ChemComm

Chemical Communications

www.rsc.org/chemcomm

Volume 49 | Number 75 | 28 September 2013 | Pages 8267–8414

ISSN 1359-7345

RSC Publishing

COMMUNICATION

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1359-7345(2013)49:75;1-R

ChemComm

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View Article Online
View Journal | View Issue

Cite this: *Chem. Commun.*, 2013, **49**, 8311

Received 2nd May 2013,
Accepted 3rd June 2013

DOI: 10.1039/c3cc43265c

www.rsc.org/chemcomm

"Integrated" and "insulated" boronate-based fluorescent probes for the detection of hydrogen peroxide†‡

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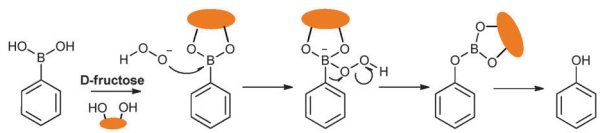
Integrated and insulated boronate-based fluorescent probes have been evaluated for the detection of hydrogen peroxide in the presence of saccharides.

Hydrogen peroxide (H_2O_2) is the simplest peroxide (a compound with an oxygen–oxygen single bond) and it is a strong oxidizing agent, which has been widely employed as a bleach and as a cleaning reagent to reduce BOD[1] and COD[2] from industrial wastewater. Hydrogen peroxide, one of reactive oxygen species (ROS), plays an important role as a signalling molecule in the regulation of a variety of biological processes, such as immune response, cell signalling,[3] Alzheimer's diseases[4] and cancer.[5] The importance of H_2O_2 has led to researchers seeking effective and applicable approaches for its detection.

Among the powerful tools available for H_2O_2 detection are synthetic fluorescent probes. Hydrogen peroxide reacts with arylboronic acids under mild alkaline conditions to generate phenols (Scheme 1).[6] Over the last decade, several groups introduced different fluorescent probes for the detection of hydrogen peroxide by utilising this property. Chang and co-workers have developed a series of boronate-based derivatives for the fluorescent detection of H_2O_2 in living systems,[7–13] and Tomapatanaget *et al.* used the H_2O_2 generated from glucose by the action of glucose oxidase to convert boronic acids to phenols in their glucose sensing system.[14]



Scheme 1 Proposed reaction mechanism between a generic aryl boronic acid/ester and H_2O_2.

We have a long-standing interest in boronic acids for saccharide and anion detection,[15–18] boronic acid derivatives rapidly and reversibly interact with saccharides in aqueous media.[19,20] During the course of our investigations, we and others have employed boronic acids as sensors where the boron atom is directly attached to a fluorphore (integrated) and separated by a Lewis basic spacer (insulated). As a model integrated boronate-based fluorescent probe we chose 2-naphthylboronic acid (**1**). 2-Naphthylboronic acid (**1**) can be employed to detect saccharides *via* fluorescence intensity changes on addition of saccharides at constant pH resulting in an "on–off" or "off–on" fluorescence switch on saccharide binding.[21] While as a model insulated boronate-based fluorescent probe we chose *N*-methyl-*o*-(aminomethyl)phenylboronic acid (**2**). *N*-Methyl-*o*-(aminomethyl)phenylboronic acid (**2**) has been previously demonstrated as an "off–on" fluorescent switch on saccharide binding, operating *via* a PET mechanism.[22]

Bearing this in mind, we decided to investigate how the reaction of H_2O_2 with two classes of boronic acid (integrated and insulated) fluorescent probes changes in the presence of saccharides. This is conceptually similar to the inspirational work by Jiang into the effect of saccharides on Suzuki homocoupling reactions.[23] Understanding the effect of saccharides on this reaction and fluorescence may be important in developing advanced intracellular probes to accurately map H_2O_2 concentrations in living systems. Probes **1** and **2** (Scheme 2) and their saccharide (D-fructose was chosen as a model saccharide since it has a high binding constant) complexes were evaluated as fluorescent indicators for hydrogen peroxide both in neutral and alkaline aqueous solutions.

In the corresponding experiments, fluorescence titrations with H_2O_2 were carried out at 25 °C in pH 7.20 buffer (52.1% methanol in water with KCl, 10 mM; KH_2PO_4, 2.752 mM; Na_2HPO_4, 2.757 mM and the pH was adjusted to 7.20 using HCl)[24] and pH 9.70 buffer (10.0% methanol in water with Na_2CO_3, 50 mM; $NaHCO_3$, 50 mM). The saccharide-boronic acid complexes were formed by mixing free boronic acid (10 μM) with D-fructose (100 mM) *in situ*.[25,26]

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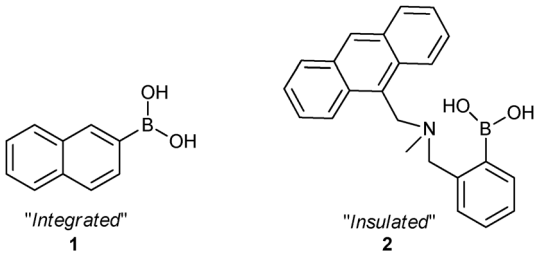
† Joseph Priestley, the discoverer of oxygen, was born in 1733. This paper is dedicated to the 280th anniversary of his birth.

‡ Electronic supplementary information (ESI) available: Detailed procedures, characterization data, and additional plots. See DOI: 10.1039/c3cc43265c



Scheme 2 Chemical structures of probe **1** and **2**.

The binding D-fructose to 2-naphthylboronic acid (**1**), in the absence of H_2O_2, causes a decrease and an increase in fluorescence intensity at pH 7.20 (Fig. S1, ESI‡) and pH 9.70 (Fig. S2, ESI‡) respectively.[21] When H_2O_2 is present, 2-naphthylboronic acid is oxidatively converted to 2-naphthol (Scheme 1), and the fluorescence is reduced at 340 nm and red-shifted to 410 nm from pH 7.2 to 9.7, when excited at 290 nm (Fig. S4, ESI‡).

Fig. 1 summarises the reaction scheme and the fluorescence intensity changes F_T (in the presence of H_2O_2)/F_0 (in the absence of H_2O_2) for fluorescent probe **1** and the **1**-D-fructose complex in pH 7.20 and 9.70 buffer. It is known that on saccharide binding and formation of a cyclic boronate ester, the pK_a of the boronic acid is enhanced, or in other words the 'ester' is more acidic than the 'acid'.[26] Therefore, binding with D-fructose increases the electrophilicity of boron in probe **1**, making it more easily oxidised by H_2O_2. This observation was borne out by our experiments. As shown in Fig. 1(b), the fluorescence decrease over the observed time (55 min)

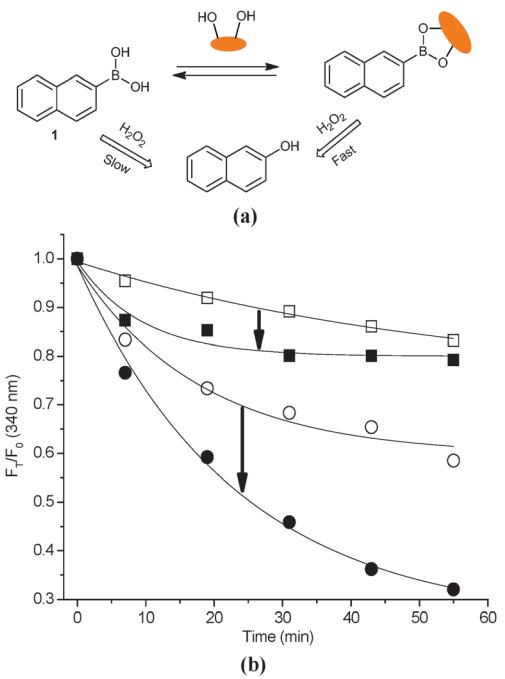


Fig. 1 (a) Proposed strategy of probe **1** and **1**-D-fructose complex for sensing of H_2O_2; (b) time curve of fluorescence intensity changes with probe **1** (10 μM) and D-fructose (100 mM) in aqueous H_2O_2 (0.10 mM) at different pH values. (Empty square – **1** and solid square – **1**-D-fructose in pH 7.20; empty circle – **1** and solid circle – **1**-D-fructose in pH 9.70.) The mixture was incubated in pH 7.20 PBS buffer and pH 9.70 Na_2CO_3/$NaHCO_3$ buffer at 25 °C, respectively. Fluorescence intensities at 340 nm were measured with excitation at 290 nm.

of probe **1** (10 μM) was *ca.* 0.85 (F_T/F_0) while there was a larger change for the **1**-D-fructose complex, *ca.* 0.80 (F_T/F_0) upon expose to H_2O_2 (0.10 mM) over the same period in neutral buffer solution. Under alkaline conditions, the **1**-D-fructose boronate ester complex reacted even faster with H_2O_2 (0.10 mM) (F_T/F_0 = *ca.* 0.30) than the unbound boronic acid **1** (F_T/F_0 = *ca.* 0.60). The responses of **1** and the **1**-D-fructose complex towards H_2O_2 became more rapid at high pH since not only is the binding between boronic acid and saccharide enhanced, but also H_2O_2 is also more reactive at higher pH.

Fluorescence was confirmed to be due to the generation of 2-napthol by comparison with spectroscopic properties of an authentic sample. *i.e.* The emission maxima for 2-naphthol (10 μM, pK_a = 9.51) was found at 340 nm in pH 7.20 PBS buffer while the maxima shifted to 410 nm in pH 9.70 alkaline buffer (Fig. S4, ESI‡). These results demonstrate the proton-induced fluorescence switching of 2-naphthol[27] at pH 7.20 and the electron donation of the negative oxygen to the fluorophore causing the red-shift at pH 9.70.

In the case of *N*-methyl-*o*-(aminomethyl)phenylboronic acid (**2**), a significant "off–on" signal response has been seen on binding with D-fructose (Fig. S5, ESI‡) due to a PET mechanism.[28] However, when the arylboronic acid moiety of probe **2** was transformed into a phenol upon adding H_2O_2, the fluorescence was further reduced due to the stronger PET from the amine in the boron free system (Fig. S5–S7, ESI‡).

The reaction scheme and fluorescence intensity changes for fluorescent probe **2** and the **2**-D-fructose complex in pH 7.20 and 9.70 buffer are given in Fig. 2. From Fig. 2(b), it can be seen that the fluorescence intensity ratios with time (F_T/F_0) of **2**-D-fructose complex slowly change to *ca.* 0.72, while for the saccharide free system a bigger change F_T/F_0 = *ca.* 0.55 on addition of 0.05 mM H_2O_2 over three hours in pH 7.20 PBS buffer (probe **2**, 10 μM). The difference between **2** and the **2**-D-fructose complex towards H_2O_2 became much larger in pH 9.70 buffer solution with F_T/F_0 = *ca.* 0.05 within 20 min upon addition of only 0.02 mM H_2O_2 (Fig. S8, ESI‡).[29] More importantly, the signal to noise ratio output of probe **2** was enhanced through binding with saccharide allowing for colorimetric detection (bright blue to colourless) in the reaction with H_2O_2 (Fig. 3, Fig. S9, ESI‡).

The fluorescence of **2** is turned on by saccharide binding, since boronic ester formation causes an enhanced interaction between the neighbouring amine and the boron atom (mediated *via* an inserted solvent molecule).[17,30,31] This N–B interaction hinders the reaction between boron and H_2O_2 in the presence of saccharides resulting in a slower decrease in fluorescence (*cf.* saccharide free system).

In conclusion, D-fructose effects the reaction of integrated (compound **1**) and insulated (compound **2**) boronic acid-based fluorescent probes with hydrogen peroxide in opposite directions. Integrated boronic acid fluorophores (compound **1**) display enhanced reactivity with H_2O_2 in the presence of D-fructose. While the PET fluorophore systems (compound **2**) display reduced reactivity with H_2O_2 in the presence of D-fructose. The insulated PET systems are particularly interesting, (compound **2**) because, in the presence of D-fructose the initial fluorescence intensity is much higher and produces a blue visible fluorescence, which implies that they could be used as temporal fluorescent probes to map both intracellular H_2O_2 and saccharide concentrations. The insulated systems also produce

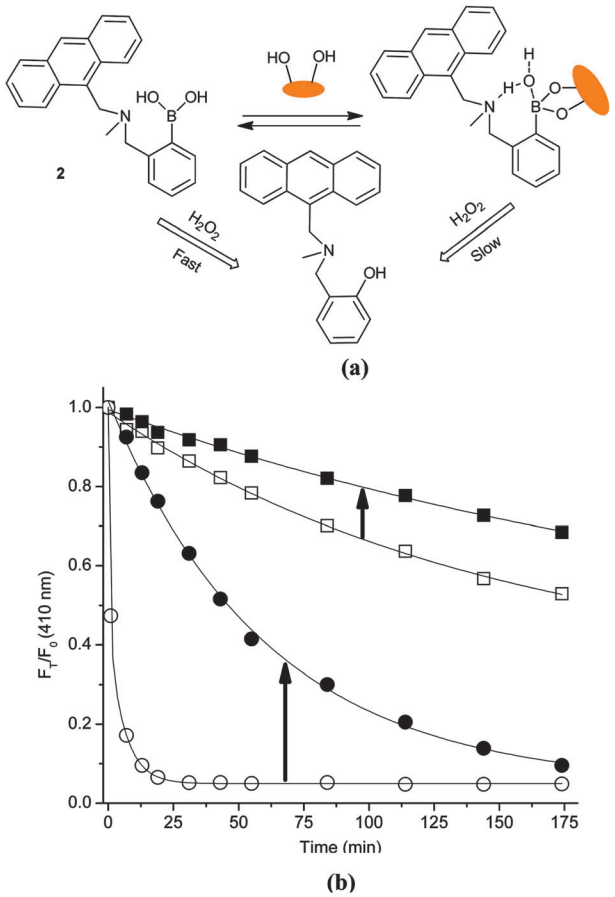


Fig. 2 (a) Proposed strategy of probe **2** and **2**-D-fructose complex for sensing of H_2O_2; (b) time curve of fluorescence intensity changes with probe **2** (10 μM) and D-fructose (100 mM) in aqueous H_2O_2 (0.05 mM for the first three samples and 0.02 mM for last sample). (Solid square – **2**-D-fructose and empty square – **2** in pH 7.20; solid circle – **2**-D-fructose and empty circle – **2** in pH 9.70.) The mixture was incubated in pH 7.20 PBS buffer and pH 9.70 Na_2CO_3/$NaHCO_3$ buffer at 25 °C. Fluorescence intensities at 410 nm were measured with excitation at 370 nm.

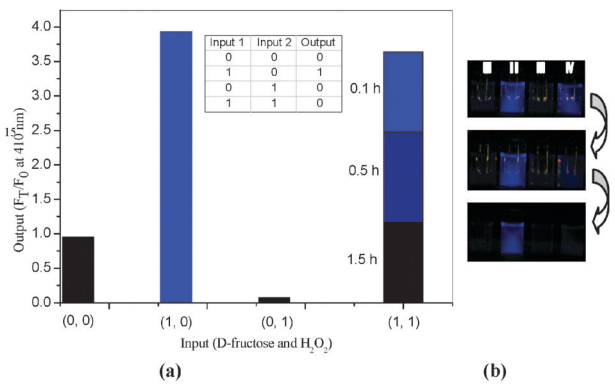


Fig. 3 (a) Column spectral and truth table with D-fructose (100 mM) and H_2O_2 (0.05 mM) as inputs; (b) fluorescent colourimetric detection using naked eye with I (probe only); II (+D-fructose 100 mM); III (+ H_2O_2 0.10 mM); IV (+D-fructose 100 mM + H_2O_2 0.10 mM) after 0.1 h, 0.5 h, 1.0 h. The mixture was incubated in pH 9.70 Na_2CO_3/$NaHCO_3$ buffer at 25 °C. Fluorescence intensities at 410 nm were measured with excitation at 370 nm.

a larger fluorescence response to low concentrations of H_2O_2. Therefore, we believe that insulated (PET) systems are the fluorescent probes of choice for use as imaging agents and sensors for H_2O_2.

We are currently exploring the development of longer wavelength insulated systems for biologically relevant imaging applications.

TDJ and XS are grateful for financial support from China Scholarship Council (CSC) and University of Bath Full Fees Scholarship. The Catalysis And Sensing for our Environment (CASE) network is thanked for research exchange opportunities.

Notes and references

1 Biochemical oxygen demand (BOD) is the amount of dissolved oxygen needed by aerobic biological organisms in a body of water to break down organic material present in a given water sample at certain temperature over a specific time period.
2 In environmental chemistry, the chemical oxygen demand (COD) test is commonly used to indirectly measure the amount of organic compounds in water.
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