



Contents lists available at ScienceDirect

Chinese Chemical Letters

journal homepage: www.elsevier.com/locate/cclet



Communication

A fluorometric and mitochondrion-targetable probe for rapid, naked-eye test of hypochlorite in real samples

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ARTICLE INFO

Article history:

Received 16 November 2017

Received in revised form 23 January 2018

Accepted 29 January 2018

Available online xxx

Keywords:

Colorimetric probe

Fluorescent probe

Rhodamine

Hypochlorite

Mitochondrion-targetable

ABSTRACT

Hypochlorite anion is a ubiquitous reactive molecule in the terminal disinfection systems, inflammatory stress and immune systems. Thus, rapid and visual monitoring ClO^- in water and biological samples is very meaningful for water quality safety and toxicity assessment of contaminants. Herein, a colorimetric and fluorometric probe (Rh-CIO) based on rhodamine B fluorophore and thiophene-2-carbohydrazide has been unveiled and successfully utilized for ClO^- detection in water samples and HeLa cells. Upon addition of ClO^- , color changes of solution from colorless to pink were immediately visible to the naked-eyes, meanwhile, brilliant red fluorescence was observed under excited at UV light (365 nm). Rh-CIO displayed high selectivity and sensitivity for ClO^- , and the detection limit was $7 \mu\text{mol/L}$ calculated from the fluorescence titration. With the aid of its merits including rapid response to ClO^- within 10 s, Rh-CIO and its test paper could successfully detect ClO^- in water. Additionally, HeLa cells image co-stained with Rh-CIO and Rh123 demonstrated that Rh-CIO possessed excellent and fast cell-membrane permeability and mitochondrion-targetability. It was clearly confirmed that Rh-CIO would be a promising probe for rapid tracking of ClO^- in water samples and in mitochondria of living cells.

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Sodium hypochlorite (NaClO), as well known as famous bactericide and oxidizing agent, play vital roles in many fields such as drinking water disinfection [1,2], dye decoloration [3,4], odor elimination [5], oxidation contaminants in soils [6,7], and so on. Especially, in water treatment, e.g., reclaimed water and drinking water, sodium hypochlorite is now among the most widely used disinfectant, owing to its residual protection, low cost and ease of use [8–10]. The free chlorine residual can guarantee to kill bacteria and inactivate viruses, but it also can react with the constituents present to form disinfection by-products (DBPs) during water disinfection [11–13]. Some DBPs (e.g., *N*-nitrosamines, trihalomethanes, haloacetic acids, haloacetonitriles, etc.) are of growing concern because they have been recently identified as probable human carcinogens and deformity [14,15]. Therefore, a reasonable control on free chlorine residual in water disinfection will be of great significance to guarantee for the health of

populations. Besides that, hypochlorous acid produced by the enzyme myeloperoxidase in inflammatory stress [16] plays a critical role as one of the most important reactive oxygen species (ROS) in defending from the invasion of contaminants [17]. On the contrary, uncontrolled endogenous hypochlorite will further induces oxidation damage of the extracellular components, which may involve in some diseases, such as arthritis [18], kidney disease [19], lung injury [20], atherosclerosis [21] and cancer [22]. As well known, both hypochlorous acid and hypochlorite anion exist *in vivo* at almost equal concentrations. Measurement of hypochlorous acid and hypochlorite constitutes a fundamental aspect to evaluate the stress state exposed to different hazardous contaminants. Given the health risk of ClO^- , it is necessary to test and monitor hypochlorite residues both in real water samples and in biological specimens.

Right now, colorimetric and fluorometric methods are available for detecting and monitoring hypochlorite residues in water [23,24]. Colorimetric methods that utilize color changes to quantitatively assess residual hypochlorite against a visual comparator standards or by usage of a colorimeter make themselves very adept in facilitating hypochlorite detection in real water samples but not *in vivo*. Fluorometric methods can offset

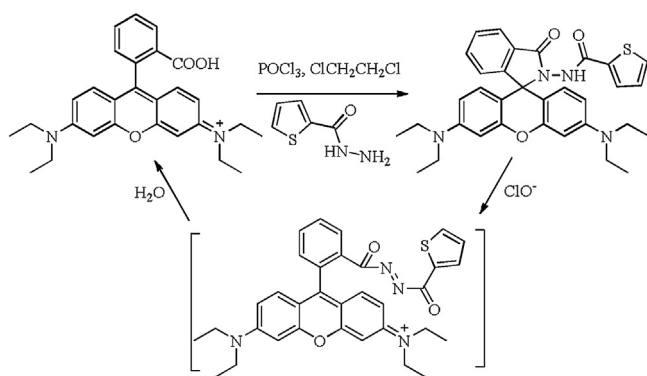
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<https://doi.org/10.1016/j.ccl.2018.01.054>

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Scheme 1. Synthetic route of Rh-C10.

this imperfection in monitoring hypochlorite produced from intracellular micro-environment of flora and fauna. Developing colorimetric and fluorometric probes for hypochlorite is still highly demanded yet challenging, though a few probes for specific detection of hypochlorite have been reported [25–29]. Recently, rhodamine dyes have been the preferred chromophore and fluorophore in the design of colorimetric and fluorometric probes, owing to its brilliant red color, high quantum yields and excellent photophysical properties [30–33]. However, most of the reported rhodamine-based probes for ClO^- displayed either modest selectivity or delayed response time. Thus, it is still urgently needed to develop rapidly responsive, highly selective and sensitive fluorescent probes for ClO^- , which can be used in both colorimetric and fluorometric tests.

Herein, a rapidly responsive, selective and sensitive fluorescent probe for hypochlorite based on rhodamine B fluorophore has been developed and utilized to detect hypochlorite in real sample and living cells. A two-step-in-one-pot synthesis of Rh-C10 was very concise and highly efficient. Reaction of rhodamine B with POCl_3 followed by thiophene-2-carbohydrazide afforded Rh-C10 with 62% yield (Scheme 1). It is reported that hydrazide can be oxidized by hypochlorite and hydrolyze into carboxylic acid in aqueous solution [34]. Recently, rhodamine dyes have been widely employed to design ‘turn-on’ fluorescent probes for various analytes [35–40], owing to their outstanding ring-opening transformations from colorless and non-fluorescent spirolactam forms to rhodamine fluorophores with intensive absorptions and emissions in the visible spectrum range

[30,31]. Thereupon, thiophene-2-carbohydrazide has been introduced into rhodamine B fluorophore through the spirolactam connection pattern to develop color and fluorometric probe Rh-C10 for hypochlorite.

Anion selectivity studies were first performed in Tris-HCl buffer solution ($\text{C}_2\text{H}_5\text{OH}/\text{H}_2\text{O}=4/6$ (v/v), pH 7.4). The presence of 100 equiv. anions and reactive species such as ONOO^- , Cl^- , HPO_4^{2-} , H_2PO_4^- , SO_4^{2-} , NO_3^- , CO_3^{2-} , S^{2-} , $\text{S}_2\text{O}_3^{2-}$, H_2O_2 , NO and $\cdot\text{OH}$, did not cause any color changes of solution, which meant that the ring-opening reaction of spirolactam was not brought about in presence of these species, as show in Figs. 1a and b. Upon addition of 100 equiv. ClO^- , the solution color changed from colorless to pink and was immediately visible to the naked-eyes, and a red fluorescence was observed under excited at UV 365 nm light. Meanwhile, a new absorption peak at 556 nm and fluorescent peak at 578 nm appeared, which indicated that hypochlorite could induce a ring-opening reaction of rhodamine moiety, and the product rhodamine B had already generated, which was verified by HRMS (Fig. S1 in Supporting information). Furthermore, in Tris-HCl buffer solution ($\text{C}_2\text{H}_5\text{OH}/\text{buffer}=4/6$ (v/v), pH 7.4), the presence of 100 equiv. various metal ions, such as K^+ , Ca^{2+} , Na^+ , Mg^{2+} , Fe^{2+} , Fe^{3+} , Cu^{2+} , Zn^{2+} , Al^{3+} , Cd^{2+} , did not cause any observable changes of absorption and emission spectra (Fig. S2 in Supporting information). The effect of competitive anions was also studied by adding hypochlorite to Rh-C10 solution in the presence of anions and reactive species, such as ONOO^- , Cl^- , HPO_4^{2-} , H_2PO_4^- , SO_4^{2-} , NO_3^- , CO_3^{2-} , S^{2-} , $\text{S}_2\text{O}_3^{2-}$, H_2O_2 , NO , $\cdot\text{OH}$, which were prepared and calibrated according to the methods reported in supplementary. The presence of anions and reactive species did not affect absorption and emission spectral responses between Rh-C10 and ClO^- (Fig. S3 in Supporting information). The good selectivity and anti-interference for ClO^- indicated that Rh-C10 would be a potential tool as both colorimetric and fluorometric probe for ClO^- test in practice.

In order to further assess pH effect, the absorption and emission spectral changes of Rh-C10 for pH were studied (Fig. S4 in Supporting information). When the pH value was above 6.0, there was no absorption peak in $\text{C}_2\text{H}_5\text{OH}-\text{H}_2\text{O}$ (4/6, v/v) solution of Rh-C10, indicating that Rh-C10 was suitable for usage at neutral and basic conditions. With the decrease of pH value, a new absorption peak at 556 nm was gradually enhanced, indicating that spirolactam of Rh-C10 underwent a ring-opening reaction in the protonation of Rh-C10. Meanwhile, Rh-C10 showed 300-folds

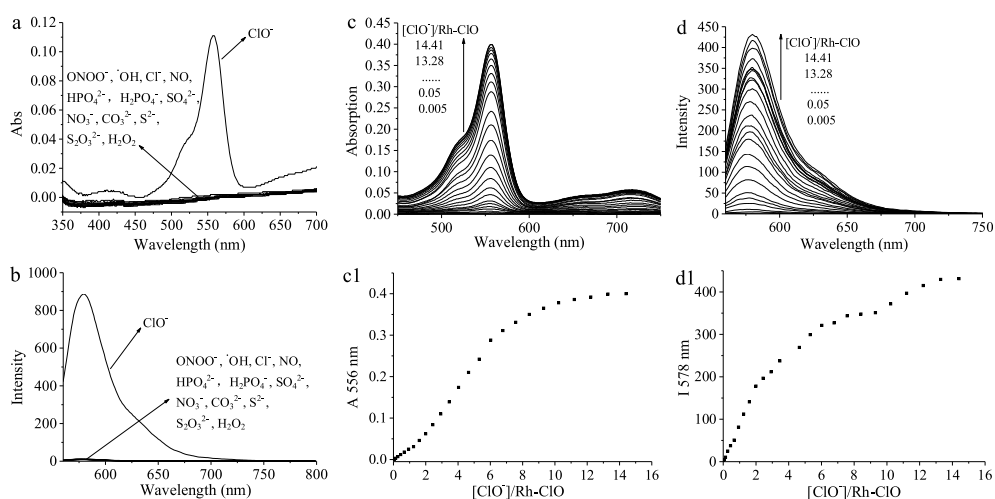


Fig. 1. Absorption (a) and emission (b) spectra of Rh-C10 in the presence of different anions. Changes in the absorption (c) and emission spectra (d) of Rh-C10 (20 $\mu\text{mol/L}$) in $\text{C}_2\text{H}_5\text{OH}/\text{H}_2\text{O}$ (4/6, v/v) at pH 7.4 (0.1 mmol/L Tris-HCl buffer) with increasing of NaOCl solution from 0 to 300 $\mu\text{mol/L}$. (e, f) The response of absorption intensity (556 nm) and emission intensity (578 nm) in the concentration of NaOCl from 0 to 300 $\mu\text{mol/L}$.

enhancement of fluorescent intensity with attenuation of pH values from 6.0 to 3.5. The pKa value of probe Rh-C1O was 4.76 (± 0.02), calculated from fluorescence titration data. Upon addition of 100 equiv. ClO⁻ into Rh-C1O solution for 3 min, there were not clear changes in absorption spectra of Rh-C1O over a wide range of pH value from 3.5 to 13.0. By contrast, the emission spectra of Rh-C1O remained unchanged in pH range from 6.0 to 12.5. When the pH further decreased (3.5–6.0), the emission intensity at 578 nm reduced slowly, which was consistent with that performance of rhodamine B in the same pH range.

Hypochlorite titration experiments were carried out in the solution of ethanol and Tris-HCl buffer (C₂H₅OH/H₂O = 4/6 (v/v), pH 7.4). With increasing of NaClO concentration, the solution of Rh-C1O exhibited intensive magenta color and a new absorption peak at 556 nm increases gradually (Fig. 1c). Meanwhile, the emission peak at 578 nm gradually enhanced (Fig. 1d). The absorption intensity at 556 nm was linear function with NaClO concentration over the range of 0–120 $\mu\text{mol/L}$ (Fig. c1). By contrast, in the range of NaClO concentration from 0 to 40 $\mu\text{mol/L}$, there was a linear enhancement of emission intensity at 578 nm (Fig. d1). The response mechanism involved the oxidation reaction between the thiophene-2-carbohydrazide moiety of Rh-C1O and hypochlorite at neutral pH condition (Scheme 1). ClO⁻ could oxidate acylhydrazine group and induce a ring-opening process in rhodamine moiety of Rh-C1O. Then hydrolyzed product rhodamine B was subjected to ESI-MS analysis in which the peak at *m/z* 443.2329 was found (Fig. S1).

The detection limit (DL) was calculated from the absorption and fluorescence titration data (Fig. S5 in Supporting information). Over the ClO⁻ range from 40 $\mu\text{mol/L}$ to 220 $\mu\text{mol/L}$, a good linear regression curve between hypochlorite concentration and the function of normalized maximum and minimum absorption was fitted ($R=0.993$), and the point at which this line crossed the ordinate axis was taken as the detection limit and equal approximately 3×10^{-5} mol/L (DL_{abs}). According to fluorescent titration of Rh-C1O, there was also a good linear regression curve in the range of ClO⁻ from 9 $\mu\text{mol/L}$ to 90 $\mu\text{mol/L}$ ($R=0.991$). The detection limit of Rh-C1O was determined to be 7×10^{-6} mol/L (DL_f). Compared with DL_{abs}, the detection limit of fluorescence titration (DL_f) was much lower, which indicated that fluorescent detection would be more highly sensitive. The reaction time between Rh-C1O and ClO⁻ was investigated in spectrofluorophotometer (Fig. S6 in Supporting information). Time-dependent intensity at 578 nm of Rh-C1O was recorded in absence and presence of ClO⁻. In the absence of NaClO, Rh-C1O showed a negligible background fluorescence that was stable under the assay conditions. Upon addition of 30 $\mu\text{mol/L}$ NaClO, the intensity of Rh-C1O at 578 nm achieved the maximum within 5 s, and then decreased slightly. The fluorescent intensity leveled off after 8 s of adding NaClO. With further addition of NaClO (0.05 mmol/L, 0.1 mmol/L, 0.15 mmol/L, respectively), the intensity of Rh-C1O leveled off within 8 s. Rh-C1O exhibited a fast response with NaClO,

which should be ascribed to the electron-withdrawing characteristic of thiophene-2-carbohydrazide moiety in Rh-C1O. The electron-withdrawing characteristic of substituent could lead to an accelerating effect on the HOCl-mediated oxidation-hydrolysis of hydrazide in aqueous solution [41].

In order to investigate the practical application of Rh-C1O for the detection of hypochlorite anion, aqueous solutions of Rh-C1O (2.0×10^{-5} mol/L) and filter paper strips test were performed. Rh-C1O in ethanol was added into vials of different anions in aqueous solution, respectively. When the color of vial changed from colorless to pink, it indicated that there was hypochlorite anion in the second vial (Fig. 2a). To ease usage of probe Rh-C1O, test-paper has been prepared and used to detection ClO⁻ in real sample. After test strips were immersed in ClO⁻ solution with various concentrations for 10 s, respectively, it could be found there was an obvious color deepening with the increasing concentration of hypochlorite solutions (0, 0.02, 0.06, 0.2 mol/L) (Fig. 2b). These results indicated that Rh-C1O could be a handy tool to be used as a colorimetric probe in the fast detection of hypochlorite in aqueous solution.

As above mentioned, hypochlorite anion produced in inflammatory stress plays a vital role in defending from the invasion of pathogens. To further examine the possibility of application in living cells, fluorescent images of HeLa cells stained with Rh-C1O were obtained by a laser-scanning confocal microscopy. Upon excitation at 559 nm, dim red intracellular fluorescence was displayed in red channel (red channel BF: 565–665 nm) (Fig. 3a). Stimulated by addition of sodium hypochlorite (50 $\mu\text{mol/L}$) for 5 min, stronger fluorescence was observed and distributed in subcellular locations of cells (Fig. 3b). In order to further validate organelle-targeting of Rh-C1O in HeLa cells, co-localization experiments were studied by HeLa cells co-stained with Rh-C1O and Rh 123, a mitochondrial tracker. After stained with 5 $\mu\text{mol/L}$ Rh-C1O and 2 $\mu\text{mol/L}$ Rh123, HeLa cells respectively illustrated green and red fluorescence in channels 1 and 2 (Fig. 3c, ch1 and ch2). The well merged image of ch2 and ch1 suggested that Rh-C1O could specifically accumulate in mitochondria of HeLa cells. The Pearson's coefficient and Mander's overlap coefficient were up to 0.82 and 0.91, respectively. Moreover, a high correlation-ship demonstrated in intensity scatter plot of ch1 and ch2 indicated Rh-C1O existed predominantly in mitochondria of HeLa cells (Fig. 3d). Selected a red rectangular section on intensity scatter plot allowed us to highlight corresponding white pixels on simulate images in accordance with the overlay image (Fig. 3e). These results demonstrated that Rh-C1O could potentially be used as a nondestructive indicator for hypochlorite monitoring in toxicological experiments.

In summary, Rh-C1O, as a colorimetric and fluorometric probe, has been easily obtained from the reaction of rhodamine B with thiophene-2-carbohydrazide. Probe Rh-C1O displayed a high selectivity and sensitivity to hypochlorite anion in aqueous solution. The detection limit of Rh-C1O was 7 $\mu\text{mol/L}$, fitted by

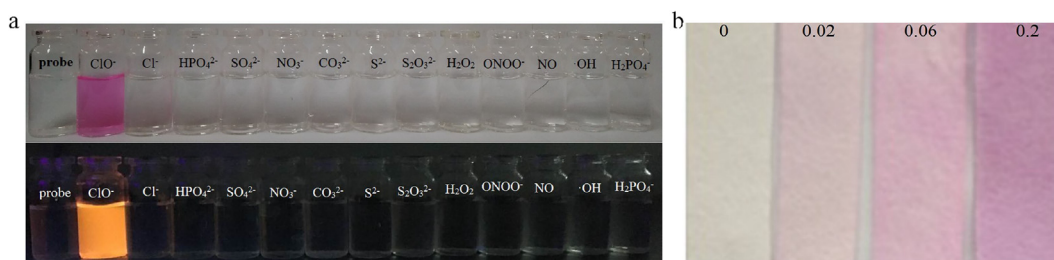


Fig. 2. (a) Color detection of absorption and emission on hypochlorite among different anions in aqueous solution (0.05 mol/L, C₂H₅OH/H₂O (4/6, v/v)). (b) Test papers soaked with Rh-C1O was immersed in 0, 0.02, 0.06 and 0.2 mol/L NaClO in distilled water, respectively.

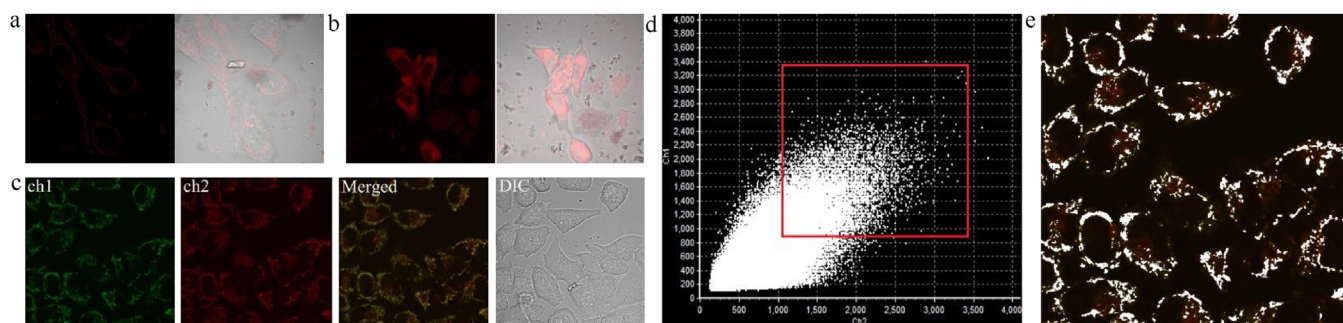


Fig. 3. Fluorescent image of HeLa cells stained with Rh-CIO (5.0 $\mu\text{mol/L}$) in absence (a) and presence (b) of sodium hypochlorite. (c) Rh-CIO co-localizes to mitochondria in HeLa cells. HeLa was stained with Rh 123 (2.0 $\mu\text{mol/L}$, Channel 1 (ch1): λ_{ex} 488 nm, λ_{em} 500–540 nm) and Rh-CIO (5.0 $\mu\text{mol/L}$, Channel 2 (ch2): λ_{ex} 559 nm, λ_{em} 565–665 nm) with sodium hypochlorite solution (50 $\mu\text{mol/L}$) for 5 min at 37 °C. Merged is overlay image of ch1 and ch2. DIC is differential interference contrast image. (d) Intensity scatter plot of ch1 and ch2. (e) Simulate image, the white pixels represent by the scatter plot is highlighted by selecting the points with a red rectangular selection.

fluorescent titration. Response time of Rh-CIO with hypochlorite anion was as short as ten seconds. Moreover, probe Rh-CIO and paper test were successfully used to determine ClO^- in real water samples. Finally, co-localization experiments of HeLa cells further confirmed that Rh-CIO could detect exogenous ClO^- in mitochondria of living cells. These results suggested that the new probe would lead to the opportunities not only for determining ClO^- in water and biological samples, but also for the evaluation of oxidation stress induced by contaminants.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 21302080) and Program Funded by Liaoning Province Education Administration (No. L2014010).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.ccllet.2018.01.054>.

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