Analyst

COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: *Analyst*, 2017, **142**, 2104

Received 17th March 2017,
Accepted 5th May 2017

DOI: 10.1039/c7an00467b

rsc.li/analyst

An arylboronate locked fluorescent probe for hypochlorite†

Leilei Shi,‡[a] Xin Li,‡[b] Min Zhou,[a] Faheem Muhammad,[a] Yubin Ding *[a,c] and Hui Wei *[a]

An unusual arylboronate based fluorescent probe R1 was synthesized for the selective and sensitive detection of ClO^-. A detailed mechanistic study revealed that R1 reacted with ClO^- through an oxidation to chlorination mechanism, and the arylboronate moiety in R1 acted as a "lock" to eliminate the effects of pH fluctuations. With this design strategy, R1 was successfully used to detect as low as 6.4 nM of ClO^- over other ROS species in a wide pH range from 4.5 to 9.0.

Introduction

Reactive oxygen species (ROS) (such as hydrogen peroxide, superoxide radical, singlet oxygen, hydroxyl radical, and hypochlorite anion) play critical roles in biological functions like cell signaling and homeostasis. A high level of ROS would lead to oxidative stress conditions, the damaging effects of which are the contributing factors of many diseases.[1] Among them, the hypochlorite anion (ClO^-) has attracted particular attention due to its importance in immune systems against microorganisms and inflammation.[2] The dysregulated production of ClO^- in living systems may cause severe adverse effects and even diseases (such as cardiovascular diseases,[3] nephropathy,[4] neurodegenerative diseases,[5] and cancers[6]). Thus, the development of effective detection methods for monitoring ClO^- levels in living systems is of great importance.

Organic molecule based fluorescent probes have been demonstrated to be ideal tools for detecting various kinds of analytes such as metal ions, anions, small molecules, and biomacromolecules.[7] Recently, numerous fluorescent probes have been successfully developed for ClO^- detection. Compared to other methods using chemiluminescent,[8] colourimetric,[9] or electrochemical[10] probes, these fluorescent probe based methods have advantages including low cost, easy fabrication, and high sensitivity. However, the selective recognition of ClO^- from the other coexisting ROS species with a fluorescent probe is still a challenging task.[11] Particularly, it is important to differentiate ClO^- from H_2O_2 because ClO^- is endogenously produced by the myeloperoxidase (MPO) catalyzed reaction of H_2O_2 and Cl^-.

Conventionally, arylboronate based fluorescent probes have been designed for the selective detection of H_2O_2.[12] However, one recent report showed that arylboronate could react faster with ClO^- than with H_2O_2.[13] Yoon *et al.* have exploited this phenomenon to develop a specific fluorescent probe containing both arylboronate and thiolactone moieties for ClO^- detection.[11e] They showed that among the ROS tested only ClO^- reacted with the arylboronate and then hydrolyzed the thiolactone moieties to generate detectable fluorescence signals. This example indicated that the arylboronate group may be developed as a powerful recognition unit to distinguish ClO^- from H_2O_2, as it may react with both ClO^- and H_2O_2 but give different final products. However, selective and sensitive arylboronate based fluorescent probes for ClO^- are still very rare.

Moreover, an ideal fluorescent probe for living systems should also be robust to environmental fluctuations such as pH changes, because the pH values of the extracellular matrix, intracellular environment, and subcellular organelles are all different. We envisioned that one strategy to address this problem is to introduce a "locked" moiety in the detection system, which would only be removed at the site of interest. With these thoughts in mind, we herein report a fluorescent probe **R1**, in the structure of which the arylboronate group functions as both the ClO^- recognition unit and the "lock" to eliminate the effects of pH fluctuations (Scheme 1). The reac-

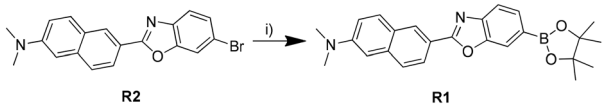
[a]*Department of Biomedical Engineering, College of Engineering and Applied Sciences, Collaborative Innovation Center of Chemistry for Life Sciences, Nanjing National Laboratory of Microstructures, Nanjing University, Nanjing, Jiangsu 210093, China. E-mail: weihui@nju.edu.cn, ybding@njau.edu.cn; http://www.weilab.nju.edu.cn; Fax: +86-25-83594648; Tel: +86-25-83593272*

[b]*Division of Theoretical Chemistry and Biology, School of Biotechnology, KTH Royal Institute of Technology, SE-10691 Stockholm, Sweden*

[c]*Jiangsu Key Laboratory of Pesticide Science, College of Sciences, Nanjing Agricultural University, Nanjing, Jiangsu 210095, China*

†Electronic supplementary information (ESI) available: Experimental details and additional figures. See DOI: 10.1039/c7an00467b

‡These authors contributed equally.



Scheme 1 Synthesis of fluorescent probe **R1**. (i) Bis(pinacolato)diboron, CH_3COOK, $Pd(dppf)Cl_2$, 1,4-dioxane, reflux.

tion mechanisms between **R1** and ClO^- and H_2O_2 were investigated in detail. We found that compared with the unlocked molecule **R1-OH**, **R1** worked well in a much wider pH range from 4.5 to 9.0. Besides, probe **R1** was determined to be quite stable, and it was successfully used to selectively detect as low as 6.4 nM of ClO^- over other ROS species in a ratiometric way.

Results and discussion

Probe **R1** was synthesized by the Miyaura borylation of the bromide precursor **R2** in a yield of 87% and characterized by NMR and mass spectrometry (MS) (Scheme 1 and Fig. S1–S3†). **R1** was stable and can be stored at room temperature for several weeks without obvious decomposition. When excited at 370 nm, **R1** emitted a strong blue/cyan fluorescence with a peak centered at ~481 nm.

The reactivity and selectivity of **R1** toward ClO^- detection was then accessed in comparison to other ROS species. To our surprise, the addition of excess H_2O_2 into **R1** solution did not induce significant fluorescence changes. Instead, among various tested ROS (such as $^1O_2$, $O_2^{\bullet-}$, $NO^\bullet$, $ROO^\bullet$, $HO^\bullet$, H_2O_2, $ONOO^-$, and ClO^-), only the addition of ClO^- to **R1** solution resulted in a vivid emission colour change from blue/cyan to yellow under UV irradiation (Fig. 1a, inset). More surprisingly, we found that the reaction between **R1** and H_2O_2 in EtOH resulted in a clear new product point as revealed by thin-layer chromatography (TLC) analysis, and the ^1H NMR signals of the new product's aromatic protons were shifted upfield (Fig. S4†). Further NMR and MS analyses confirmed the new product as **R1-OH** (Scheme 2, Fig. S5 and S6†). Then, the emission spectrum of purified **R1-OH** was compared with probe **R1**, **R1** + H_2O_2 and **R1** + ClO^- in EtOH/H_2O (4 : 1, v : v). As shown in Fig. 1b, the emission peaks of **R1-OH**, **R1** and **R1** + H_2O_2 were mainly in the blue-cyan colour region, while that of **R1** + ClO^- was in the green-yellow region.[14] In addition, the titration of **R1** solution with H_2O_2 only resulted in a ~4 nm blue shift of the fluorescence emission at ~481 nm (Fig. S7†), which was not large enough to induce significant emission colour changes. That is to say, despite the fact that H_2O_2 was able to oxidize **R1** into **R1-OH**, the emission spectrum of the oxidized product remained in the same blue-cyan region as **R1**, while the addition of ClO^- resulted in different reaction products with vivid emission colour changes. Fluorescence spectroscopic measurements revealed that the addition of ClO^- to **R1** solution resulted in almost complete fluorescence quenching at ~481 nm and the development of a new emission peak at ~532 nm (Fig. 1b). Therefore, the ratio of fluorescence intensities at 532 and 481 nm can be used as a ratiometric reporting signal for the quantitative assessment of the specificity of **R1**. As shown in Fig. 1a, the F_{532}/F_{481} value was more than ten times higher for ClO^- than that for all the other ROS including H_2O_2. These results demonstrated the high selectivity of **R1** for ClO^- detection.

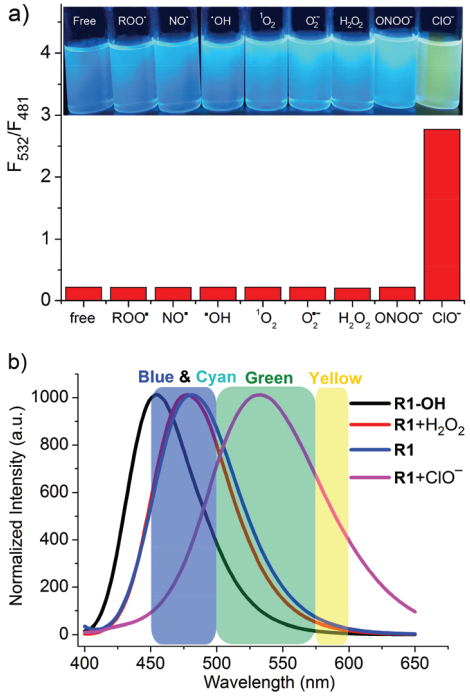
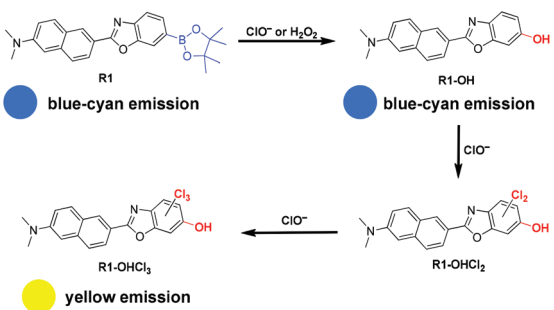


Fig. 1 Selectivity of **R1** toward ClO^- in EtOH/H_2O (4 : 1, v : v). (a) Fluorescence intensity ratio of **R1** (1 μM) at F_{532}/F_{481} after the addition of 60 equiv. of various ROS species. Inset: a photograph showing the fluorescence change of **R1** (10 μM) upon the addition of 60 equiv. of various ROS species in EtOH/H_2O (4 : 1, v : v) under a portable UV lamp irradiation; (b) normalized emission spectra of purified **R1-OH**, **R1** + H_2O_2 (60 equiv.), **R1** and **R1** + ClO^- (60 equiv.) in EtOH/H_2O (4 : 1, v : v). The colour boxes indicate the spectral range of the corresponding colour.



Scheme 2 Proposed reaction mechanisms of **R1** upon the addition of H_2O_2 and ClO^-. Note, chlorination reaction could also occur at the naphthalene ring.

The unexpected selectivity of **R1** toward ClO^- urged us to investigate the reaction mechanisms between **R1** and ClO^-. Therefore, the titration of **R1** solution with ClO^- was per-

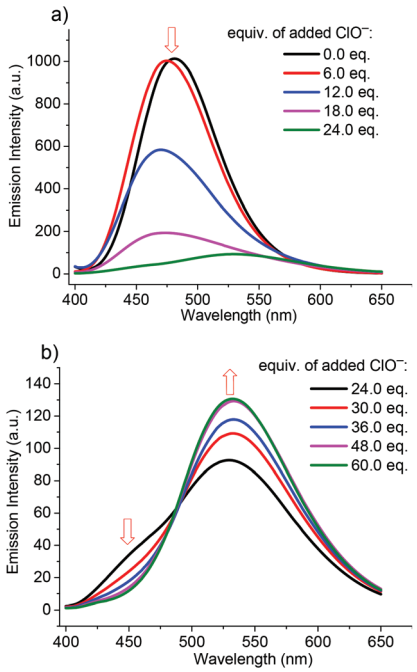


Fig. 2 Fluorescence titration profile of **R1** (1 μM) with an increasing amount of ClO^- (0–60 equiv.) in EtOH/H_2O (4 : 1, v : v). (a) 0–24 equiv. of ClO^-; (b) 24–60 equiv. of ClO^-. λ_{ex} = 370 nm.

formed using a fluorescence spectrophotometer. Interestingly, two sets of reaction processes were observed. As shown in Fig. 2, the addition of 0–24 equiv. of ClO^- to **R1** solution resulted in a ~10 nm blue shift and then quenching of the fluorescence emission at ~481 nm, whereas further addition of 24–60 equiv. of ClO^- led to the development of a new emission peak at ~532 nm. For the second interaction process, the distinctive isoemissive point at ~489 nm indicated the formation of new species during the second reaction process. Based on the fluorescence titration data, the detection limit of probe **R1** for ClO^- was then determined to be 6.4 nM based on $3\sigma/s$, where σ is the standard deviation of 10 blank measurements and s is the slope of F_{532}/F_{481} as a function of ClO^- concentration (Fig. S8 and S9†). Similarly, multi-step reactions were also observed during the titration of **R1** with ClO^- using a UV-visible spectrophotometer (Fig. S10†). We noticed that the reaction of **R1** with 6 equiv. of ClO^- or 60 equiv. of H_2O_2 resulted in almost identical blue shifts (~6 nm for ClO^- and ~4 nm for H_2O_2) in the fluorescence emission spectra, indicating the generation of the same product, that is **R1-OH**. Moreover, the addition of ClO^- to the purified **R1-OH** generated a similar emission peak to **R1** + ClO^- (Fig. S11†). These results suggested that **R1-OH** should be the final product of the reaction between **R1** and H_2O_2, but an intermediate for the reaction between **R1** and ClO^- (the first reaction step). This conclusion was further supported by the NMR titration data (*vide infra*).

To elucidate the final reaction products of **R1** + ClO^-, the reaction was carefully investigated by NMR and MS. As discussed above, since we have determined **R1-OH** to be an intermediate for the reaction between **R1** and ClO^-, we envisioned that the initial addition of ClO^- to the solution of **R1** might cause boronate oxidation similar to that by H_2O_2. Indeed, the boronate oxidation reaction was observed during the initial titration of **R1** in DMSO-d_6 with ClO^-, as the boronate protons at ~1.32 ppm fully disappeared and the peak at ~3.07 ppm assigned to the –N–$(CH_3)_2$ protons of **R1** was shifted upfield to ~3.06 ppm upon the addition of 1 equiv. of ClO^- (Fig. 3). Upon the successive addition of 2–4 equiv. of ClO^-, two new peaks assigned to –N–$(CH_3)_2$ were then developed at ~3.05 and ~3.02 ppm, accompanied by the disappearance of the peak at 3.06 ppm (Fig. 3), indicating that two new products (**R1-OHCl$_2$** and **R1-OHCl$_3$**, *vide infra*) were generated after the boronate oxidation reaction. Complicated proton signals in the low-field region of the ^{1}H NMR spectra were observed upon the addition of ClO^- (Fig. S12†), indicating a mixture of new products. Further addition of more than 4 equiv. of ClO^- resulted in precipitation in the NMR tube and no further NMR data could be obtained. Luckily, we successfully isolated the final main reaction product of **R1** + ClO^-, and discovered only 6 aromatic protons from its ^{1}H NMR data (Fig. S13†). It was possible that the addition of ClO^- to the solution of **R1** resulted in chlorination reaction at the aromatic rings. This conclusion was further supported by the MS data. The MS data of **R1** after the reaction with different equiv. of ClO^- showed that two main products were formed (*i.e.*, **R1-OHCl$_2$** (*m*/*z*: 370.9, $[M-H]^-$) and **R1-OHCl$_3$** (*m*/*z*: 404.9, $[M-H]^-$)). Moreover, the percentage of **R1-OHCl$_3$** increased along with the addition of more equiv. of ClO^- (Scheme 2, Fig. S14†). The above results suggested that the addition of ClO^- first played the same role as H_2O_2 to oxidize **R1** into **R1-OH**, and then chlorinated **R1-OH** to **R1-OHCl$_2$** and eventually **R1-OHCl$_3$** (Scheme 2).

To test if the arylboronate of **R1** could function as a "lock" to eliminate pH induced signal fluctuations, a comparison study between **R1** and **R1-OH** was carried out. Interestingly, though **R1-OH** does not have the arylboronate group, it can be selectively chlorinated by ClO^- to produce the yellow emissive product. Therefore, **R1-OH** could also be used as a highly specific fluorescent probe for ClO^- detection (Fig. S15†). However, the fluorescence of **R1-OH** was greatly influenced by pH, which was possibly due to the protonation/deprotonation

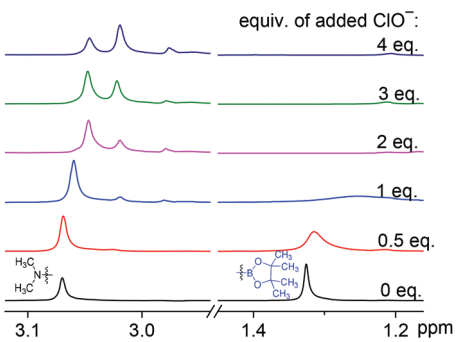


Fig. 3 High-field region of the ^{1}H NMR spectra of **R1** (20 mM) in DMSO-d_6 upon successive addition of ClO^- solution.

reaction at the phenol group of **R1-OH** (Fig. S16†). In contrast, **R1** was able to detect ClO^- robustly in a wide pH range from 4.5 to 9.0 (Fig. S17†). Thus, the arylboronate unit in **R1** functioned as a "lock" for effectively preventing the pH induced fluorescence fluctuation of **R1**.

The proposed multistep reaction mechanism drew our attention into an important issue in designing fluorescent probes, which is the response speed. It is well known that reaction based fluorescent probes usually suffer from the slow response speed, which limits their sensing performance. For probe **R1**, only about 2 min were required to complete the reaction at room temperature, which fully met the sensing requirements (Fig. S18†). In addition to its merits, such as fast response speed, high sensitivity, selectivity and pH tolerance for ClO^- detection, **R1** was highly photostable. The photostable test showed that the fluorescence intensity of **R1** at 481 nm did not show obvious decay upon continuous irradiation under 365 nm UV light for 4 h (Fig. S19†). These results indicated that **R1** was a promising fluorescent probe for ClO^- detection.

Conclusions

In conclusion, a ratiometric fluorescent probe **R1** was developed for specific ClO^- detection. Detailed mechanistic studies revealed that **R1** reacted with ClO^- through an oxidation to chlorination mechanism. Moreover, the arylboronate moiety in **R1** acted as a "lock" to prevent the pH influence on its ClO^- sensing behavior. **R1** was not only highly stable and robust but also exhibited high sensitivity and selectivity for ClO^-. This work unravelled the mechanism of the specific response of an arylboronate contained fluorophore to ClO^- over other ROS including H_2O_2, and demonstrated an effective strategy for designing highly robust probes by introducing a "lock" moiety.

Acknowledgements

We thank the National Natural Science Foundation of China (no. 21405081), the Natural Science Foundation of Jiangsu Province (no. BK20140593 and no. BK20130561), the 973 Program (no. 2015CB659400), the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), the Shuangchuang Program of Jiangsu Province, the Six Talents Summit Program of Jiangsu Province, Open Funds of the State Key Laboratory for Chemo/Biosensing and Chemometrics (2014002), Open Funds of the State Key Laboratory of Electroanalytical Chemistry (SKLEAC201501), Open Funds of the State Key Laboratory of Analytical Chemistry for Life Science (SKLACLS1704), the Fundamental Research Funds for the Central Universities (KYZ201750), and the Thousand Talents Program for Young Researchers for financial support. We thank Professor Yong Liang, Professor Bingling Li, Professor Yongshu Xie, Dr Jing Liu, and Dr Shiliang Tian for helpful discussions.

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14 It was also surprising to find that the maximum emission peak of **R1-OH** was obviously blue shifted (481 to 454 nm) relative to the unpurified **R1** + H_2O_2 system. This may be due to the presence of complicated ingredients in the solution of **R1** + H_2O_2 than the pure **R1-OH**.