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ARTICLE

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Cite this: DOI: 10.1039/x0xx00000x

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

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We have developed a novel fluorescent probe (RBT) based on rhodamine B and benzothiazole hydrazine units for the detection of hypochlorite in living cells and real water samples with excellent selectivity and sensitivity. In the presence of hypochlorite in a mixture solution of MeCN–PBS (v/v = 3:7, pH = 7.4) at room temperature, the fluorescence intensity of RBT increased by 350 fold with the color change from colorless to red. The detection limit of the probe for hypochlorite is 1.06×10^{-9} M. Moreover, RBT was successfully used to image endogenous hypochlorite in living cells and detect hypochlorite in real water samples.

Introduction

Hypochlorous acid (HOCl) or hypochlorite ($^{-}$OCl) is one of the most important reactive oxygen species resulted from the reaction of hydrogen peroxide and chloride ions by the catalysis of myeloperoxidase (MPO) in living organisms.[1] Endogenous hypochlorite plays a vital role in defending invasion of pathogens.[2] However, uncontrolled generation of hypochlorite is closely associated with some diseases, such as arthritis, kidney disease, lung injury, atherosclerosis, osteoarthritis and cancer.[3] Therefore, detecting HOCl/$^{-}$OCl in vitro and in vivo is of great interest. On the other hand, hypochlorite is widely used as household bleach and disinfection of drinking water. High concentration of hypochlorite is a potential health hazard to human and animals, arousing eye or nose irritation and stomach discomfort. Considering the adverse health effects of hypochlorite, it is necessary to detect and monitor $^{-}$OCl residues in real water samples.

Fluorescence probe has become a powerful tool for detecting trace amounts of species, such as metal ions,[4-11] biothiols[12-17] and anions,[18-21] in environmental and biological samples, because of its high sensitivity, selectivity, real-time and simplicity. To date, numerous fluorescence probes for specific detection of $^{-}$OCl have also been reported.[22-37] A large number of probes based on rhodamine platform have been known due to their excellent photophysical properties, high quantum yields, good water solubility and high photostability. The sensing mechanism of the rhodamine-based dyes involves the opening of the spirolactam ring to give a pink color along with a fluorescence 'turn-on' response.[38] Among them, the chemoselective conversion of rhodamine 6G hydrazide or rhodamine B and aryl hydrazide to the corresponding carboxylic acid by an oxidation–hydrolysis mechanism provides a facile reaction-based approach to specific detection of HOCl/$^{-}$OCl over other ROS and RNS in aqueous solutions.[39-45] However, most of the reported hydrazide-based probes showed either modest selectivity, necessity for large amounts of organic co-solvents, or prolonged reaction time. Therefore, there is still need to further develop selective, sensitive and fast-response fluorescent probes for HOCl/$^{-}$OCl, which can be obtained through convenient synthesis.

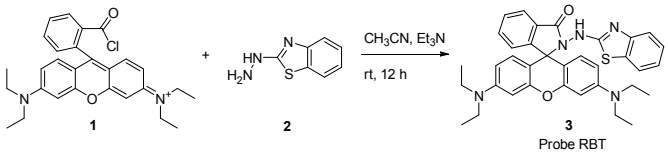
As a continuation of our work on the fluorescent probes for pH,[46-49] metal ions,[50-52] biothiol[53-57] and HOCl/$^{-}$OCl,[58] we here report a new fluorescent probe based on rhodamine B and benzothiazole hydrazine for sensing hypochlorite in living cells and real water samples (Scheme 1).

Experimental

Apparatus and chemicals

Thin-layer chromatography (TLC) was conducted on silica gel 60 F_{254} plates (Merck KGaA) and column chromatography was conducted over silica gel (mesh 200–300). ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were acquired on a Bruker

Avance 400 spectrometer, with DMSO-*d6* used as a solvent and tetramethylsilane (TMS) as an internal standard. High-resolution mass spectrometry (HRMS) involved a Q-TOF6510 spectrograph (Agilent). UV–vis spectra were measured by a Hitachi U-4100 spectrophotometer. Fluorescence measurements were performed on a Perkin-Elmer LS-55 luminescence spectrophotometer. Quartz cuvettes with a 1-cm path length and 3-mL volume were used for all measurements. The pH was determined with a model PHS-3C pH meter. Unless otherwise stated, all reagents were purchased from Aladdin, J&K or Sinopharm Chemical Reagent Co. and used without further purification. Twice-distilled water was used throughout all experiments. The spectroscopic properties of probe RBT were investigated and all samples were performed in a mixture solution (MeCN : PBS, v/v = 3 : 7, pH = 7.4).



Scheme 1 Synthesis of probe **RBT**

Synthesis of 2-(benzo[d]thiazol-2-ylamino)-3',6'-bis(diethylamino)spiro[isoindoline-1,9'-xanthen]-3-one, (compound 3, probe RBT)

The mixture of rhodamine B chloride (**1**) (0.2987 g, 0.6 mmol), triethylamine (2 mL) and 2-hydrazinylbenzo[*d*]thiazole (**2**) in CH_3CN (50 mL) was stirred for 12 h at room temperature. The completion of the reaction was monitored by TLC (PE: EA = 4:1). The crude product was purified by column chromatography on silica gel to afford the desired compound **3** (probe RBT) in 25% yield, red solid; m.p. 117℃-118℃. FT-IR (KBr): 3434, 2969, 2929, 2871, 1722, 1613, 1547, 1516, 1271, 1224, 1121 cm^{-1}. ^1H NMR (400 MHz, d_6-DMSO), δ: 1.05 (t, J = 7.2 Hz, 12H), 3.29 (q, J = 7.2 Hz, 8H), 6.31 (s, 2H), 6.38 (d, J = 8.4 Hz, 2H), 6.51 (br, 2H), 7.02 (br, 1H), 7.16-7.22 (m, 2H), 7.35 (d, J = 6.4 Hz, 1H), 7.54-7.67 (m, 3H), 7.90 (d, J = 7.2 Hz, 1H), 9.78 (s, 1H); ^{13}C NMR (100 MHz, d_6-DMSO), δ: 12.8, 44.1, 66.1, 97.7, 105.2, 108.3, 119.4, 121.3, 122.2, 123.3, 124.8, 125.9, 129.3, 129.8, 130.0, 131.1, 134.3, 148.9, 151.3, 152.1, 153.9, 165.6, 170.1 ppm; TOF-HRMS: $C_{35}H_{36}N_5O_2S$ $[M+H]^+$: 590.2590, Found 590.2597.

Preparation of the test solutions

Probe RBT was dissolved in MeCN for a stock solution (1 mM). Test solutions were prepared by displacing 50 μL of the stock solution into a 10 mL volumetric flask. The solution was diluted to 10 mL in a mixture of PBS (0.01 M, pH = 7.4) and MeCN (7 : 3, V/V). Small aliquots of each testing species solution were added. The resulting solutions were shaken well and incubated for 1 h at room temperature before recording spectra.

Cell culture and imaging

RAW264.7 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and were cultured as routine in DMEM medium containing 10% fetal bovine serum. All cells were maintained at 37 ℃ under humidified conditions and 5% CO_2. RAW264.7 cells were passed on small glasses and incubated for 24 h, then incubated with LPS (0.1 μg mL^{-1}) for 12 h. Before the staining experiment, cells were washed 3 times with PBS, incubated with probe RBT (1 μM) for 1 h, then washed 3 times with PBS and underwent imaging measurement with a confocal microscope (Zeiss LSM780, Carl Zeiss Canada) at excitation 550 nm. The emission of the red channel was 555-700 nm.

Results and discussion

UV-vis absorption response of probe RBT to ClO^-

In the absence of ClO^-, RBT (10.0 μM) had almost no absorbance, whereas the maximum absorption at 554 nm was increased with ClO^- increase (Fig. 1), which confirmed that the interaction between RBT and ClO^- led to ring-opening of spirocycle of rhodamine hydrazide.

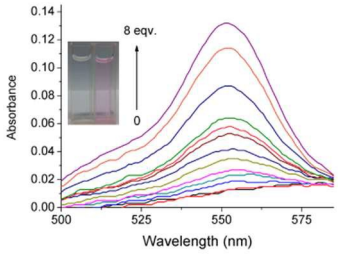


Fig. 1 Absorption spectra of probe RBT (10.0 μM) with different ClO^- concentration (0, 5, 10, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80 μM) in MeCN – PBS (v/v = 3:7, pH = 7.4) solution at room temperature.

Selectivity studies

Selectivity is the most important requirement for all kinds of detection methods. Subsequently, to evaluate the selectivity of probe RBT to ClO^-, we evaluated the response of probe RBT to other reactive oxygen species (ROS) and reactive nitrogen species (RNS) by fluorescence spectra. As shown in Fig. 2, the addition of 10 equiv. of ClO^- resulted in a significant enhancement of the fluorescence intensity (with an enhancement factor over 350-fold) positioned at 575 nm. However, the addition of HO•, $ONOO^-$, NO, H_2O_2, *t*-BuOOH, *t*-$BuOO^•$, $^1O_2$ and some cations and anions had no obvious effect on the fluorescence emission (Fig. 2 and Fig. S1, ESI).

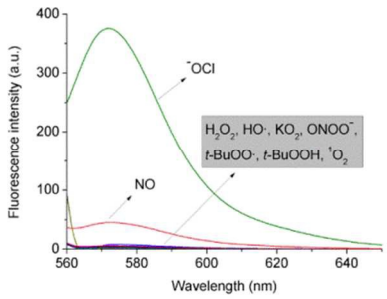


Fig. 2 Fluorescence spectra of probe RBT (5 μM) to ClO^- and other ROS/RNS (10 equiv). Condition: MeCN – PBS (v/v = 3:7, pH = 7.4; λex: 550 nm (slit widths: 12 nm/3 nm).

Fluorescence response of probe RBT to ClO^-

The following titration experiments were implemented with the addition of ClO^- at pH 7.4 for probe RBT. The fluorescent titration profiles of RBT (5 μM) with ClO^- (0–35 μM) are shown in Fig. 3a. The titration of ClO^- into RBT gave a strong fluorescence enhancement at 575 nm with the increase of the ClO^- concentration. The enhancement of fluorescence intensity in ClO^- titrations saturated at the addition of around 5 equiv. of ^-OCl (Fig. 3b). The results can be attributed to the ring-opening rhodamine acid formed by the reaction of probe RBT and ClO^-. A color change is also observed by naked eye. The high-resolution mass spectra (HRMS) of the reaction mixture of RBT with ClO^- also demonstrated the formation of rhodamine acid which showed a clear peak (m/z) at 443.2354 (calcd. 443.2329, Fig. S2, ESI).

In addition, probe RBT shows an excellent linearity between the fluorescence intensity and the concentration of ClO^- from 15 to 25 μM (Fig. 3c). The detection limit was calculated to be 1.06×10^{-9} M, which also showed a highly sensitive feature (Table S1, ESI). This finding indicates that the probe can be utilized to detect HOCl/ClO^- quantitatively.

The effect of pH on the fluorescence

We then proceeded to evaluate the photophysical behavior of probe RBT (5 μM) and its ability to react with ClO^- in a series of buffers with different pH values ranging from 4.7 to 8.3. The results showed that the emission spectra of probe RBT itself were essentially pH-insensitive across a wide range of pH values. However, the fluorescent responses of probe RBT toward ClO^- were pH-dependent; the maximal sensing responses of probe RBT upon treatment with 50 μM ClO^- were observed above pH 7.4. Consequently, the pH value of 7.4 was chosen in this work (Fig. S3, ESI).

Effect of reaction time

Time-dependent modulations in the fluorescence spectra of probe RBT were monitored in the presence of 4 equiv. of OCl^-. The results showed that the reaction was completed within 40 min for OCl^-, indicating that the probe reacted rapidly with OCl^- under the experimental conditions (Fig. S4, ESI).

Detection of ClO^- in living cells

Firstly, cytotoxicity and photostability of probe RBT was evaluated. After RAW 264.7 cells were incubated with different concentration of the probe (0.5, 1, 5 and 10 μM) for 12 h, viabilities of cells did not change, revealing that the probe has no cytotoxicity (Fig. S5, ESI). After RAW 264.7 cells were incubated with probe RBT (1.0 μM) for 1 h followed by continuously irradiation for 180 s, the fluorescence intensities were almost no change during the test time (0, 10, 30, 60, 90, 120, 150 and 180 s). Therefore, the probe is stable in tested condition (Fig. S6, ESI).

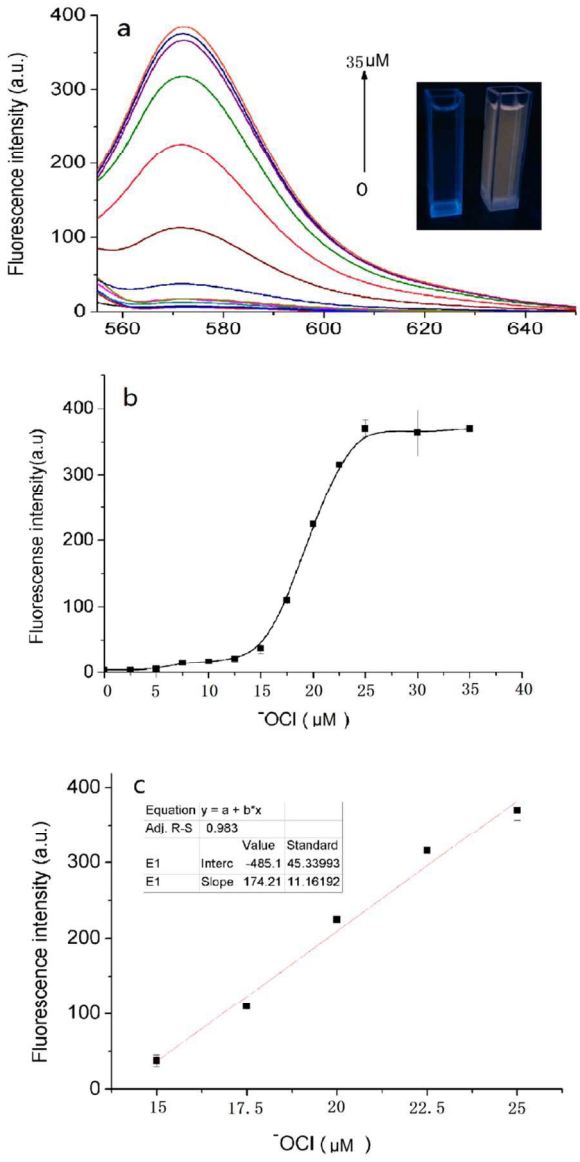


Fig. **3** (a) Fluorescence spectra of probe RBT (5 μM) with the addition of ClO^- (0, 2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5, 20.0, 25.0, 30.0, 35.0 μM); (b) Fluorescence intensity changes of probe RBT with the addition of ClO^- (0–35μM); (c) Linearity of fluorescence intensity of probe RBT with ClO^- from 15 to 25 μM; Condition: MeCN – PBS (v/v = 3:7, pH = 7.4; λex: 550 nm (slit widths: 12 nm/3 nm).

Probe RBT was then exploited to sense endogenous HOCl/ClO^- in RAW264.7 cells because macrophage cells activate the

generation of HOCl/ClO$^-$ after exposure to LPS. There was almost no fluorescence in the absence of LPS. In contrast, strong fluorescence was observed after treatment with LPS followed by the incubation with the probe (Fig. 4). Therefore, probe RBT can be sued to image endogenous HOCl/ClO$^-$ in living RAW264.7 cells.

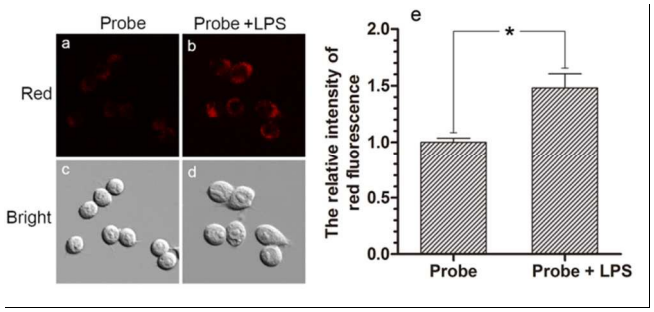


Fig. 4 Confocal fluorescence images of RAW 264.7 cells. The fluorescence images were shown in (a) and (b), RAW 264.7 cells were stimulated with 0.1 mg·mL^{-1} lipopolysaccharide (LPS) for 12 h, then incubated with 1 μM probe for 1 h. (e) The fluorescence intensity calculated by ImageJ. Experimental conditions: The probe was excited by 555 nm and the emission was collected by red channel (555-700nm). Data are presented as mean ± SE, *P≤0.05, n = 3.

Detection of ClO$^-$ in natural water samples

Hypochlorite is used in many industrial processes and also in daily life. Therefore, the detection of hypochlorite in natural water samples is of interest. The water samples were obtained from tap water, Daming Lake and bottled purity water. After the probe treated with the water samples, the fluorescence intensities were determined. The ClO$^-$ in these samples was not detected. Then the water samples were spiked with standard ClO$^-$ solution at different concentration levels. The probe was able to measure the concentrations of spiked ClO$^-$ with less error (Table S2, ESI). Therefore, probe RBT can potentially be used for the detection of ClO$^-$ in natural water samples.

Conclusions

In summary, we have developed a fluorescent probe RBT based on rhodamine B and benzothiazole hydrazine units for the detection of hypochlorite in living cells and real water samples. The probe showed a dramatic color change from colorless to red and fluorescence enhancement (350 fold) upon addition of ClO$^-$ in a buffer solution of MeCN–PBS (v/v = 3:7, pH = 7.4). The probe had a low limit of detection (1.06×10^{-9} M). Moreover, RBT could be used to image endogenous hypochlorite in living cells and detect hypochlorite in real water samples.

Acknowledgments

This study was supported by the Natural Science Foundation of Shandong Province (ZR2014BM004).

Notes and references

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Electronic Supplementary Information (ESI) available: Supplementary Figures S1-S5. See DOI: 10.1039/b000000x/

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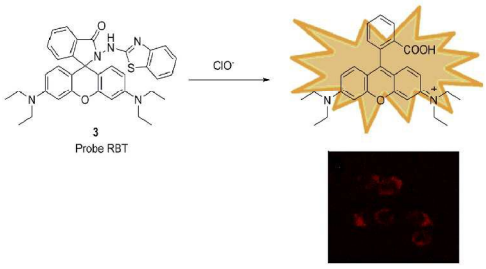

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