, we present the design and synthesis of a new arylboronate-based fluorescent probe **1** (Scheme 1), and demonstrate its potential for detecting $ONOO^-$ with fast response (within five seconds) and high selectivity over a series of ROS including H_2O_2. With the probe, the imaging of the exogenous and stimulation-induced $ONOO^-$ in RAW264.7 murine macrophages has successfully been realized.



Scheme 1 Proposed sensing mechanisms of probe **1** for $ONOO^-$.

Probe **1** was designed by installing a boronate binding group to 2-position of 4-(diethylamino)benzene-malononitrile conjugate, a potential fluorophore. It was expected that the probe is poorly fluorescent due to the C=C isomerization-induced fluorescence quenching;[17] however, the oxidative hydrolysis of **1** by $ONOO^-$ followed by the intramolecular cyclization would lead to the highly fluorescent iminocourmarin **5**. Also, we speculated that the electron-rich diethylaminobenzene group of the probe could partly decrease the electrophilicity of the adjacent boronate, which would probably inhibit the nucleophilic attact of H_2O_2 toward boronate B center to some extent, but has little affect on the strongly nucleophilic $ONOO^-$. If so, an improved selectivity toward $ONOO^-$ over H_2O_2 would be realized.

Probe **1** could easily be synthesized through a simple three-step synthetic procedue, and its structure was confirmed by ^1H NMR, ^{13}C NMR, and HRMS spectra (ESI†). With probe **1** in hand, we first examined the reactivity of **1** toward $ONOO^-$ in Tris-HCl buffer (100 mM, pH 7.4, containing 20% methnol) at 25 °C. As shown in Fig. 1A, in the absence of $ONOO^-$, the solution of **1** (5 μM) showed a very poor fluorescence, indicating the efficient C=C isomerization-induced fluorescence quenching; upon treated with $ONOO^-$ (10 equiv), a dramatic fluorescence enhancement of 28-fold at 486 nm and an obvious fluorescence color change from dark to green were observed. Notably, the time-dependent fluorescence intensity changes of **1** in the presence of $ONOO^-$ revealed that the reaction could be completely finished within five seconds (Fig. 1B), confirming the fast fluorescence response ability of **1** toward $ONOO^-$. In this regard, probe **1** is indeed superior to most of the reported $ONOO^-$ fluorescence probes and comparable to HKGreen-4 reported recently.[10a]

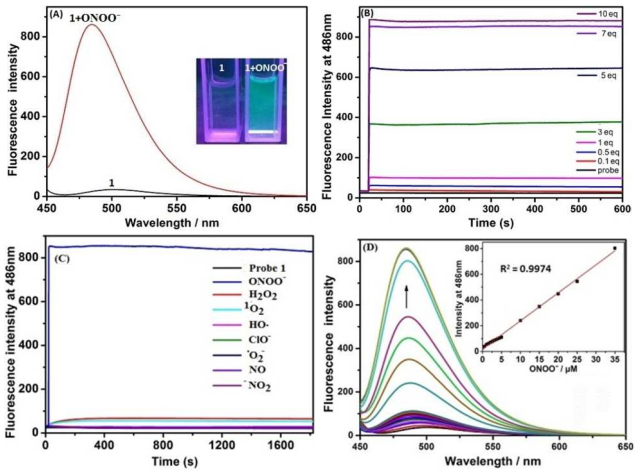


Fig. 1 (a) Fluorescence spectra of **1** (5 μM) in the absence and presence of 10 equiv of $ONOO^-$. (b) Time-dependent fluorescence intensity changes at 486 nm of **1** (5 μM) treated with varied concentrations of $ONOO^-$. (C) Time courses of the responses of **1** (5 μM) to $ONOO^-$ (50 μM) and other ROS (100 μM) for 30 min. (D) Fluorescence spectra of **1** (5 μM) treated with $ONOO^-$ (0 – 50 μM). Inset: the corresponding linear relationship between the fluorescent intensity and $ONOO^-$ concentrations. Spectra were recorded after incubation with different concentrations of $ONOO^-$ for 1 min. Conditions: Tris-HCl buffer (100 mM, pH 7.4, containing 20% methanol); λ_{ex} = 436 nm; λ_{em} = 486 nm; Slits: 5/10 nm; voltage: 500 V; T: 25 °C.

In order to confirm the reaction mechanism, HRMS experiment of **1** treated with $ONOO^-$ was performed, wherein the peak at *m/z* 242.1286 corresponding to the product iminocourmarin **5** was clearly observed (Fig. S1, ESI†). Also, we synthesized iminocoumarin **5** by treatment of **1** with $ONOO^-$ solution, and its chemical structure was unambiguously confirmed by ^1H NMR, ^{13}C NMR, and HRMS spectra (ESI†). Moreover, the absorption and fluorescence spectra profiles of iminocourmarin **5** were in accordance with those of **1** treated with $ONOO^-$ (Fig. S2, ESI†).

To evaluate the selectivity of probe **1** for $ONOO^-$, we subsequently tested the fluorescence response of **1** (5 μM) toward a series of biologically relevant ROS, including H_2O_2, $^1O_2$, $^{\bullet}OH$, ClO^-, $O_2^{\bullet-}$, NO, and NO_2^-, with the high concentration of 100 μM and in a lengthened reaction time of 30 min. As shown in Fig. 1C, in these competitive species, only H_2O_2 and $^1O_2$ elicited a slight fluorescence enhancement of 2.0-fold and 1.8-fold, respectively. In fact, in view of the similar reaction mechanism, the slight interference by H_2O_2 is understandable. As for the case of $^1O_2$, we speculated that the slight interference should be a result of the residual H_2O_2 because $^1O_2$ was synthesized by addition of the HClO into a solution of H_2O_2. Overall, the selectivity of **1** for $ONOO^-$ is high, which is probably due to, as mentioned previously, the decreased electrophilicity of the boronate binding group. In addition, we also tested the fluorescence response of **1** toward some biologically relevant anions and cations, such as Cl^-, Br^-, I^-, SO_4^{2-}, HCO_3^-, $H_2PO_4^-$, Na^+, K^+, Mg^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, and Fe^{2+}, as well as biothiols and glucose (Fig. S3, ESI†). As expected, all these species did not give any obvious fluorescence change of **1**.

Next, we performed the fluorescence titration experiment of **1** for $ONOO^-$ in the same condition. As shown in Fig. 1D, upon treatment with the increasing concentrations of $ONOO^-$, the fluorescence intensity of **1** at 486 nm gradually increased, and when the amount of $ONOO^-$ was more than 10 equiv, the spectra saturation was reached. In this case, a linear calibration graph of the fluorescent intensities (I_{486}) to $ONOO^-$ concentrations from 0 μM to 35 μM was obtained (R^2 = 0.9974), and the detection limit was measured to be 48 nM based on S/N =3. Also, we evaluated the effect of pH on the fluorescence response of **1** to $ONOO^-$ (Fig. S4, ESI†). It was found that probe **1** had the smallest fluorescence in a wide pH range of 2–12, and displayed the obvious fluorescence enhancement for $ONOO^-$ in the region of 7–10. Thus, probe **1** could function properly at physiological pH.

Finally, we evaluated the capability of probe **1** for the imaging of $ONOO^-$ in RAW264.7 murine macrophages, which are known to produce $ONOO^-$ upon stimulation with interferon-γ (IFN-γ) and lipopolysaccharide (LPS).[18] As shown in Fig. 2, when the cells were incubated with **1** (10 μM), they gave almost no fluorescence in green channel (Fig. 2A); when the cells were pretreated with $ONOO^-$ donor SIN-1 (3-morpholinosydnonimine),[19] and then treated with **1**, an obvious fluorescence enhancement in green channel was observed (Fig. 2B), revealing that the probe could image exogenous $ONOO^-$ in cellular environment; when the cells were stimulated with IFN-γ/LPS and then treated with **1**, the

dramatic fluorescence in green channel was also observed, indicating the the probe is capable of imaging the stimulation-induced $ONOO^-$; when the cells were pretreated with $O_2^{\bullet}$ scavenger 2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPO)[20] or NO synthase inhibitor aminoguanidine (AG)[20] during stimulation of the cells with IFN-γ/LPS, and then treated with **1**, an obvious decrease in fluorescence intensity within the cells was observed (Figs. 2D and 2E). Thus, the green fluorescence from the activated RAW264.7 murine macrophages could be attributed to the formation of $ONOO^-$ and its rapid reaction with **1** to produce fluorescent **5**.

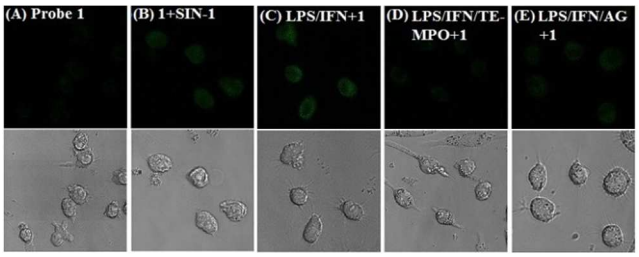


Fig. 2 Fluorescence images of RAW264.7 murine macrophages under different conditions. (A) cells were treated with **1** (10 μM, 20 min) only; (B) cells were pretreated with **1** (10 μM, 20 min), and then treated with SIN-1 (1 mM, 30 min); (C) cells were stimulated with LPS (1 μg/mL) and IFN-γ (50 ng/mL) for 12 h, and then treated with **1** (10 μM, 20 min); (E) cells were pretreated with TEMPO (300 μM) during LPS/IFN-γ stimulation, and then treated with **1** (10 μM, 20 min); (E) cells were pretreated with AG (5 mM) during LPS (1 μg/mL)/IFN-γ (50 ng/mL) stimulation, and then treated with **1** (10 μM, 20 min). Image from band path of 467-600 nm upon excitation of **1** at 458 nm.

In summary, we reported herein a new arylboronate-based fluorescent probe **1**, which could detect $ONOO^-$ with fast fluorescence response, high selectivity and sensitivity. The sensing mechanism proved to be the $ONOO^-$-triggered oxidative hydrolysis of **1** followed by an intramolecular cyclization to lead to the fluorescent iminocourmarin **5**. The imaging of the exogenous and stimulation-induced $ONOO^-$ in RAW264.7 murine macrophages was successfully achieved.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental procedures, supplemental spectra, and the ^1H–, ^{13}C– NMR, and MS spectrum. See DOI: 10.1039/b000000x/

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Graphic Abstract

An arylboronate-based fluorescent probe for peroxynitrite with fast response and high Selectivity

Jian Zhang, Yaping Li, and Wei Guo*

An arylboronate-based fluorescent probe was developed for detecting peroxynitrite *in vitro* and *in vivo* with fast response and high selectivity.