A novel rhodamine-based fluorescent probe for selective detection of ClO^- and its application in living cells imaging

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Abstract: A novel fluorescent rhodamine-based probe **L** for selective responding to ClO^- has been synthesized and characterized. The spectroscopy showed that probe **L** can detect ClO^- in aqueous solution without interaction with other interfering ions, and the detection is also evident by the color change from colorless to red purple under white light. The remarkable fluorescence enhancement showed the high selectivity and sensitivity of probe **L** for the detection of ClO^-. Furthermore, probe **L** was applied to intracellular fluorescent imaging of Hela cells treated with ClO^-, and MTT assay showed nontoxicity in living cells.

Keywords: Rhodamine B; fluorescent probe; ClO^-; Hela cells; bioimaging

Introduction

Hypochlorite/hypochlorous acid (ClO^-/HClO), one of the reactive oxygen species (ROS),[1] plays an important role in human immune defense system, such as killing a wide range of bacteria and pathogens in cells.[2, 3] Due to the good antibacterial effects and cheap price, HClO has been widely used as disinfectant for tap water.[4] However, high concentrations of hypochlorous acid solution may damage the respiratory system,[5] and many studies suggest that the oxidative stress mediated by HClO participates in kinds of pathophysiological processes such as atherosclerosis,[6, 7] apoptosis and cellular senescence, which may be potential harm to human health.[8–11]

Compared with the traditional sensing techniques such as atomic absorption spectroscopy, inductively coupled plasma-mass spectroscopy and inductively coupled plasma-atomic emission spectroscopy,[12, 13] and the recent development for the novel sensing techniques,[14–16] fluorescence chemosensors[17–20] turn out to be quite cost-efficient.

A hypochlorite fluorescent probe rhodamine 6G hydrazide has been synthesized for the detection of HClO in tap water and fluorescence imaging in Hela cells.[21] For the detection of hypochlorous acid produced by human neutrophil cells, a highly selective fluorescent probe was synthesized.[22, 23] In the pH detection of physiological environment, this probe showed higher selectivity on the HClO than other reactive oxygen species.[23, 24] These probes showed the "off-on" mechanism for the fluorescence and luminous reactions of HClO through spirolactam

ring opening.[25–27]

Because of the great importance of hypochlorous acid/hypochlorite and the fluorescent method used in cell imaging superior to many classical cell sensing methods[28–30] in view of the easy use and low cost, we herein developed a new rhodamine B hydrazide derivative as a fluorescent probe for ClO^- detection. Rhodamine fluorophores are known for their high fluorescence quantum yield, low sensitivity to pH, excellent optical properties and cell membrane penetrating ability, low toxic effects of organism,[31–33] we therefore speculate the probe is highly selective and sensitive, and thus could be applied in bioimaging in living cells.

Experiment sections

Materials and methods

All used reagents from commercial suppliers were analytical grade, untreated and used directly. Deionized water was used in the whole study. Cl^-, SiO_3^{2-}, $S_2O_3^{2-}$, $H_2PO_4^-$, NO_3^-, MnO_4^-, SO_4^{2-} were respectively prepared from NaCl, Na_2SiO_3, $Na_2S_2O_3$, NaH_2PO_4, $NaNO_3$, $KMnO_4$, Na_2SO_4 dissolved in deionized water. Ac^- and ClO^- were respectively prepared from acetic acid and sodium hypochlorite. $O_2^{\cdot-}$ was prepared by KO_2 dissolved in DMSO[34] and $^1O_2$ was prepared by the reaction of NaClO and 2 equiv. of H_2O_2.[35] $^{\cdot}OH$ was prepared by the reaction of ammonium ferrous sulfate and hydrogen peroxide.[36, 37] Phosphate buffer saline (PBS buffer) contained 2 mM potassium dihydrogenphosphate, 10 mM disodium hydrogen phosphate, 137 mM sodium chloride and 2.7 mM potassium chloride in deionized water at pH 7.4, adjusted by hydrochloric acid. The probe was dissolved in *N*, *N*-dimethylformamide as stock solution with a final

concentration at 100 μmol/L. The test solution was mixed by 2.0 mL PBS buffer (0.01 M, pH 7.4), 1.6 mL DMF and 0.4 mL stock solution.

^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Bruker Advance 400 FT spectrometer, using $CDCl_3$ as solvent with tetramethylsilane (TMS) as internal standard. Chemical shifts were reported in parts per million. Mass spectra (MS) were measured by the ESI method on an Agilent 6510 Q-TOF mass spectrometer. UV-Vis spectra were measured by a UV-2550 spectrometry (Shimadzu, Japan). Fluorescent measurements were recorded on an F-4500 FL spectrophotometer (Hitachi, Japan). All reactions were monitored by TLC (thin-layer chromatography) using 0.25 mm silica gel plates with UV indicator (Shanghai Jiapeng Technology Co. Ltd., China). The pH was determined with PHS-3B pH meter (Shanghai Ruixi equipment Co. Ltd., China). Images of Hela cells were captured by a laser confocal microscope (Nikon A1/A1R+, Japan). Melting point (uncorrected) was determined on a micro melting point apparatus (Shanghai Shenguang Instrument Co. Ltd., China).

Synthesis of probe L

The synthetic route for probe **L** is shown in Scheme 1. The reaction mixture of rhodamine hydrazide **2** (1.17 mmol, 1.0 equiv.),[38] chloroacetyl chloride (2.32 mmol, 2.0 equiv.) and triethylamine in 17.5 mL dichloromethane was stirred at room temperature for 4 h and quenched by excess triethylamine (11.67 mmol, 10.0 equiv.). The solvent was evaporated and to the residue 30 mL deionized water was added. The mixture was filtered and the filter cake was dried to give the compound **3** without further purification.[39]

To obtain probe **L**, the mixture of intermediate **3** (0.91 mmol, 1.0 equiv.), potassium carbonate (2.09 mmol, 2.3 equiv.), and *N*-methyl piperazine (0.90 mmol, 1.0 equiv.) in acetonitrile (12 mL) was heated to reflux for 18 h. The reaction was monitored by TLC. When the reaction was completed, the solvent was removed under vacuum and water was added to the residue. The crude product was extracted by ethyl acetate for three times. The combined organic layer was dried and filtered, and the filtrate was evaporated to give the crude product. The crude product was further recrystallized from acetonitrile to afford probe **L** as a white solid in 60% yield. m.p. 176.6–178.0 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 8.33 (s, 1H), 7.98–7.95 (m, 1H), 7.52–7.47 (m, 2H), 7.14 (s, 1H), 6.66 (s, 1H), 6.64 (s, 1H), 6.32 (dd, *J* = 4.7, 2.3 Hz, 4H), 3.37–3.30 (m, 8H), 2.97 (s, 2H), 2.35 (s, 4H), 2.18 (s, 3H), 1.89 (s, 4H), 1.16 (t, *J* = 7.0 Hz, 12H) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 12.66, 44.35, 45.89, 52.98, 55.20, 60.31, 66.05, 97.38, 104.10, 108.14, 123.45, 124.07, 128.34, 129.43, 133.09, 149.00, 151.42, 153.60, 165.17, 167.61 ppm. HRMS (ESI): Calcd. for $[M + H]^+$ ($C_{35}H_{45}N_6O_3$) *m/z* 597.3548, found *m/z* 597.3562.

Cell culture and imaging

Hela cells were cultured in dulbecco's modified eagle medium (DMEM, Gibco) containing 10% fetal bovine serum. All cells were cultured at 37 °C in an ESCO cell incubator (Esco Micro Pte. Ltd., Singapore). Cells were digested with 0.25% trypsin (w/v) and passaged at approximately 90% confluence every 2–3 days, and then cultured for 12 h in glass bottom culture dishes (NEST Biotech Co. Ltd., Hong Kong). The cells were washed three times by PBS buffer solution (pH 7.2). The ClO^- dissolved in DMEM was then added into the

dishes, at the final concentration at 200, 400, and 800 μM, respectively. The cells with ClO^- were incubated for 1.5 h, and washed three times by PBS buffer. And then the Hela cells were incubated for 1.5 h with probe **L** in PBS/DMF (v/v = 5: 5, pH 7.2), which reached to a final concentration at 10 μM. The Hela cells were washed by PBS buffer for three times, dissolved in 1 mL PBS and then monitored by a laser confocal microscopy (Nikon A1/A1R+, Japan). In the experiments, the control group only contained the Hela cells and DMEM. The probe group gave red fluorescence emission under confocal microscopy with the excitation wavelength at 565 nm.

Results and discussion

Synthesis of probe L

As depicted in Scheme 1, the reaction of rhodamine B and hydrazine hydrate obtained rhodamine hydrazide **2**, which then reacted with chloroacetyl chloride and triethylamine to afford the intermediate **3**. The probe **L** was obtained from *N*-alkylation of *N*-methyl piperazine with intermediate **3**. Probe **L** was characterized by ^{1}H NMR, ^{13}C NMR and HRMS. The chemical shift for the protons of CH_2CH_2 and CH_3 from *N*-methyl piperazine appeared in the ^{1}H NMR spectrum (Fig. S3.), and the new peaks at 165.17, 167.61 from the two carbonyl groups in ^{13}C NMR spectrum (Fig. S4.) confirmed the formation of probe **L**.

Spectroscopic properties of probe L and the detection of ClO^-

The solution of the probe **L** (10 μM) in PBS/DMF (5: 5, v/v, pH 7.4) was colorless and had no significant UV absorption at 400–700 nm and fluorescence at 450–700 nm. However, the color of the solution changed from colorless to purple under visible light with the addition of different concentrations of hypochlorite, and a significant absorption peak appeared at 565 nm in

UV spectrum and a strong emission at 576 nm in fluorescence spectrum were observed. We further found that the UV absorbance and fluorescence intensity of probe **L** increased significantly when ClO^- varied from 0–30 equiv., as shown in Figure 1. The linear correlations of probe **L** were shown in Fig. S1 and Fig. S2.

In order to explore probe **L** as an efficient and selective probe, the fluorescence spectrum of probe **L** was examined with different anions and reactive oxygen species (Ac^-, Cl^-, SiO_3^{2-}, SO_4^{2-}, $S_2O_3^{2-}$, $H_2PO_4^-$, NO_3^-, H_2O_2, MnO_4^-, $^{\cdot}OH$, $O_2^{\cdot-}$, $^1O_2$) in PBS/DMF solution (5:5, v/v, pH 7.4) (Figure 2). All species except hypochlorite ion only induced weak emissive peaks for probe **L** while probe **L** showed a very strong emissive peak in the presence of ClO^-. Although these interfering anions at high concentration above 14 equiv. of probe **L** did not affect the probe for sensing hypochlorite, they had an impact at low concentrations less than 14 equiv. For this reason, increasing the concentration of the probe can be helpful for the detection. And under visible light, the color change of the solution of probe **L** with these interfering anions was not observed. These fluorescence spectra results suggested that this probe might be a desirable probe for selectively detecting hypochlorite ions.

We next investigated the effect of the interfering anions on probe **L** for selective detection of ClO^-. After the addition of Ac^- (1 mM), Cl^- (1 mM), ClO_3^- (1 mM), $H_2PO_4^-$ (1 mM), MnO_4^- (20 μM), NO_3^- (1 mM), SO_4^{2-} (1 mM), $S_2O_3^{2-}$ (1 mM), SiO_3^{2-} (1 mM), $O_2^{\cdot-}$ (1 mM KO_2), $^1O_2$ (1 mM

Na_2MoO_4 + 2 mM H_2O_2) respectively to the solution of probe **L** containing 20 equiv. hypochlorite, the fluorescence intensity did not significantly change, which indicated that these anions had no effect on the selectivity of probe **L**, as illustrated in Figure 3. Interestingly, the fluorescence intensity was slightly increased in the presence of H_2O_2 (1 mM) or $^{\cdot}OH$ (100 mM Fe^{2+} + 1 mM H_2O_2). These two are active oxygen species and hypochlorite ion is also a reactive oxygen species, and we thus speculate that H_2O_2 or $^{\cdot}OH$ results in a synergistic effect on ClO^- for the fluorescence intensity of this probe.

We next explored the effect of pH value on the probe **L** for detecting ClO^-. The fluorescence intensity of the probe with 20 equiv. hypochlorite under different pH value was increased significantly. The intensity did not change markedly as pH converted from 6.76 to 7.68, whereas a variation appeared when the pH was over 7.68 (Figure 4).

Fluorescent imaging of intracellular ClO^-

Hela cells were incubated with probe **L** and hypochlorite at the concentration of 0.2, 0.4 and 0.8 mM, respectively, for intercellular ClO^- imaging of Hela cells by a laser confocal microscope. The cells only with the probe (final concentration at 10 μM) incubated at 37 °C for 1.5 h did not show fluorescence, in agreement with our previous study, while the cells and probe **L** in the presence of hypochlorite manifested strong red fluorescence. The intracellular fluorescence

images were shown in Figure 5, which indicated that the probe could penetrate into Hela cells and can detect hypochlorite ions in viable cells. In addition, Hela cells can be pierced by this probe within 1.5 h, which is more practical than other low-permeability probes. We also found the fluorescence intensity in cells increased when the concentration of hypochlorite increased.

MTT assay for toxicity of probe L

Then MTT assay has been carried out to assay the toxicity of probe **L** to cells. After incubation of Hela cells with probe **L** at 5, 10, 15, 20, 25 μM, respectively for 24 h, the cell viability remained similar to the control group, as shown in Figure 6. Higher concentration at 25 μM did not decrease the viability, however, the survival rate of Hela cells was slightly higher than that of the untreated group. Therefore, probe **L** was non-toxic to Hela cells and thus expected to be safely applied in cell marking and clinical use in physiological conditions.

Conclusions

In summary, the synthesized rhodamine-based probe **L** for fluorescent recognition of ClO^- in living cells has been developed in the present study. The probe is highly selective and sensitive to ClO^- without the interference from other anions, and also has the property of fast color change, easy detection, and direct observation through naked eyes. Probe **L** is nontoxic and has good penetrability to Hela cells, and has been successfully applied to fluorescent imaging of living

cells by sensing hypochlorite.

Acknowledgements

We would like to thank the Southwest University Undergraduate Training Programs for Innovation and Entrepreneurship, China (20152902005), the Fundamental Research Funds for the Central Universities, P. R. China (XDJK2016A015), Technology and Program Project of the Science and Technology Department of Sichuan Province, China (2015FZ0070) for financial support.

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Schemes and figures captions:

Scheme 1. The synthetic route of probe **L**. Reagents and conditions: (a) ethanol, reflux, 24 h; (b) Et_3N, CH_2Cl_2, r.t., 4 h; (c) K_2CO_3, CH_3CN, reflux, 18 h.

Figure 1. The UV spectrum (a) and fluorescence spectrum (b) of **L** (10 μM) in PBS/DMF solution (v/v = 5: 5, pH 7.4) in the presence of ClO^- (0–30 equiv.). λ_{ex} = 565 nm, λ_{em} = 576 nm.

Figure 2. The fluorescence spectra of **L** (10 μM) in PBS/DMF solution (v/v = 5: 5, pH 7.4) with 20 equiv. ClO^- or other anions, λ_{ex} = 565 nm. The anions were Ac^- (1 mM), Cl^- (1 mM), ClO_3^- (1 mM), $H_2PO_4^-$ (1 mM), MnO_4^- (20 μM), NO_3^- (1 mM), SO_4^{2-} (1 mM), $S_2O_3^{2-}$ (1 mM), SiO_3^{2-} (1 mM), $O_2^{\cdot-}$ (1 mM KO_2), $^1O_2$ (1 mM Na_2MoO_4 + 2 mM H_2O_2), H_2O_2 (1 mM) or $^{\cdot}OH$ (100 mM Fe^{2+} + 1 mM H_2O_2).

Figure 3. Fluorescence intensity of probe **L** (10 μM) in PBS/DMF solution (v/v = 5: 5, pH 7.4) with 20 equiv. ClO^- ion with or without the interfering ions, λ_{ex} = 565 nm, λ_{em} = 576 nm.

Figure 4. Fluorescence intensity at 576 nm of probe **L** in different pH PBS/DMF solution (v/v = 5: 5) before and after the addition of 20 equiv. ClO^-. λ_{ex} = 565 nm, λ_{em} = 576 nm.

Figure 5. Fluorescent imaging of ClO^- in Hela cells incubated with probe **L**. (a–c) Hela cells only incubated with 10 μM probe for 1.5 h at 37 °C. (d–f) Hela cells incubated with 0.2 mM ClO^- in

the growth medium for 1.5 h and then incubated with 10 μM probe for 1.5 h at 37 °C. (g–i) Hela cells incubated with 0.4 mM ClO^- in the growth medium for 1.5 h and then incubated with 10 μM probe for 1.5 h at 37 °C. (j–l) Hela cells incubated with 0.8 mM ClO^- in the growth medium for 1.5 h and then incubated with 10 μM probe for 1.5 h at 37 °C; (a), (d), (g) and (j) are the fluorescent images. (b), (e), (h) and (k) are the images of the bright field. (c), (f), (i) and (l) are merged images for (a) and (b), (d) and (e), (g) and (h), (j) and (k), respectively. Scale bar: 100 μm.

Figure 6. The viability of Hela cells after cultured with different concentrations of probe **L** (5, 10, 15, 20, 25 μM) for 24 h.