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Ratiometric Fluorescent Probes for Hydrogen Peroxide from a Focused Library

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Hydrogen peroxide (H_2O_2), the simplest peroxide, is a precursor molecule of other reactive oxygen species (ROS), such as the hydroxyl radical ($^{\bullet}OH$).[1] In living cells, H_2O_2 is generated as a byproduct from various cellular aerobic oxidation reactions such as glucose oxidation by glucose oxidase (GOx).[2] H_2O_2 widely causes oxidative damage on proteins and nucleic acids, and it is assumed that the accumulation of this damage is responsible for the aging process.[3] H_2O_2 also has physiological functions as a cellular signaling molecule in many cases.[4] Therefore, precise detection of H_2O_2 is crucial for understanding H_2O_2-related biochemical mechanisms.

The most widely accepted optical method for measuring H_2O_2 is the horseradish peroxidase (HRP)-catalyzed oxidation of fluorogenic substrates.[5] This method is considered sensitive and precise, but rather expensive because it requires purified enzymes. Recently, Chang and his co-workers developed boronate-based fluorescent probes that enabled the direct detection of H_2O_2.[6] Usually, these sensors are designed to quench fluorescence due to the presence of an electron-deficient boronic acid ester group. When this boronate group reacts with H_2O_2 to yield a hydroxyl moiety,[7] the fluorescence of the probe is drastically increased by regeneration of a "push–pull" pair in the fluorophore. However, quantitative assay of H_2O_2 is still challenging because of this "night and day" property of the sensors in many applications.[8] For practical needs, the simultaneous recording of two measurable fluorescence intensities that are of seesaw-type ratiometric change would be a better alternative for the quantitative detection of H_2O_2.

Boronate-based ratiometric sensors for H_2O_2, which utilize the Förster resonance energy-transfer (FRET)-based mechanism,[9] and an intramolecular charge-transfer (ICT)-based strategy have been recently reported.[10] However, the emission ratio change based on FRET is limited (less than 8-fold).[9] An ICT-based sensor using H_2O_2-mediated deprotection of carbobenzyloxy (Cbz) boronate exhibits 75-fold emission ratio changes.[10b] However, the probe's fluorescence change rate is much slower ($t_{1/2} > 45$ min) than a directly conjugated aryl boronate sensor without the Cbz moiety ($t_{1/2} < 30$ min),[9] because the fluorescence change should occur upon the H_2O_2-mediated oxidation of boronate followed by the benzyl deprotection reaction. Therefore, we wanted to develop directly conjugated aryl boronate-based probes (Figure 1) that would enable dramatic ratiometric sensing of H_2O_2 in a short time. Although the electronic state change of boronate-based H_2O_2 probes can be rationally designed from the electron push–pull strategy,[11] it is hard to predict whether the resulting H_2O_2 probes would generate fluorescence upon reaction with H_2O_2. Therefore, we aimed to find ratiometric fluorescent probes for H_2O_2 through a focused, combinatorial approach.

For the best chance of finding a 'hit' molecule to sense H_2O_2, we used formylphenyl boronate ester (**1**–**3**) as one combinatorial block for the styryl dye synthesis.[12] For the structural diversity of fluorophores, we used five different *N*-methylpicolinium and *N*-methylquinolinium blocks (**A**–**E**) (see Figure S1 in the Supporting Information). The Knoevenagel condensation reaction between 3-formylphenyl boronate esters and 5-*N*-methylpicolinium and *N*-methylquinolinium iodides provided 15 fluorescent styryl probes. As shown in Figure 1a, each condensation reaction produced a fully conjugated fluorophore. Each product was purified and separated through flash silica gel column chromatography and preparative HPLC (prep-HPLC). It turned out that 12 of 15 dyes have a quantum yield considerably higher than 0.010. Each probe's observed mass, absorption wavelength, emission wavelength, and quantum yield are reported in Table S1 in the Supporting Information.

To find ratiometric fluorescent H_2O_2 probes, we investigated the spectral fluorescence changes of styryl dyes (each 50 μM) upon addition of H_2O_2. As expected, most of these dyes showed a distinct fluorescence change with H_2O_2, as shown in Figure 1. Among them, **1A**, **1B**, **1C**, and **3D** underwent redshifts within 30 min of incubation with H_2O_2. Probes **1A** and **1C**, especially, showed a redshift of over

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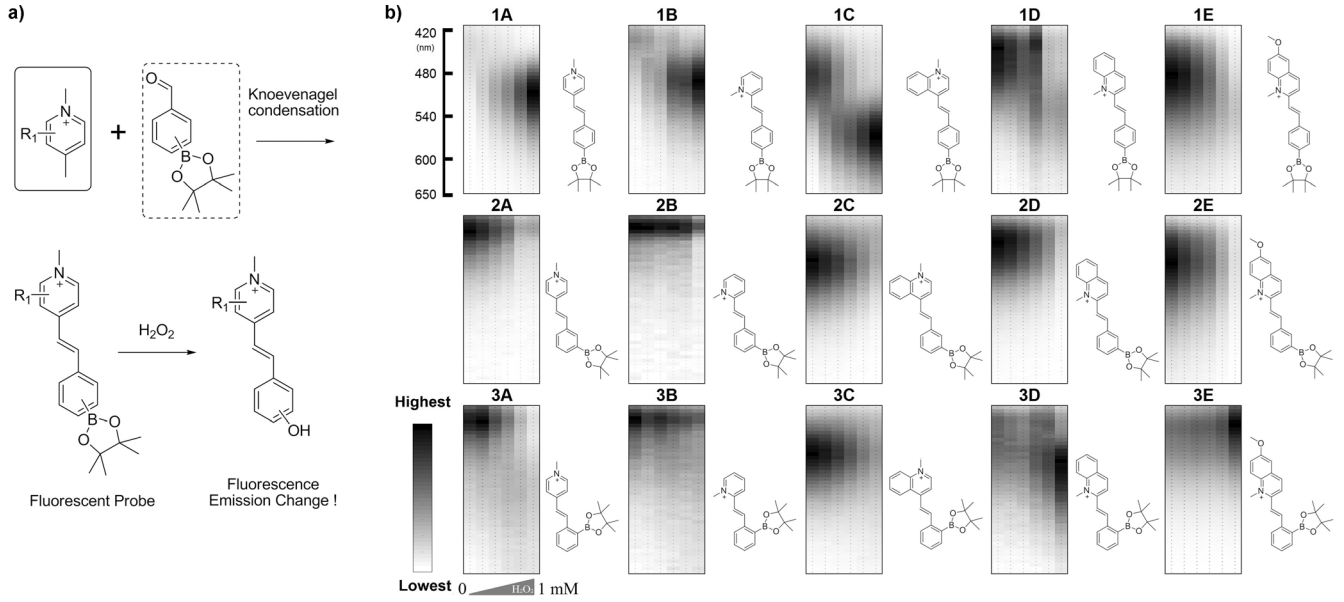

Figure 1. a) Focused library synthesis of fluorescent probes for H_2O_2. Detailed procedures are described in the Supporting Information. b) Fluorescence response of probes (50 μM) at each fluorescence emission wavelength after 30 min incubation with H_2O_2 (0, 50, 100, 200, 500, 1000 μM). Fluorescence intensity at each wavelength was depicted as an artificial color standard, which is shown as a bar graph. The highest and lowest fluorescence values were determined in each probe's fluorescence data. $\lambda_{ex}=375$ nm.

70 nm at the maximum wavelength in the fluorescence emission spectrum and the UV/Vis spectrum upon addition of H_2O_2 (Figure 2). Although they have the same *para*-substituted boronic acid ester as **1A** and **1C**, **1D** and **1E** showed quenched fluorescence by H_2O_2 addition. All five styryl fluorophores (**2A**–**E**) with *meta*-positioned boronate showed decreased fluorescence after H_2O_2 addition. Among *ortho*-positioned boronate probes, **3D** and **3E** showed an increase in fluorescence with H_2O_2, whereas three other probes (**3A**, **3B**, and **3C**) showed fluorescence quenching with H_2O_2. Therefore, the fluorescence change of each probe, upon treatment with H_2O_2, is largely affected by the position of boronate and its conjugated structure with the *N*-methylpicolinium and *N*-methylquinolinium blocks.

To demonstrate the utility of our probes, we selected **1A** and **1C** because of their superior photophysical properties, including fluorescence intensity and changes in the intensity ratio upon addition of H_2O_2, compared to other probes. First, we checked the reactivity of **1A** and **1C** towards other ROSs. **1A** and **1C** showed se-

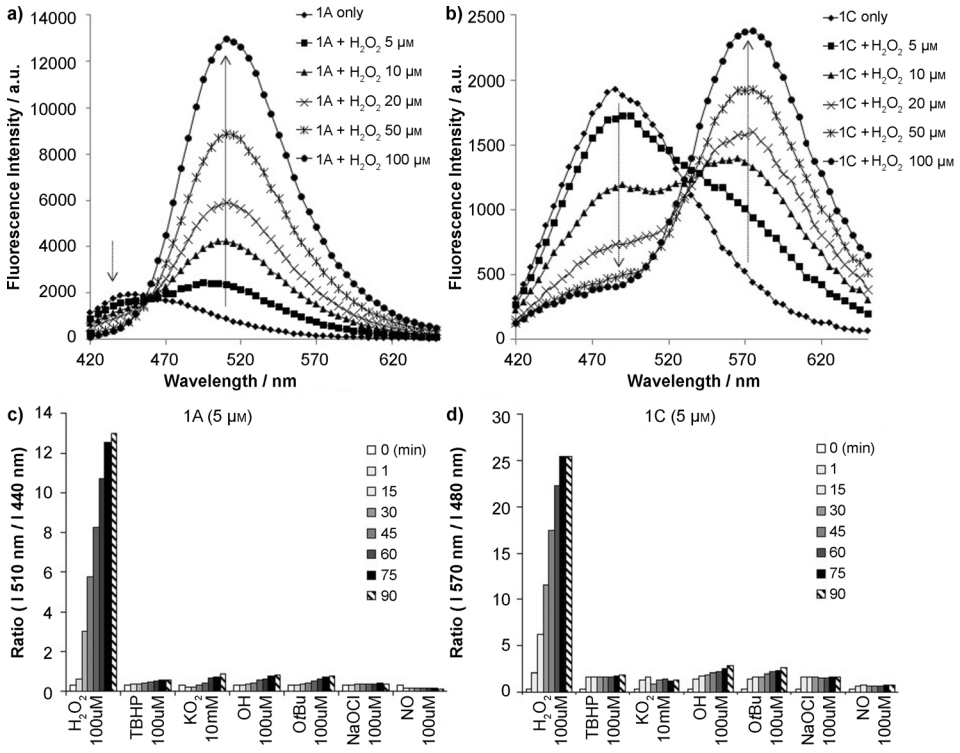

Figure 2. Fluorescence-emission titration spectra of **1A** (5 μM, a) and **1C** (5 μM, b) after 30 min incubation with various concentrations of H_2O_2. Fluorescence ratio changes of **1A** (5 μM, c) and **1C** (5 μM, d) toward ROSs at the indicated time. The generation methods of these ROSs and absorption spectra changes are described in the Supporting Information, Figure S2. $\lambda_{ex}=375$ nm.

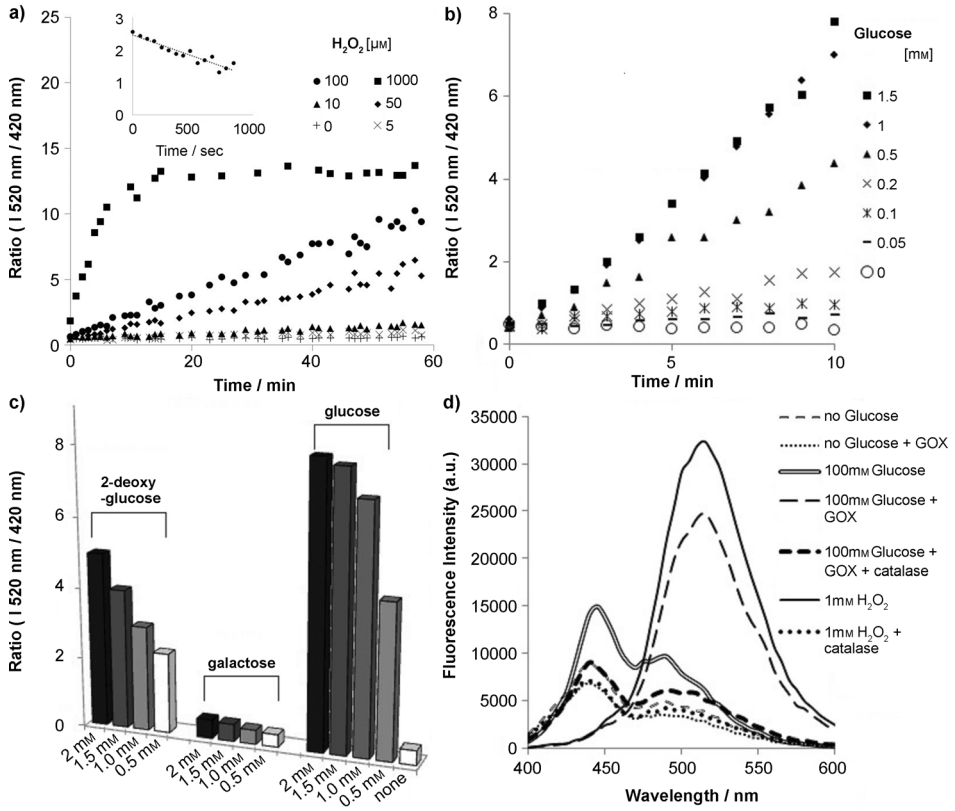

Figure 3. a) Ratiometric fluorescence emission changes ($I_{520\ nm}/I_{420\ nm}$) upon reaction of **1A** (5 μM) with various concentrations of H_2O_2 with time. The k_{obs} was calculated from the slope of the inset plot of fluorescence intensity changes of **1A** with 1 mM of H_2O_2, *x*-axis: $\ln\{(I_{520\ nm}/I_{420\ nm})_\infty-(I_{520\ nm}/I_{420\ nm})\}$, *y*-axis: reaction time. b) Real-time monitoring of glucose oxidation by GOx (1 μM) with **1A** (5 μM). c) Reactivity of GOx (1 μM) toward 2-deoxy-D-glucose, D-galactose, and D-glucose. GOx/**1A** (5 μM) was incubated with these sugars (0.5–2 mM) for 10 min. d) Fluorescence emission spectra of **1A** (10 μM) under various conditions. The mixture was incubated for 30 min at room temperature. $\lambda_{ex}=375$ nm.

lectivity towards H_2O_2 only, over other ROSs, within a 10 min incubation period (Figure 2c,d). Other ROSs showed a negligible fluorescence response with **1A** and **1C**. Next, we measured the kinetics of **1A** with various concentrations of H_2O_2.

As shown in Figure 3a, probe **1A** reacts rapidly with 1 mM H_2O_2 and its pseudo-first-order rate constant (k_{obs}) is $1.3\times10^{-3}\,sec^{-1}$ in 1 mM of H_2O_2 ($t_{1/2}<5$ min).[10b,c] Over 60 min of observation in neutral aqueous buffer solution revealed no change in the fluorescence intensity ratio of the spontaneous oxidation of probe **1A** without H_2O_2. Encouraged by **1A**'s high selectivity for and fast response to H_2O_2, we used the probe to monitor glucose oxidation by GOx in real time. Probe **1A** (5 μM), GOx (1 μM), and glucose (0.05 mM to 1.5 mM) were mixed in a cuvette, and changes in the fluorescence emission ratio ($I_{520\ nm}/I_{420\ nm}$, excitation wavelength 375 nm) were recorded every minute for 10 min. As expected, after the addition of GOx into glucose solution (1 mM), the probe's fluorescence ratio ($I_{520\ nm}/I_{420\ nm}$) increased dramatically from 0.3 to 8 by H_2O_2 produced from glucose oxidation (Figure 3b). Further, the emission ratio changes with time were obviously dependent on the glucose concentration (remaining) between 0.1 and 1 mM (Figure 3b). When glucose concentration was higher than 1 mM, the slope did not increase significantly because only a limited pool of molecular oxygen (ca. 0.3 mM) could be depleted in the aqueous glucose solution.[13] This result implies that our probe system has the potential utility of precisely sensing the glucose level of diabetes patients in a sample with a glucose concentration of less than 1 mM.[14]

Notably, the fluorescence change of probe **1A** was totally blocked in this reaction by the addition of catalase (Figure 3d), which catalyzes the decomposition of H_2O_2 in the solution. This is solid evidence that the fluorescence change of the probe was caused by H_2O_2, and not by the direct oxidation reaction between GOx and the probe.

Using probe **1A**, we could also check the selectivity of GOx for other saccharide molecules, namely, 2-deoxy-D-glucose and galactose. Figure 3c indicates that GOx oxidized 2-deoxy-D-glucose as well, but more slowly than D-glucose, producing H_2O_2.[15] On the other hand, galactose showed a negligible change in fluorescence because galactose is not a good substrate for GOx, as reported earlier.[15] Further, probe **1A** was successfully utilized to detect glucose in human urine. Human urine was collected from a healthy volunteer and tested. By using probe **1A** and GOx, enhanced and redshifted fluorescence emission spectra were obtained from glucose-containing (20–100 μM) urine samples, as seen in Figure 4 and Figure S4 in the Supporting Information. Moreover, we could quantify a small amount of glucose (19.7 ± 2.4 μM, see Figure 4b and Figure S4b in the Supporting Information) in human urine (diluted 10-fold) by using standard addition plots (see Figure S5 in the Supporting Information). Coincidentally, the fluorescence spectrum of healthy human urine[16] was very similar to that of probe **1A** itself (see Figure S3 in the Supporting Information). Therefore, we analyzed data of urine samples using a fluorescence intensity of 510 nm (see Figure S4g in the Supporting Information for fluorescence ratio change spectra). Since the concentration of glucose in the urine of a diabetic patient is above 1 mM, our probe, **1A**, would be useful in diagnosing diabetes.

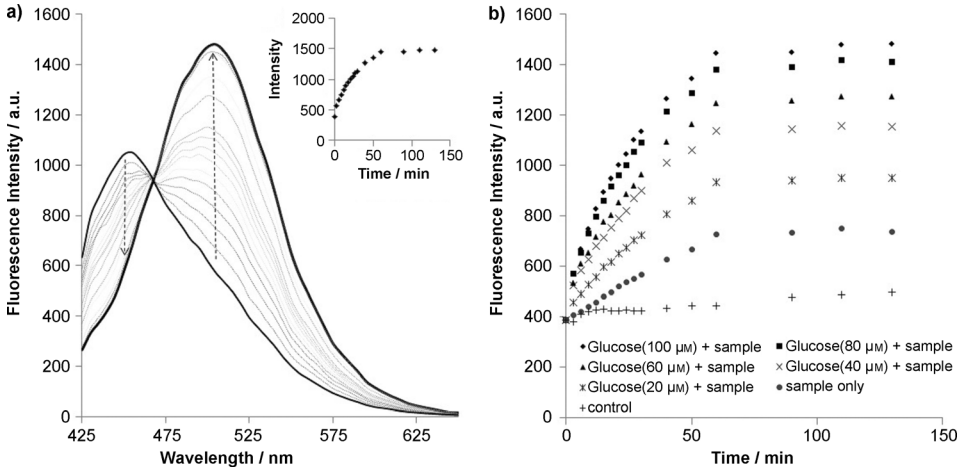

Figure 4. a) Real-time monitoring of fluorescence changes of **1 A** (100 μM) upon addition of GOx (0.5 μM) and glucose (100 μM) in 10-fold diluted human urine. Inset graph, *x*-axis: elapsed time from 3 to 130 min, *y*-axis: fluorescence intensity at 510 nm. b) Real-time monitoring of glucose (20, 40, 60, 80 and 100 μM) oxidation by GOx (0.5 μM) with **1A** (100 μM) in 10-fold diluted human urine. See the Supporting Information for detailed experimental procedures. All experiments were performed in phosphate buffer solution (10 mM, pH 7.4). λ_{ex} = 375 nm.

By the same token, upon addition of cholesterol oxidase, the increase in the fluorescence intensity of **1A** was proportional to the amount of cholesterol in the buffer (Figure S6 in the Supporting Information).[17] Furthermore, many oxidases oxidize biomolecules, such as lactate,[18] xanthine[19] and choline[20] with high selectivity. Because all these oxidases produce H_2O_2 as a byproduct during oxidation, it is very likely that one can detect the activity of these oxidases, as well as the presence and the quantity of specific biomolecules by using our probe. Probe **1A** would be a low-cost method relative to HRP-catalyzed oxidation—a method that requires additional enzymes.

To the best of our knowledge, this is the first example of a boronate-based fluorescence probe that was used for real-time monitoring of the activities of glucose oxidase and cholesterol oxidase. Moreover, probe **1A** was successfully utilized to quantify a small amount of glucose in human urine by using standard addition plots. Our probe is also strongly chromogenic (see Figure S2c in the Supporting Information) when reacting with H_2O_2, and therefore, H_2O_2 generation in an enzyme reaction with a specific metabolite can be detected by the naked eye in the real-life environment.

In summary, we have developed ratiometric fluorescent probes for H_2O_2 from a focused library. Upon the addition of H_2O_2, probe **1A** shows a redshift of over 100 nm in the maximum fluorescence emission wavelength and an increase in ratio of over 30-fold. Compound **1A** was successfully utilized for the real-time monitoring of glucose oxidation by GOx.

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