Rapid-Response Fluorescent Probe for Hydrogen Peroxide in Living Cells Based on Increased Polarity of C−B Bonds

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ABSTRACT: Hydrogen peroxide (H$_2$O$_2$) as a reactive oxygen species (ROS) plays a crucial role in oxidative stress and signal transduction of organisms. Currently, a fluorescence probe has proven to be a powerful tool for the H$_2$O$_2$ analysis. However, the common problem is the slow response, causing difficulty in tracking H$_2$O$_2$ in situ. Herein, we describe a novel aggregation-induced emission (AIE) fluorescence probe based on increased polarity of C−B bonds that is well suited for monitoring H$_2$O$_2$ rapidly and selectively. Importantly, the probe was successfully applied to visualize H$_2$O$_2$ levels in living cells, which provides a rapid-response and highly selective fluorescence tool for monitoring of the H$_2$O$_2$ levels in biological process.

Hydrogen peroxide (H$_2$O$_2$) as an important marker for oxidative stress and a second messenger in signal transduction plays vital roles in the regulation of various physiological processes. Overgeneration of the H$_2$O$_2$ has been implicated in many pathological conditions, including cardiovascular diseases, diabetes, and cancer. Monitoring H$_2$O$_2$ level in living cells is thus very important. Fluorescence methods have proven to be powerful techniques due to their fast response, high sensitivity, and ability to afford high spatial resolution through microscopic imaging. At present, various fluorescence probes have been developed and applied for the H$_2$O$_2$ analysis. However, a common problem often encountered by the fluorescent probe for H$_2$O$_2$ is slow response. When the probe reacted with the H$_2$O$_2$, the recognition reaction was completed after a long time, and it has become an important yet complex issue for monitoring the H$_2$O$_2$ concentration level in living cells. This is mainly due to H$_2$O$_2$ in the cells possessing the following characteristics: (i) the H$_2$O$_2$ easily crosses the cell membrane; (ii) H$_2$O$_2$ reacts with other substances to generate new ROS; (iii) short half-life of H$_2$O$_2$. If you want to study the true concentration of H$_2$O$_2$ in living cells at specific areas, the slow response probe can not meet the demand. Therefore, development of a rapid and highly selective method for H$_2$O$_2$ in living cells is of great significance.

As is well-known, boronate ester has been recognized as a specific recognition element for H$_2$O$_2$ based on the chemospecific boronate-to-phenol switch to respond to H$_2$O$_2$. In order to better fit monitoring of the H$_2$O$_2$ in the biological process, the slow response based on the boronic acid ester and H$_2$O$_2$ need to be solved. The reaction process of boronate-to-phenol switch would appear to be either a homolytic or an electrophilic substitution in the benzene ring, in which the boronate group rather than the usual hydrogen is replaced. Currently, a great variety of tetraphenylethylene (TPE) based fluorescent sensing materials have been developed and successfully applied in the field of biology. The question is whether the π-electron conjugate structure of the TPE can be used to improve the polarity of C−B bonds to realize rapid monitoring of H$_2$O$_2$ through AIE. The luminescence mechanism of the TPE probe was different from that of the traditional dye probe. In order to confirm the reaction mechanism of the probe, a computational study was performed and the results implied that polarity of C−B bonds was increased and was more prone to be replaced (Figure S1). As far as we know, no reports to date have been published on the use of AIE to realize rapid monitoring H$_2$O$_2$ in living cells.

EXPERIMENTAL SECTION

Materials and Reagents. All chemicals were available commercially and the solvents were purified by conventional methods before use. (4-Bromophenyl) (phenyl)methanone and 4,4′,4′,5,5′,5′′-octamethyl-2,2′-bi(1,3,2-dioxaborolane) were purchased from Shanghai Taitian Chemical Reagent Company. Titanium tetrachloride, zinc powder, potassium carbonate, and 1,1-bis(diphenylphosphino)ferrocene]dichloropalladium(II) were purchased from Aladdin Industrial. Reactive oxygen species were as follows: hypochlorite (NaOCl), H$_2$O$_2$, and...
tert-butylhydroperoxide (TBHP) were delivered from 10, 30, and 70% aqueous solutions, respectively. Hydroxyl radical (•OH) was generated by reaction of 1 mM Fe^{2+} with 200 μM H_{2}O_{2}. Nitric oxide (NO) was used from stock solution prepared by sodium nitroprusside. Singlet oxygen (¹O₂) was prepared by the ClO⁻/H₂O₂ system. Superoxide (O₂⁻•) was delivered from KO₂ in DMSO solution. Peroxynitrite was used prepared by the ClO⁻/H₂O₂ system. Superoxide (O₂⁻•) was prepared by the ClO⁻/H₂O₂ system.

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**Instruments.** ¹H NMR spectra were recorded with Bruker NMR spectrometers at 300 MHz. The mass spectra were measured by ABI 4000 MSD. The fluorescence spectra measurements were recorded on a FLS-920 Edinburgh fluorescence spectrometer. Confocal fluorescent images were measured on a Leica TCS SPS, confocal laser scanning microscope with an objective lens (×40). The excitation wavelength was 405 nm (5 mW). HPLC measurement was carried out with the LC-20AD solution system from Shimadzu (Kyoto, Japan). HPLC conditions employed for this work were as follows: mobile phase, methanol; ow rate, 0.1 mL/min; room temperature; fluorescence detection, 400 nm.

**Synthesis of 1,2-Bis(4-bromophenyl)-1,2-diphenylethene (TPE-Br).** 4-Bromophenone (5.22 g, 20 mmol) and zinc powder (3.92 g, 60 mmol) were added into a 250 mL three-necked flask. The mixture was allowed to warm to 25 °C for 1 h and then refluxed overnight. The reaction was quenched with a 10% potassium carbonate aqueous solution and extracted with DCM. The organic layer was washed with deionized water and dried over anhydrous magnesium sulfate. The residue was puri fi ed by silica gel column chromatography, using petroleum ether and ethyl acetate (20:1) as eluent. The frac tion was degassed and flushed with dry argon three times, after which THF (60 mL) was injected. The mixture was cooled to −5 to 0 °C by an ice-salt bath, then titanium tetrachloride (3.32 mL, 30 mmol) was added slowly. The mixture was allowed to warm to 25 °C and kept there for 0.5 h and then ref uxed at 75 °C. After ref uxing overnight, the reaction was quenched with a 10% potassium carbonate aqueous solution and extracted with DCM. The organic layer was washed with deionized water and dried over anhydrous magnesium sulfate. After filtration and solvent evaporation, the residue was puri fi ed by silica gel column chromatography, using petroleum ether and ethyl acetate (v/v = 20:1) as eluent. ¹H NMR (400 MHz, CDCl₃) δ 7.26–7.20 (m, 4H, Ar H), 7.15–7.09 (m, 6H, Ar H), 7.01–6.96 (m, 4H, Ar H), 6.90–6.85 (m, 4H, Ar H). ¹³C NMR (100 MHz, CDCl₃) δ 143.01, 142.91, 142.48, 142.38, 140.39, 132.99, 131.31, 131.21, 131.01, 130.23, 128.44, 128.18, 127.94, 127.06, 126.95, 120.93, 120.79. MS data, m/z: 490.9838 (M + H).

**Preparation of 1,2-Diphenyl-1,2-bis[4-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenylethenyl]anthene (TPE-BO).** To a 50 mL flask was added, TPE-Br (0.982 g, 2 mmol), bis(pinacolato) diboron (1.27 g, 5 mmol), potassium acetate (1.374 g, 14 mmol), Pd(dppf)Cl₂ (80 mg, 0.11 mmol), and 30 mL of anhydrous DMF. The reaction was heated at 85 °C for 24 h under nitrogen. The dark brown reaction was cooled to room temperature, solvent evaporated, and then extracted with DCM. The organic layer was washed with deionized water and dried over anhydrous magnesium sulfate. After fi ltration and solvent evaporation, the residue was puri fi ed by silica gel column chromatography, using petroleum ether and ethyl acetate (v/v = 15:1) as eluent. ¹H NMR (300 MHz, CDCl₃) δ 7.54 (d, J = 1.5 Hz, 2H), 7.51 (d, J = 1.5 Hz, 2H), 7.06–7.10 (m, 6H), 7.05–6.99 (m, 8H), 1.32 (s, 24H). ¹³C NMR (100 MHz, CDCl₃) δ 146.80, 146.62, 143.61, 143.45, 141.34, 134.23, 134.14, 131.41, 130.76, 127.79, 127.66, 126.62, 126.55, 83.78, 24.99. ¹¹B NMR (400 MHz, CDCl₃) δ 6.10. HRMS (m/z): [(M + H)+] calc for C_{38}H_{42}B_{2}O_{4}, 585.3354; found, 585.3328.

**Cell Culture.** RAW.264.7 cells were cultured in DMEM containing 10% fetal bovine serum, 1% penicillin, and 1% streptomycin at 37 °C (w/v) in a 5% CO₂/95% air incubator MCO-15AC (Sanyo, Tokyo, Japan). The concentrations of counted cells were adjusted to 1 × 106 cells mL⁻¹ for confocal imaging in high-glucose DMEM (4.5 g of glucose/L) supplemented with 10% fetal bovine serum (FBS), NaHCO₃ (2 ng/L), and 1% antibiotics (penicillin/streptomycin, 100 U/mL). Cultures were maintained at 37 °C under a humidified atmosphere containing 5% CO₂.

**MTT Assay.** RAW.264.7 cells (106 cell mL⁻¹) were dispensed within replicate 96-well microtiter plates to a total volume of 200 μL well⁻¹. Plates were maintained at 37 °C in a 5% CO₂/95% air incubator for 24 h. Then RAW.264.7 cells were incubated for 12 h upon different probe concentrations of 10⁻⁸, 10⁻⁷, 10⁻⁶, 10⁻⁵, 10⁻⁴, and 0 M. MTT solution (5 mg mL⁻¹, PBS) was then added to each well. After 4 h, the remaining MTT solution was removed, and 150 μL of DMSO was added to each well to dissolve the formazan crystals. Absorbance was measured at 490 nm in a Triturus microplate reader.

**Confocal Imaging.** RAW.264.7 cells were passed and dispersed on 18 mm glass coverslips at 37 °C, 5% CO₂ 1 day before imaging. Then cells were incubated with probe TPE-BO (1.0 mM) for 15 min. The medium was removed and cells were washed with PBS (10 mM, pH 7.4) three times. Fluorescent images were acquired on a Leica TCS SPS confocal laser-scanning microscope with an objective lens (×40). The excitation wavelength was 405 (5 mW) and 488 nm (15 mW), respectively. Following incubation, the cells were washed three times with DMEM without FBS and imaged.

**RESULTS AND DISCUSSION**

The general scheme for synthesis of the probe TPE-BO is illustrated in Scheme S1. The chemical structure of TPE-Br and TPE-BO was characterized with ¹H NMR, ¹³C NMR, and mass spectra. This probe consists of a TPE molecule (Scheme 1) and boronic pinacol ester moiety. The reaction mechanism of probe toward H₂O₂ is outlined in Scheme 1. In the presence of H₂O₂, the phenylboronic ester moiety of the probe can convert into the phenol group. Accordingly, hydrophilic and hydrophobic properties of the probe TPE-BO is changed, the aggregation will occur based on the AIE feature of compounds TPE-OH. In

Scheme 1. Chemical Structure of the Probe TPE-BO and the Product (TPE-OH) of the DPE Reaction with H₂O₂ and Proposed Mechanism of the Fluorescence Probe for Detection of H₂O₂

[Diagram of Scheme 1]
this way, the probe TPE-BO can be employed for the fluorescence turn on detection of H$_2$O$_2$ in living cells. The photophysical properties of the probe were tested, and the results were shown in Figure S2. It can be seen that the excitation peak was around 400 nm, and the emission peak was around 500 nm. A computational study was employed to study the characteristics of the chemical bonds between the boronic ester moiety and TPE from the aspects of electronegativity, which implied that the polarity of the chemical bond is larger and is easily attacked (Figure S1). As expected, the probe TPE-BO can rapidly identify hydrogen peroxide and the fluorescence kinetic response of the probe to H$_2$O$_2$, shown in Figure 1. It can be seen that the fluorescence intensity increased rapidly after addition of the H$_2$O$_2$. The time of fluorescence intensity reaches maximum value rapidly, which indicated that the probe was a rapid-response fluorescent tool for H$_2$O$_2$.

The changes of fluorescence spectra of probe with H$_2$O$_2$ in various concentrations were monitored. Figure 2a shows the fluorescence spectra of probe TPE-BO before and after addition of different amounts of H$_2$O$_2$. As anticipated, after incubation with H$_2$O$_2$ at room temperature, the fluorescence intensity of probe TPE-BO increased gradually. Figure 2b shows the plot of the relative fluorescence intensity ($F/F_0$) at 500 nm as a function of concentration of H$_2$O$_2$. The fluorescence intensity of probe TPE-BO increases almost linearly with the concentration of H$_2$O$_2$ in the range of 10.0–200.0 μM, as displayed in Figure 2b, and a correlation coefficient of 0.9940. Accordingly, the detection limit of H$_2$O$_2$ was estimated to be 0.52 μM ($n = 11$ and S/N = 3). Good linear correlation was obtained, which indicated that probe was able to qualitatively and quantitatively determine the level of H$_2$O$_2$.

To prove probe TPE-BO selectivity for H$_2$O$_2$, fluorescence responses to other reactive oxygen species were examined. As shown in Figure 2c, significant fluorescence enhancement was observed only after incubation with H$_2$O$_2$. Other ROS, such as singlet oxygen, hydroxy radical, superoxide anion, nitric oxide, and so on, induced only negligible fluorescence enhancement for probe TPE-BO under the same conditions. Thus, the probe shows high selectivity toward H$_2$O$_2$. Moreover, the formation of TPE-OH after incubation of probe TPE-BO with H$_2$O$_2$ (see Scheme 1) is confirmed by the mass spectral and HPLC analysis. The mass signal, corresponding to the molecular weight of TPE-OH, was detected after the solution of probe was incubated with H$_2$O$_2$, and the results of the HPLC analysis also indicated the formation of TPE-OH (Figure 2d).

Finally, we assessed the application of the probe for monitoring and imaging of H$_2$O$_2$ in living cells (Figure 3).

**Figure 1.** Fluorescence emission spectra of the probe TPE-BO before (1) and after (2) addition of the H$_2$O$_2$ (a), and the fluorescence kinetic response of the probe to H$_2$O$_2$ (b). Experimental conditions: performed with 10.0 μM probe TPE-BO and 200.0 μM H$_2$O$_2$.

**Figure 2.** Fluorescence emission spectra of probe as a function of H$_2$O$_2$ concentration (a), the plot of fluorescence intensity ($F/F_0$) against the concentration of hydrogen peroxide (b), fluorescence responses of TPE-BO (10.0 μM) after incubation with 100.0 μM of hydrogen peroxide and 200.0 μM of other reactive oxygen species (c), chromatograms (d) of the solution of TPE-BO (a) and the product of the TPE-BO (b) after incubation of H$_2$O$_2$ on an ODS column monitored at the wavelength of 400 nm. Experimental conditions: column, 250 mm × 4.6 mm I.D.; mobile phase, methanol; flow rate, 0.1 mL/min.

**Figure 3.** Confocal fluorescence images of living mice macrophages (RAW264.7) under different conditions with probe TPE-BO. Mice macrophages were incubated with 1.0 mM probe. (a) Overlay of (b) and (c). (b) Probe-loaded cells fluorescence image. (c) Bright-field image of the samples. (d) Overlay of (e) and (f). (e) Probe-loaded cells fluorescence image upon stimulation with PMA. (f) Bright-field image of the samples.

RAW 264.7 macrophages were incubated with the probe (5.0 μM) at room temperature. Figure 3a–c is the overlay of the Figure 3a and b, probe-loaded cells fluorescence image and bright-field image, respectively, which indicated that the probe was able to image endogenous levels of H$_2$O$_2$ in the living cells. Then we tested the ability of the probe to detect endogenous H$_2$O$_2$ produced by exogenous stimulation. Phorbol-12-myristate-13-acetate (PMA) was used to induce H$_2$O$_2$ generation through a cellular inflammation response. Figure 3d–f is the overlay of the Figure 3e and f, probe-loaded cells
fluorescence image and bright-field image upon stimulation with PMA, respectively. It can be seen that the fluorescence intensity increased distinctly upon stimulation with PMA, this mainly due to large amounts of H$_2$O$_2$ was produced after the stimulation, which indicated that the probe could image of H$_2$O$_2$ in living cells. In addition, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] as, say, in RAW 264.7 macrophages, was performed to evaluate the cytotoxicity of probe (Figure S3). Figure S3 showed that the cell viability was more than 95% which indicated that the probe TPE-BO showed almost no cytotoxicity or side effects in living cells.

## CONCLUSIONS

In conclusion, aiming at the widespread reaction speed problem of the H$_2$O$_2$ probe, we have designed and synthesized a novel AIE fluorescent probe TPE-BO with activity based on increased polarity of C–B bonds, which can rapidly respond and offers highly selective monitoring of H$_2$O$_2$ in living cells. The present study provides a fluorescent tool for rapid monitoring of H$_2$O$_2$ in living cells, and we believe that the strategy for improving the electrophilic reaction activity to achieve the rapid fluorescence response will be broadly applicable to the quantitative monitoring of active small molecules in biological systems.

## ASSOCIATED CONTENT

1 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b02194.

Computational details, supporting analytical data, and additional scheme and figures (PDF).

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### Notes

The authors declare no competing financial interest.

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