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Fluorescent Detection of Hypochlorous Acid from Turn-On to FRET-Based Ratiometry by a HOCl-Mediated Cyclization Reaction

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Abstract: Hypochlorous acid (HOCl), a reactive oxygen species (ROS), plays a significant biological role in living systems. However, abnormal levels of HOCl are implicated in many inflammation-associated diseases. Therefore, the detection of HOCl is of great importance. In this work, we describe the HOCl-promoted cyclization of rhodamine-thiosemicarbazides to rhodamine-oxadiazoles, which is then exploited as a novel design strategy for the development of a new fluorescence turn-on HOCl probe **2**. On the basis of the fluorescence resonance energy transfer (FRET) signaling mechanism, **2** was further converted into **1a** and **1b**, which represent the first paradigm of FRET-based ratiometric fluorescent HOCl probes. The outstanding features of **1a** and **1b** include well-resolved emission peaks, high sensitivity, high selectivity, good functionality at physiological pH, rapid response, low cytotoxicity, and good cell-membrane permeability. Furthermore, these excellent attributes enable us to demonstrate, for the first time, the ratiometric imaging of endogenously produced HOCl in living cells by using these novel ratiometric probes. We expect that **1a** and **1b** will be useful molecular tools for studies of HOCl biology. In addition, the HOCl-promoted cyclization reaction of rhodamine-thiosemicarbazides to rhodamine-oxadiazoles should be widely applicable for the development of different types of fluorescent HOCl probes.

Keywords: fluorescent probes · FRET · oxadiazole · ratiometry · sensors

Introduction

Reactive oxygen species (ROS) mediate a wide variety of biological processes.[1] Hypochlorous acid (HOCl), a biologically significant ROS, is produced by the peroxidation of chloride ions catalyzed by the enzyme myeloperoxidase (MPO) in activated leukocytes.[2] HOCl is also a critical microbicidal agent in natural defense.[3] However, abnormal levels of HOCl are implicated in many inflammation-related diseases, including cardiovascular diseases,[4] damage of human red blood cells,[5] lung injury,[6] rheumatoid arthritis,[7] and cancer,[8] so the detection of HOCl is of great importance. A number of methods such as electroanalysis,[9] potentiometry,[10] spectrophotometry,[11] and chemiluminescence[12] for the detection of HOCl have been developed. However, fluorescence sensing is advantageous because of its high sensitivity and selectivity.[13] In addition, in conjunction with microscopy, it is very useful for bio-imaging applications in living cells. Therefore, the construction of small-molecule fluorescent probes for the specific detection of HOCl has received intense attention.[14] However, most of the known fluorescent probes respond to HOCl with changes only in fluorescent intensity. A major limitation of intensity-based probes is that fluorescence measurement is affected by variations in the sample environment and probe distribution. By contrast, ratiometric fluorescent probes allow the measurement of emission intensities at two different wavelengths; this should provide a built-in correction for environmental effects and can also increase the dynamic range of fluorescence measurement.[15] Our group previously reported a ratiometric fluorescent OCl^- probe operating through an intramolecular charge transfer (ICT) signaling mechanism.[16] The ratiometric fluorescent OCl^- probe was designed on the basis of the specific hypochlorite-mediated transformation of oximes to aldehydes. Unfortunately, this oxime-based ratiometric OCl^- probe only functions at a high pH value (pH 9.0), so it is not suitable for applications in living cells. To our best knowledge, ratiometric fluorescent probes suitable for ratiometric imaging of HOCl in living cells have not yet been reported. Therefore, there is a need to develop ratiometric fluorescent probes for HOCl with favorable characteristics for imaging applications by a new design strategy, which should be compatible with biological conditions. Since the pioneering work of Czarnik's group regarding a rhodamine-based Cu^{2+} fluorescent chemodosimeter,[17] rhodamine derivatives have been widely employed as an effective platform for the construction of small-molecule fluorescence "turn-on" probes for diverse species such as Cu^{2+}, Hg^{2+}, Fe^{3+}, Cr^{3+}, Pb^{2+}, Zn^{2+}, Ag^+, Au^{3+}, Pd^{2+}, Pt^{2+}, HOCl, and NO, through exploitation of the unique spirolactam ring-opening process of rho-

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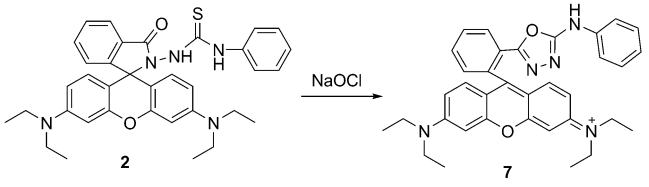
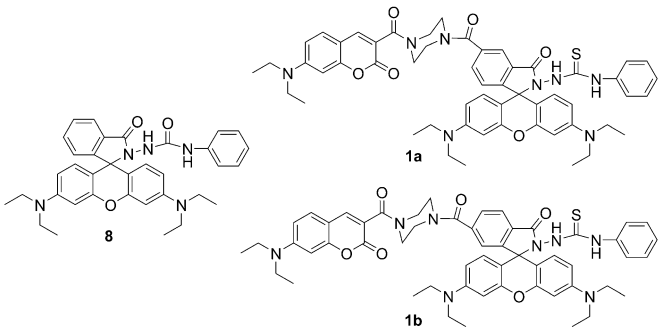
damines.[18] However, the responses of most of these rhodamine-based probes to an analyte of interest with an optical signal change only in their fluorescence intensity. Fluorescence resonance energy transfer (FRET) is a common signaling mechanism used in the design of dual-emission ratiometric fluorescent probes,[19] in which the excitation energy of the energy donor is transferred to the energy acceptor. The aim of this work was to develop FRET-based ratiometric fluorescent probes for the ratiometric imaging of HOCl in living cells. In the preliminary work, we found that the reaction of rhodamine-thiosemicarbazide **2** with NaOCl at room temperature could result in the formation of rhodamine-1,3,4-oxadiazole **7** (Scheme 1). The observation that the conversion of thiosemicarbazides into oxadiazoles can be mediated by NaOCl under very mild conditions suggests that this reaction is promising as a new platform for the development of fluorescent HOCl probes. In this contribution, we initially constructed a new fluorescence turn-on HOCl probe **2** (Scheme 1) by a novel design approach, that is, the HOCl-mediated cyclization of rhodamine-thiosemicarbazides to rhodamine-oxadiazoles. To fulfill the aim of this work, we decided to further translate the fluorescence turn-on probe **2** into novel ratiometric fluorescent HOCl probes based on a FRET signaling mechanism. The coumarin dye was selected as the energy donor to render a large emission shift between the coumarin donor and the rhodamine acceptor (Figure S1 in the Supporting Information). Thus, the 4- and 5-position regioisomers of compounds **1a** and **1b** (Scheme 2) were designed as new candidates for FRET-based ratiometric fluorescent HOCl probes. Herein, we describe the design, synthesis, spectral properties, and fluorescence imaging studies for these ratiometric fluorescent HOCl probes.



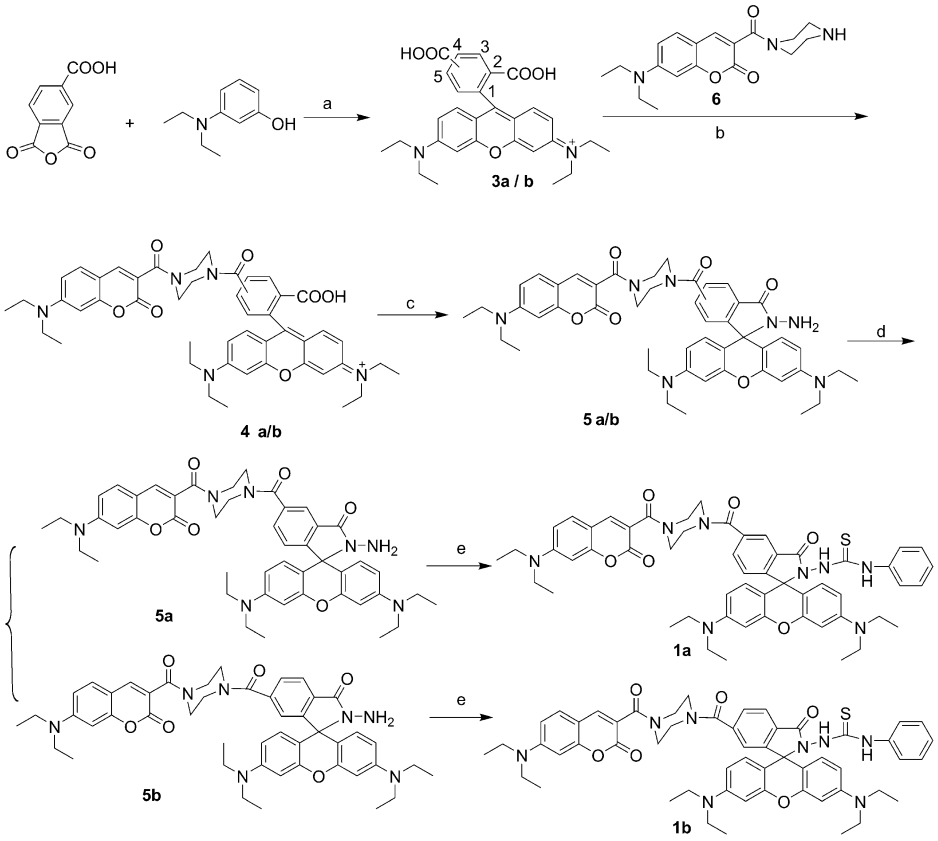
Scheme 1. Reaction of **2** with NaOCl.



Scheme 2. Structures of the new ratiometric fluorescent HOCl probes **1a** and **1b** and the control compound **8**.

Results and Discussion

Synthesis: The synthesis of the ratiometric probes **1a** and **1b** started with the preparation of the intermediate **3a/b** (Scheme 3). Condensation of 3-(diethylamino)phenol **1** with commercially available 1, 2, 4-benzenetricarboxylic anhydride **2** led to the formation of the mixture of 4′- and 5′-car-



Scheme 3. Synthesis of 4- and 5-position regioisomers of ratiometric probes **1a** and **1b**. Reagents and Conditions: a) 85% H_3PO_4, 170–180 °C; b) DCC, DMAP, CH_2Cl_2; c) $NH_2NH_2{\cdot}H_2O$, DCC, DMAP, CH_2Cl_2; d) separation by column chromatography (C_2H_5OH:CH_2Cl_2 = 1:200 to 1:20); e) phenylisothiocyanate, DMF.

boxyrhodamines **3a/b**. Condensation of **3a/b** with coumarin amine **6** by standard coupling chemistry gave the FRET dyads **4a/b**. Notably, the coupling occurred almost exclusively at the 4- or 5-position carboxylic acid. The high regioselectivity can be attributed to the lower steric hindrance of the 4- or 5-position carboxylic acid compared to the 2-position one. Reaction of **4a/b** with hydrazine hydrate gave compounds **5a/b**. Separation of the mixture of compounds **5a/b** could be readily achieved by standard column chromatography to give the individual compounds **5a** and **5b** in pure isomeric form. Alternatively, the pure isomeric forms **4a** and **4b** can be obtained by preparative thin-layer chromatographic separation (CH_2Cl_2:CH_3OH = 9:1) from the mixture of **4a/b** (Scheme S1 in the Supporting Information) or from the pure isomeric forms **3a** and **3b** (Scheme S2 in the Supporting Information), respectively. The pure isomeric forms **5a** and **5b** can also be obtained from the pure isomeric forms **4a** and **4b** (Scheme S2 in the Supporting Information). With the pure intermediates **5a** and **5b** in hand, probes **1a** and **1b** were readily synthesized by reaction of **5a** and **5b**, respectively, with phenylisothiocyanate (Scheme 3). The control compound **8** (Scheme 2) was previously synthesized.[18i] The synthesis of the intermediate **6** and energy donor **10** are shown in Scheme S3 of the Supporting Information. The energy acceptors **9a** and **b** were obtained according to Scheme S4 of the Supporting Information. Compound **2** was prepared in one step by the reaction of rhodamine B hydrazide **13** with phenylisothiocyanate (Scheme S5, Supporting Information). The compounds **2**, **1a**, and **1b** were fully characterized by ^{1}H NMR and ^{13}C NMR spectroscopy and HRMS.

Photophysical properties and sensing responses of probe 2: As shown in Figures 1A and B, the free compound **2** displayed almost no considerable absorption or emission bands above 500 nm ($\Phi < 0.001$), indicating that it exists as the ring-closed form. However, significantly, treatment of NaOCl with compound **2** induced the formation of a strong visible absorption band centered at 569 nm and an intense emission band ($\Phi_f = 0.29$) at around 590 nm (Figure 1A, B), suggesting that HOCl promotes the transformation of compound **2** from the ring-closed form to the ring-opened form. Furthermore, **2** displayed a high selectivity for HOCl over other biologically relevant species represented by hydrogen peroxide, hydroxyl radicals, superoxide, CH_3COOOH, Fe^{3+}, Cu^{2+}, Co^{2+}, Zn^{2+}, NO_2^-, NO_3^-, HCO_3^-, Val, cytosine, thymine, adenine, glucose, GSH, and vitamin C (Figure 1C). Taken together, these results indicate that compound **2** is a new fluorescence turn-on probe for HOCl. As the aim of this work was to construct new fluorescent HOCl probes, we decided to investigate the signaling mechanism of probe **2** for sensing HOCl. Obviously, the identity of the fluorescent product arising from treatment of probe **2** with HOCl is highly valuable for the fluorescence sensing mechanism. Thus, rhodamine-thiosemicarbazide **2** was reacted with NaOCl at room temperature, and the reaction product was isolated and purified through a silica gel column (CH_2Cl_2/C_2H_5OH = 10:1) to afford a dark purple solid, which was unambiguously identified to be rhodamine-1,3,4-oxadiazole **7** (Scheme 1) by studies of the ^{1}H NMR, ^{13}C NMR, ESI-MS, HRMS, and absorption and emission spectra (Figures S2–S4 in the Supporting Information). To gain some insight into the reaction mechanism, control compound **8**, which contains a semicarbazide instead of the thiosemicarbazide group, was treated with NaOCl at room temperature. However, no reaction was observed (Scheme S6, Supporting Information). Consistently, control compound **8** exhibited almost no fluorescence response toward NaOCl (Figure S5, Supporting Information). These findings indicate that the sulfur atom plays a significant role in the reaction of probe **2** with NaOCl. On the other hand, although it is known that iodine (or other oxidants) can transfer thiosemicarbazides to 1,3,4-oxadiazoles through iodine- (or other oxidant)-mediated oxidation in the presence of a base (under basic conditions) and the reaction is widely used in organic synthesis,[20] it is noteworthy that the reaction mechanism is still unclear. In addition, it has been reported that thiones can be oxi-

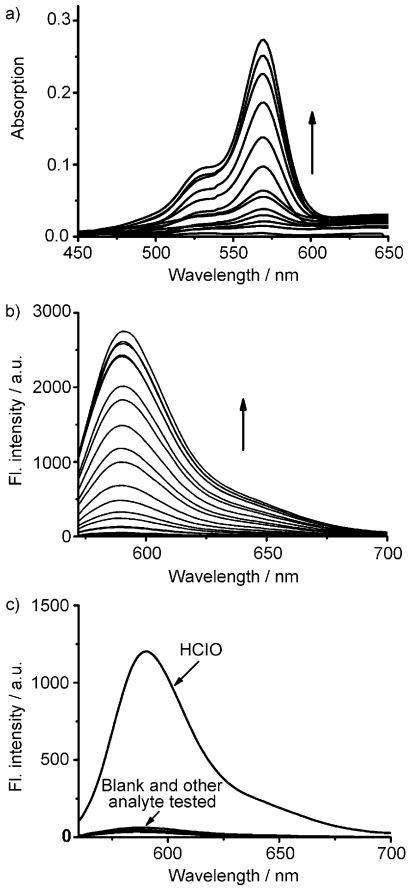


Figure 1. Absorption (a) and fluorescence (b) titration spectra of **2** (5 μM) in PBS/DMF (pH 7.4, 1:1) upon addition of increasing concentrations of NaOCl (0–30 equiv). c) Fluorescence spectra of **2** (5 μM) in the presence of various biologically relevant analytes (7 equiv) including hydrogen peroxide, hydroxyl radicals, superoxide, HOCl, CH_3COOOH, Fe^{3+}, Cu^{2+}, Co^{2+}, Zn^{2+}, NO_2^-, NO_3^-, HCO_3^-, Val, cytosine, thymine, adenine, glucose, GSH, vitamin C.

dized to sulfonic acid,[21] and NaOCl can transfer N^1-acyl-N^3-cyanoguanidines to 1,3,4-oxadiazoles through a carbodiimide intermediate.[22] On the basis of these literature reports and our findings from the studies of the control compound **8**, a possible reaction mechanism is proposed as shown in Scheme S7 in the Supporting Information. Although the detailed reaction mechanism of the HOCl-promoted transformation of thiosemicarbazides to oxadiazoles remains to be investigated in the future, the turn-on sensing mechanism of rhodamine-thiosemicarbazide **2** is evident. The free rhodamine-thiosemicarbazide **2** is non-fluorescent as it exists as the ring-closed form of the rhodamine. However, upon treatment with HOCl, rhodamine-thiosemicarbazide is converted into rhodamine-1,3,4-oxadiazole **7**, which is highly fluorescent as it exists as the ring-opened form of the rhodamine. The structure of rhodamine-1,3,4-oxadiazole **7** is well corroborated by the ^{1}H NMR, ^{13}C NMR, ESI-MS, HRMS, and absorption and emission spectra (Figures S2–S4 in the Supporting Information).

Ratiometric responses of probes 1a and 1b to HOCl: As shown in Figure 2 A, upon excitation at 414 nm, the free ratiometric probe **1b** showed the featured emission of the coumarin moiety at 473 nm but no characteristic emission of the rhodamine moiety at 594 nm, indicating that there is no intramolecular FRET in the free probe, as the rhodamine component has the ring-closed spirolactam structure. However, upon addition of NaOCl, the coumarin emission at 473 nm gradually decreased, and a new emission band corresponding to the rhodamine emission at 594 nm appeared and gradually increased in intensity, implying that intramolecular FRET occurred due to the HOCl-induced formation of rhodamine-1,3,4-oxadiazole. Notably, **1b** exhibited an outstanding emission spectral feature with the two emission peaks well resolved (a large separation of 121 nm), which can ensure the highly accurate determination of the emission intensities and ratios. The changes in the absorption spectra (Figure S6, Supporting Information) are in good agreement with the variations in the fluorescence spectra. Consistently, the emission color of the probe showed a dramatic change from blue to orange–red upon addition of NaOCl (Figure S7, Supporting Information). In addition, like probe **1b**, probe **1a** displayed the drastic spectral variations in the presence of HOCl (Figure S8, Supporting Information). The ratiometric probe **1b** showed a good linearity between the ratios (I_{594}/I_{473}) and the NaOCl concentration in the range 1.0×10^{-7} to 1.0×10^{-4} M (Figure 2 B) with a detection limit of 5.2×10^{-8} M (S/N = 3), indicating that probe **1b** is potentially useful for the quantitative determination of HOCl. The free probe is stable over a wide span of pH from 3.0–11.0 (Figure S10 A, Supporting Information). The ratiometric response of the probe to HOCl is pH-dependent, with the maximal ratio signal at pH 6.0. As the pK_a of HOCl is 7.6, we reasoned that the probe senses HOCl instead of OCl^-, in agreement with the previous reports.[14b,c] The time-course studies indicate that the probe has a very rapid ratiometric response to HOCl (Figure S11 A, Supporting Information). Furthermore, the properties of probe **1a** are quite similar to those of **1b** (Figures S9, S10 B, and S11 B, Supporting Information). As shown in Figure 3 and Figure S12 of the Supporting Information, **1b** and **1a** exhibited high selectivity for HOCl over other biologically relevant species represented by hydrogen peroxide, hydroxyl radicals, superoxide, CH_3COOOH, Fe^{3+}, Cu^{2+}, Co^{2+}, Zn^{2+}, NO_2^-, NO_3^-, HCO_3^-, Val, cytosine, thymine, adenine, glucose, GSH, and vitamin C, indicating that these ratiometric fluorescent probes are potentially useful for biological applications.

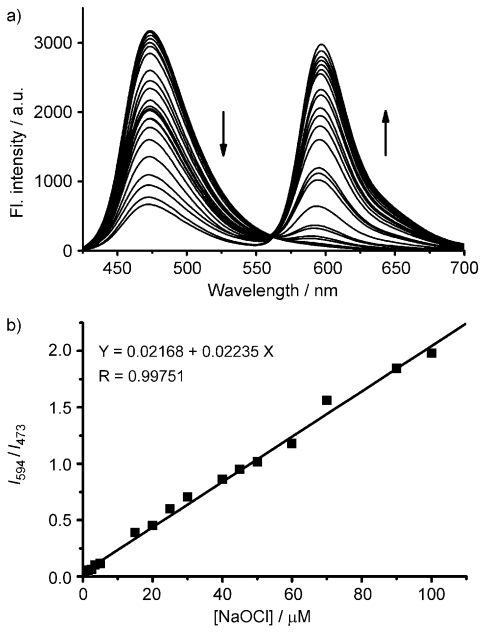


Figure 2. a) Fluorescence titration spectra of **1b** (5 μM) in PBS/DMF (pH 7.4, 1:1) upon gradual addition of NaOCl (0–30 equiv). Excitation at 414 nm. b) The emission ratio (I_{594}/I_{473}) of probe **1b** (5 μM) to various concentrations of NaOCl (1.0×10^{-7}–1.0×10^{-4} M). Excitation was provided at 414 nm, and the ratios of emission intensities at 594 and 473 nm were measured.

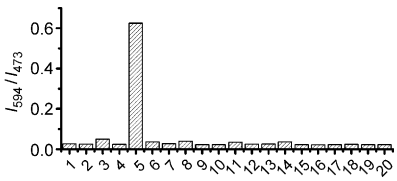


Figure 3. Emission intensity ratio (I_{594}/I_{473}) of **1b** (5 μM) in the presence of various biologically relevant analytes (5 equiv). 1) blank; 2) hydrogen peroxide; 3) hydroxyl radical; 4) superoxide; 5) HOCl; 6) CH_3COOOH; 7) Fe^{3+}; 8) Cu^{2+}; 9) Co^{2+}; 10) Zn^{2+}; 11) NO_2^-; 12) NO_3^-; 13) HCO_3^-; 14) Val; 15) cytosine; 16) thymine; 17) adenine; 18) glucose; 19) GSH; 20) vitamin C. The data were collected from the emission spectra excited at 414 nm.

Living cell imaging studies: In order to be useful as ratiometric imaging agents, ratiometric fluorescent probes should have low cytotoxicity. Thus, we investigated the potential

toxicities of **1a** and **1b** against a representative cell line: RAW264.7 macrophages. The living cells were incubated with various concentrations (5–200 μM) of the new ratiometric probes for 24 h, and then the cell viability was determined by the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays;[23] the results indicated that the ratiometric probes do not exhibit marked cytotoxicity at concentrations below 100 μM (Figure S13, Supporting Information).

To demonstrate the capability of these novel probes for HOCl ratiometric fluorescence imaging in living cells, **1a** and **1b** were incubated with three different types of cell lines: living RAW 264.7 macrophages, Bel 7702 cells, and HeLa cells. As exhibited in Figure S14 of the Supporting Information, the living RAW 264.7 macrophages incubated with **1a** (5 μM) for 0.5 h at 37 °C provided a strong blue fluorescence in the coumarin emission window (Figure S14B, Supporting Information), but almost no red fluorescence in the rhodamine emission window (Figure S14C, Supporting Information). By contrast, the living RAW 264.7 macrophages treated with **1a** and then with NaOCl gave a marked decrease in the blue emission (Figure S14E, Supporting Information) and a significant increase in the red emission (Figure S14F, Supporting Information), consistent with the HOCl-induced ratiometric fluorescent response. A similar ratiometric response was observed when **1b** was incubated with live HeLa cells in the presence of NaOCl (Figure S15, Supporting Information). Furthermore, Bel 7702 cells incubated with only **1b** (5 μM) for 30 min gave an average emission ratio value ($I_{605\pm75}/I_{450\pm35}$) of 0.09 (Figures 4A and C). By contrast, treatment of **1b**-loaded cells with NaOCl (10 μM) for 30 min elicited a significant increase in the emission ratio ($I_{605\pm75}/I_{450\pm35}$) to about 0.59 (Figures 4B and C), in good agreement with the HOCl-mediated transformation of thiosemicarbazides to oxadiazoles. Thus, these data establish that **1a** and **1b** are cell-membrane permeable and suitable for the ratiometric imaging of HOCl in the living cells. The promising results of the above ratiometric imaging of externally added HOCl in living cells encourage us to evaluate the feasibility of **1a** further for the detection of endogenously produced HOCl. When stimulated by lipopolysaccharide (LPS) and 12-myristate-13-acetate (PMA), macrophages may produce endogenous HOCl.[24,25] The RAW264.7 macrophage cells loaded with only **1a** (5 μM) displayed a strong blue emission (Figure 5B) and a slight red emission (Figure 5C). However, after the macrophage cells were incubated with LPS (1 μg mL^{-1}) for 12 h, and then further co-incubated with PMA (1 μg mL^{-1}) and **1a** (5 μM) for 60 min, a marked decrease in the blue emission (Figure 5E) and a dramatic enhancement in the red emission (Figure 5F) were observed. These data indicate that **1a** is capable of the ratiometric fluorescent imaging of endogenously produced HOCl. To the best of our knowledge, this represents the first report of the ratiometric fluorescent imaging of endogenously produced HOCl in macrophage cells.

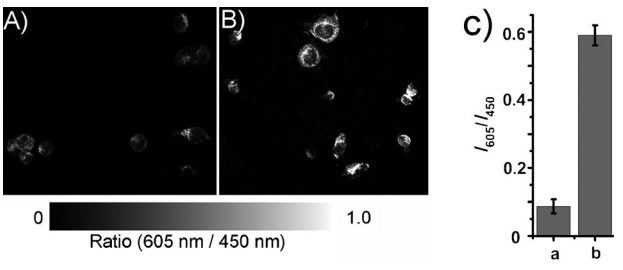


Figure 4. Confocal ratio images ($I_{605\pm75}/I_{450\pm35}$) of HOCl in Bel 7702 cells with $\lambda_{ex}=408$ nm. A) ratio image ($I_{605\pm75}/I_{450\pm35}$) of the cells incubated with **1b** (5 μM) only; B) ratio image ($I_{605\pm75}/I_{450\pm35}$) of **1b**-loaded cells after treatment with NaOCl (10 μM). C) Average ratios ($I_{605\pm75}/I_{450\pm35}$) of the cells incubated with **1b** (5 μM) only (a) and **1b**-loaded cells after treatment with 10 μM NaOCl (b). For a color version, see Figure S17 in the Supporting Information.

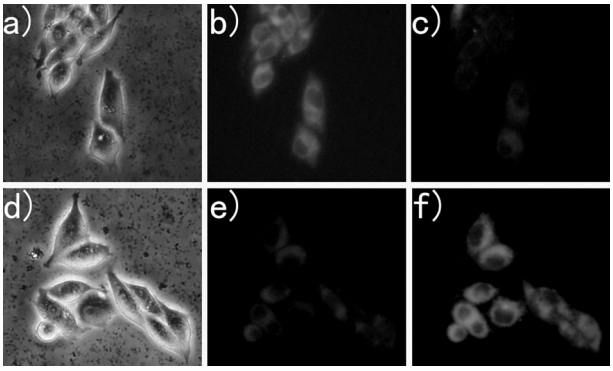


Figure 5. Images of endogenously produced HOCl in living RAW 264.7 macrophages treated with probe **1a**. a) DIC image of the RAW 264.7 macrophages incubated with only **1a** (5 μM) for 60 min; b) fluorescence image of (a) from blue channel; c) fluorescence image of (a) from red channel; d) fluorescence image of RAW 264.7 macrophages treated with LPS (1 μg mL^{-1}) for 12 h, and then further co-incubated with PMA (1 μg mL^{-1}) and **1a** (5 μM) for 60 min; e) fluorescence image of (d) from blue channel; f) fluorescence image of (d) from red channel. For a color version, see Figure S18 in the Supporting Information.

Conclusion

In summary, we have described the HOCl-promoted cyclization of rhodamine-thiosemicarbazides to rhodamine-oxadiazoles, which was then exploited as a novel design strategy for the development of a new fluorescence turn-on HOCl probe, **2**. On the basis of the FRET signaling mechanism, **2** was further transformed into **1a** and **1b**, which, to our best knowledge, represent the first paradigm of FRET-based ratiometric HOCl probes. The outstanding features of **1a** and **1b** include the well-resolved emission peaks, high sensitivity, high selectivity, good functionality at physiological pH, rapid response, low cytotoxicity, and good cell-membrane permeability. Furthermore, these excellent attributes enable us to demonstrate, for the first time, the ratiometric imaging of endogenously produced HOCl in living cells by using these novel ratiometric probes. We expect that **1a** and **1b**

will be useful molecular tools for studies of HOCl biology. In addition, the HOCl-promoted cyclization of rhodamine-thiosemicarbazides to rhodamine-oxadiazoles should be widely applicable for the development of a variety of fluorescent HOCl probes.

Experimental Section

Synthesis of compound 7: A solution of NaOCl (18.8 mg, 0.25 mmol) in H_2O (1 mL) was added slowly to a solution of **2** (30.0 mg, 0.05 mmol) in C_2H_5OH/H_2O (9:1, 40 mL). Subsequently, the reaction mixture was stirred at room temperature for 2 h, and then the solvents were removed under reduced pressure. The resulting residue was purified on a silica gel column ($CH_2Cl_2/C_2H_5OH = 10:1$) to afford compound **7** as a dark purple solid (14.1 mg, isolated yield: 50.6%). M.p. 166–168 °C; ^{1}H NMR (400 Hz, $CDCl_3$): $\delta = 1.30$ (t, 12H), 3.53–3.58 (q, 8H), 6.72 (d, $J = 2.0$ Hz, 2H), 6.76–6.78 (d, $J = 9.6$ Hz, 2H), 6.85–6.89 (t, 1H), 7.11–7.13 (d, $J = 9.2$ Hz, 2H), 7.14–7.18 (t, 2H), 7.24–7.26 (d, $J = 7.6$ Hz, 1H), 7.60–7.64 (t, 1H), 7.67–7.70 (d, $J = 8.0$ Hz, 3H), 8.33–8.35 (d, $J = 8.0$ Hz, 1H), 10.50 ppm (bs, 1H); ^{13}C NMR (100 Hz, $CDCl_3$): $\delta = 12.64$, 46.04, 96.45, 113.92, 114.15, 117.97, 121.80, 123.62, 128.63, 129.16, 129.89, 129.99, 130.22, 130.75, 131.50, 138.62, 155.51, 157.76, 158.11 ppm; ESI-MS *m/z* 558.2 $[M]^+$; HRMS (EI) *m/z* calcd for $C_{35}H_{36}O_2N_5$ ($[M]$): 558.2864; found: 558.2844; *m/z* calcd for $C_{35}H_{35}O_2N_5$ ($[M-H]$): 557.2785; found: 557.2784.

Synthesis of 1a: Phenylisothiocyanate (12.7 mg, 0.09 mmol) was added dropwise to a solution of compound **5a** (50.4 mg, 0.06 mmol) in DMF (1 mL) with stirring. Subsequently, the reaction mixture was heated to 50 °C and stirred at that temperature for 12 h. After being cooled to room temperature, the solvent was removed under reduced pressure. The resulting residue was purified on a silica gel column ($CH_2Cl_2/C_2H_5OH = 50:1$) to give compound **1a** as a yellow powder (45.3 mg, yield: 77.6%). M.p. 220–224 °C; ^{1}H NMR (400 Hz, $CDCl_3$): $\delta = 1.12$–1.16 (t, 12H), 1.20–1.23 (t, 6H), 3.31–3.90 (20H), 6.30 (s, 2H), 6.46–6.51 (s, 5H), 6.60–6.62 (d, $J = 8.4$ Hz, 1H), 7.02–7.04 (d, $J = 7.6$ Hz, 2H), 7.08–7.10 (d, $J = 7.6$ Hz, 1H), 7.14–7.18 (2H), 7.24 (s, 1H), 7.30–7.34 (m, 2H), 7.70–7.72 (2H), 7.92 (s, 1H), 8.05 ppm (s, 1H); ^{13}C NMR (125 Hz, $CDCl_3$): $\delta = 12.19$, 12.32, 44.17, 44.75, 67.17, 96.55, 98.01, 103.11, 107.48, 108.21, 109.32, 114.94, 122.19, 124.91, 125.16, 125.79, 127.55, 127.99, 129.18, 129.90, 132.98, 136.11, 137.43, 145.83, 149.15, 151.37, 151.67, 153.97, 157.12, 159.05, 165.13, 165.99, 168.89, 182.20 ppm; ESI-MS *m/z* 947.3 $[M+H]^+$; HRMS (ESI) *m/z* calcd for $C_{54}H_{59}N_8O_6S$ ($[M+H]^+$): 947.4273; found: 947.4258.

Synthesis of 1b: **1b** was prepared from pure compound **5b** according to the above method for the synthesis of **1a**. M.p. 175–178 °C; ^{1}H NMR (500 Hz, $CDCl_3$): $\delta = 1.16$ (t, 12H), 1.21–1.23 (t, 6H), 3.34–3.80 (20H), 6.29 (s, 2H), 6.46 (s, 5H), 6.59–6.61 (dd, $J = 9.0$, 2.0 Hz, 1H), 7.03–7.05 (d, $J = 8.0$ Hz, 2H), 7.10–7.13 (t, 2H), 7.17–7.20 (t, 2H), 7.30 (d, $J = 2.0$ Hz, 2H), 7.50 (s, 1H), 7.64 (s, 1H), 7.87 (s, 1H), 8.08 ppm (s, 1H); ^{13}C NMR (125 Hz, $CDCl_3$): $\delta = 12.40$, 12.53, 44.46, 44.99, 67.46, 96.92, 107.72, 108.63, 109.53, 113.94, 115.24, 125.08, 126.12, 127.38, 127.78, 128.33, 130.03, 130.37, 137.60, 150.56, 151.98, 154.29, 157.42, 159.18, 166.16, 168.99, 182.66 ppm; ESI-MS *m/z* 947.1 $[M+H]^+$; HRMS (ESI) *m/z* calcd for $C_{54}H_{59}N_8O_6S$ ($[M+H]^+$): 947.4273; found: 947.4256.

RAW 264.7 macrophage imaging using probe 1a: Raw 264.7 murine macrophages were obtained from Xiangya hospital and cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in an atmosphere of 5% CO_2 and 95% air at 37 °C. For imaging studies, the cells were plated on 6-well plates and allowed to adhere for 24 h. Immediately before the experiments, the cells were washed with phosphate-buffered saline (PBS) buffer solution. Subsequently, the cells were incubated with probe **1a** (5 μM) for 30 min at 37 °C, and then washed three times with PBS. After incubation with 10 μM NaOCl for another 30 min at 37 °C, the Raw 264.7 murine macrophages were rinsed three times with PBS, and the fluorescence images were acquired using an Olympus inverted fluorescence microscope equipped with a cooled CCD camera (Figure S14, Supporting Information). For the detection of endogenously produced HOCl, the living RAW 264.7 macrophages were treated with LPS (1 μg mL^{-1}) for 12 h, and then further co-incubated with PMA (1 μg mL^{-1}) and **1a** (5 μM) for 60 min. Prior to imaging, the cells were washed three times with PBS (1 mL), and the fluorescence images were acquired using an Olympus fluorescence microscope equipped with a cooled CCD camera (Figure 5).

Bel 7702 cells confocal fluorescence imaging using probe 1b: Bel 7702 cells were cultured in DMEM supplemented with 10% FBS in an atmosphere of 5% CO_2 and 95% air at 37 °C. One day before imaging, the cells were passed and plated on 18 mm glass coverslips coated with poly-L-lysine (50 μg mL^{-1}, Sigma). After 24 h, the cells were incubated with 5 μM **1b** (in the culture medium containing 1% DMF) for 0.5 h at 37 °C, and then washed three times with PBS. After incubation with 10 μM NaOCl for another 0.5 h at 37 °C, the cells were rinsed three times with PBS. The glass coverslips were attached to slides before imaging. Confocal fluorescence images of intracellular NaOCl in the cells were recorded using a Nikon 90i C1 Si laser scanning confocal microscope (Figure 4). The excitation wavelength of the laser was 408 nm. Emissions were centered at 450 ± 35 nm and 605 ± 75 nm (double channels).

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