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A simple boronic acid-based fluorescent probe for selective detection of hydrogen peroxide in solutions and living cells

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ABSTRACT

An approach of high sensitivity and selectivity for hydrogen peroxide (H_2O_2) detection is highly demanded due to its important roles in regulating diverse biological process. In this work, we introduced an easily synthesized fluorescent "turn off" probe, **BNBD**. It is designed based on the core structure of 4-chloro-7-nitrobenzofurazan as a fluorophore and incorporated with a specific H_2O_2-reactive group, aryl boronate, for sensitive and selective detection of H_2O_2. We demonstrated its selectivity by incubating the probe with other types of ROS, and measured the limit of detection of **BNBD** as 1.8 nM. **BNBD** is also conducive to H_2O_2 detection at physiological conditions. We thus applied it to detect both exogenous and endogenous changes of H_2O_2 in living cells by confocal microscopy, supporting its future applications to selectively monitor H_2O_2 levels and identify H_2O_2-related physiological or pathological responses from live cells or tissues in the near future.

1. Introduction

H_2O_2, one of the most important reactive oxygen species (ROS), plays a vital role in regulating a myriad of molecular signaling events and maintaining cellular homeostasis [1,2]. H_2O_2 of picomolar concentration [3] functions as an omnipresent intracellular messenger in biological process, including immune response, host defense, pathogen invasion, etc. [4–13]. However, mounting evidences also show that excessive levels of H_2O_2 could lead to DNA, RNA and protein damages, which subsequently results in advent of many diseases, such as cardiovascular disease [14], cancer [15], diabetes [16] and neurodegenerative disorders [17]. Consequently, a sensitive and specific method that allows for sensitive and selective monitoring H_2O_2 is highly demanded.

Powerful approaches of H_2O_2 detection have thus been developed, including spectrophotometry [18], titrimetry [19], electrochemistry [20], fluorometry [21] and chemiluminescence [22]. Fluorometry and photoluminescence are widely used in the detection of metal ions, anions and neutral molecules [23,24]. Among these methods, fluorescent probes exhibit high sensitivity and allow for detection of H_2O_2 with high spatial and temporal resolution in living cells, making them particularly appealing tools [25]. A number of fluorescent probes for H_2O_2 detection have been developed [26,27], which makes use of distinct chemical reactions, such as the conversion of diketone to acid [28], sulfonic acid hydrolysis [29], and the reaction of arylboronates to phenols [30]. Among these probes, aryl boranate has been found to provide superior selectivity for H_2O_2 even in the presence of other types of ROS. This remains a challenge for some probes such as those developed based on sulfonic ester hydrolysis with low sensitivity toward H_2O_2 [29]. In contrast, aryl boronate is capable of specifically reacting with H_2O_2 and generating quinone methide in mild alkaline conditions. Herein, considering the position of substituents affecting the fluorescence properties of compounds [31], we designed three ortho-, meta-, para-substituted aryl boronic acid derivatives and found that para-substituted aryl boronic acid derivative is a suitable selective "turn-off" fluorescent probe in this study. Next we evaluated its capability in H_2O_2 detection for live cell monitoring.

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2. Materials and methods

All the chemicals were purchased from Adamas-Beta (Shanghai, China). $CDCl_3$ was purchased from Cambridge Isotope Laboratory. Milli-Q water was supplied by a Milli-Q Plus System (Millipore Corp., Breford, USA). Fluorescence was measured on a Shimadzu RF-5301 spectrophotometer with both excitation and emission slits set at 5.0 nm. ^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectra were measured on Bruker AV 300 spectrometer (Bruker, Zurich, Switzerland). High resolution mass spectrometry (HRMS) spectra were recorded on an Agilent 6500 (Agilent, CA, USA). Fluorescent emission spectra were collected on PerkinElmer LS 55 (PerkinElmer, MA, USA).

2.1. Compound 2

4-Chloro-7-nitro-1,2,3-benzoxadiazole (**1**) (502 mg, 2.5 mmol) was dissolved in ethanol (13 mL) followed by addition of sodium acetate (310 mg, 3.75 mmol) at room temperature. Methyl piperazine (374 mg, 3.75 mmol) was then added by drops and the mixture was stirred overnight. After suction filtration, the solid was washed by ethanol to concentrate compound **2** as orange solid powder (590 mg, 89.4%). ^{1}H NMR (300 MHz, DMSO) δ (ppm): 8.435–8.403 (d, J = 9.6 Hz, 1H), 6.643–6.612 (d, J = 9.4 Hz, 1H), 4.065 (s, 4H), 2.471–2.505 (m, 4H), 2.178 (s, 3H). ^{13}C NMR (126 MHz, DMSO) δ 145.78, 145.30, 145.24, 136.73, 121.60, 104.13, 54.67, 49.73, 45.71.

2.2. Compound 3

To a solution of compound **2** (109 mg, 0.39 mmol) dissolved in acetonitrile (20 mL), sodium acetate (55 mg, 0.66 mmol) was added. Subsequently, 2-(bromomethyl) phenylboronic acid (136 mg, 0.63 mmol) was quickly added. After being stirred overnight, the mixture was filtered and the solid was washed with acetonitrile several times to produce the compound **3** as orange solid (2.67 g, 99.4%). ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 8.634–8.604 (d, J = 9.0 Hz, 2H), 7.844–7.815 (m, 1H), 7.626–7.541 (m, 2H), 6.838–6.808 (d, J = 9.0 Hz, 1H), 5.014 (s, 2H), 4.794–4.744 (d, J = 6.0, 2H), 4.288–4.208 (t, J_1 = 11.4 Hz, J_2 = 12.6 Hz, 2H), 3.880–3.812 (t, J_1 = 11.1 Hz, J_2 = 9.3 Hz, 2H), 3.689–3.647 (d, J = 12.6 Hz, 2H), 3.161 (s, 3H). ^{13}C NMR (126 MHz, MeOD) δ 145.12, 144.52, 144.45, 135.57, 135.15, 132.80, 130.54, 128.95, 125.02, 104.83, 69.14, 58.77, 45.21, 42.91. HRMS $C_{18}H_{21}BN_5O_5$ $[M + Na]^+$ calcd 420.1564, found 420.1565.

2.3. Compound 4

The synthesis of compound **4** was the same as compound **3**. ^{1}H NMR (300 MHz, DMSO) δ (ppm): 3.586–3.624 (d, J = 11.4 Hz, 2H), 3.768 (s, 2H), 4.231 (s, 2H), 4.786 (s, 2H), 6.786–6.815 (d, J = 8.7 Hz, 1H), 7.449 (s, 1H), 7.534 (s, 1H), 7.845–7.867 (d, J = 6.6 Hz, 1H), 8.599–8.628 (d, J = 8.7 Hz, 1H), 11.187 (s, 2H). ^{13}C NMR (126 MHz, DMSO) δ 145.26, 145.17, 145.03, 139.26, 136.70, 136.43, 134.83, 132.93, 128.38, 126.54, 123.62, 105.49, 68.17, 58.34, 45.78, 43.47. HRMS $C_{18}H_{21}BN_5O_5$ $[M]^+$ calcd 398.1630, found 398.1685.

2.4. Compound 5 (*BNBD*)

Compound **2** (199 mg, 0.75 mmol) was dissolved in ethanol (10 mL) under heat, then sodium acetate (78 mg, 0.94 mmol) and 4-(bromomethyl) phenylboronic acid was added quickly and stirred overnight under reflux. After the reaction, the mixture was filtered and the solid was washed with ethanol to deliver the **BNBD** as yellowish solid (220 mg, 60.4%). ^{1}H NMR (300 MHz, DMSO) δ (ppm): 8.655–8.625 (d, J = 9 Hz, 2H), 8.276 (s, 1H), 7.956–7.931 (d, J = 7.5 Hz, 2H), 7.560–7.585 (d, J = 7.5 Hz, 2H), 6.836–6.806 (d, J = 9 Hz, 1H), 4.784 (s, 2H), 4.732 (d, 2H), 4.281 (t, J = 10.2 Hz, 2H), 3.814 (t, 2H), 3.663 (d, J = 11.7 Hz, 2H),3.173 (s, 3H). ^{13}C NMR (75 MHz, $CDCl_3$) δ 145.19, 145.10, 144.96, 136.61, 134.86, 132.55, 132.28, 128.96, 123.51, 105.47, 67.76, 67.40, 58.33, 56.35, 45.99, 43.47. HRMS $C_{18}H_{21}BN_5O_5$ $[M]^+$ calcd 398.1630, found 398.1685.

2.5. Confocal microscopy

A549 cells (1×10^4 cells/well) were seeded in a 35 mm cell culture glass dish. After 24 h, cells were treated with **BNBD** (10 μM) for 1 h, following by incubation with H_2O_2 for 20 min. Finally, florescent images of cells were acquired on a LSM-700 Microscope (Zeiss, Jena, German) with an objective lens (×40) using a green filter (excitation wavelength: 488 nm).

3. Results and discussions

The synthesis route of our developed probe is concise and involves merely two steps. We started with a core structure of 4-chloro-7-nitro-1, 2, 3-benzoxadiazole (NBD-Cl), a commercially available fluorophore (***compound 1***), and then introduced the N-methyl piperazine to substitute the −Cl group on the benzene ring (***compound 2***). Phenylboronic acid group subsequently reacted with compound 2 and formed a tertiary amine as shown in Scheme 1. The boronic acid group substituted at different sites of the benzene ring resulted in the production of three positional isomers (***compound 3–5***). The yield can reach up to 60.4%. The acquired ^{1}H NMR and ^{13}C NMR spectra of the products can be found in supplementary information (Dick).

Subsequently, we investigated the capability of the three probes (compound 3, 4, 5/**BNBD**) in H_2O_2 detection under different pH values. We first recorded the fluorescent spectra of the three probes, and found they all showed a maximum fluorescent emission at 535 nm under the excitation at 465 nm. Interestingly, upon exposure to H_2O_2 the three probes exhibited distinct behaviors. For instance, probe 3 can be oxidized by H_2O_2 in PBS (phosphate buffered saline) buffer at pH = 7.8, which concomitantly reduces the fluorescence emission intensity to ~1/3 of that prior to the reaction by fluorescent quenching due to intramolecular charge transfer (ICT) effect. However, negligible changes to fluorescent intensities are observed when the redox reactions occur in buffers at pH 5.4 and 7.0 (Fig. S9). In contrast, probe 4 exhibits marked fluorescent changes upon exposure to H_2O_2 in PBS buffers only under pH = 5.4 (Fig. S10). As for **BNBD**, When it was incubated with H_2O_2 in PBS under mild alkaline condition, the fluorescent intensity was effectively quenched to nearly 1/10 of that prior to the reaction. Obvious fluorescence quenching was also noted in buffers of pH = 7.0 rather than that of pH = 5.4 (Fig. S11). Consequently, the para-position substituted compound, **BNBD**, is the most robust probe that undergoes oxidation by H_2O_2 in both neutral and alkaline aqueous conditions and thus most amenable for detection in living systems compared with the other two compounds.

Such difference could be explained by a tentative mechanism (Fig. 1). **BNBD** contains a para-phenylboronic acid group as an electron-donating group and a 7-nitro group as an electron-withdrawing group, forming a push-pull system to cause strong ICT effect, and thus BNBD shows strong green fluorescence [32]. **BNBD** is prone to form a quinone methide with the release of boronic acid upon the oxidation of H_2O_2 when the boronate group is in the *para* site of the benzene ring. Concominantly, compound 2 is produced as a final product, delivering much weaker fluorescence due to inhibition of the ICT effect and thus resulting in fluorescence quenching. In contrast, the positions of the boronate in compound 3 and 4 are unfavorable for the production of quinone methide and boronic acid, and thus show inferior capability to quench the fluorescence compared with **BNBD**. We confirmed the structure of the final product produced by **BNBD** and H_2O_2 as compound 2 by ^{1}H NMR and HRMS, which has been further validated by comparison with authentic standards.

Next, we selected **BNBD** to test its performance as a H_2O_2 probe due