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A ratiometric fluorescence sensor for HOCl based on FRET platform and application in living cells

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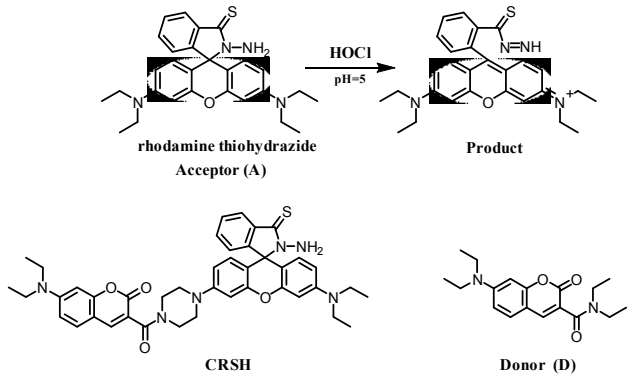
Abstract: Studies showed that the intravascular pH of neutrophils fell to 4.5-5.0 with stimulation for a few minutes. Under this condition, myeloperoxidase was activated to catalyze H_2O_2 and Cl^- to form hypochlorous acid (HOCl). Therefore, it is of significance to develop fluorescence probes for sensing HOCl in acid condition. In this work, we reported a ratiometric probe CRSH based on fluorescence resonance energy transfer (FRET) platform for detecting HOCl under acid condition. Probe CRSH exhibited excellent sensitivity, high selectivity and rapid response toward HOCl and is suitable for imaging endogenous HOCl in the living cells.

1. Introduction

Hypochlorite/hypochlorous acid (HOCl/$^-$OCl) widely used in our daily life is known to be a biologically important reactive oxygen species (ROS).[1] Endogenous HOCl is mainly produced from the reaction of H_2O_2 and chloride ion catalyzed by myeloperoxidase (MPO), and plays a significant role in many biologically vital processes.[2-5] However, current evidence suggested that uncontrolled HOCl/$^-$OCl production may be associated with various diseases, such as arthritis, kidney disease, lung injury, cardiovascular diseases, asthma and even cancers.[6-11] Development of methods for sensing HOCl/$^-$OCl is very significant to understand the role of HOCl in living organism. Therefore, lots of sensitive and selective analytical methods have been exploited for conducting such research.[12-15] Among these methods, fluorescent probes have innate advantages due to their high selectivity, excellent sensitivity, and fast response time. So a number of fluorescent probes were developed for the detection of HOCl/$^-$OCl.[16-25]

Studies reported that the intravascular pH of neutrophils fell to values as low as 4.5-5.0, when they were stimulated for a few minutes. Under this condition, myeloperoxidase was activated, and effectively catalyzed the reaction of H_2O_2 and Cl^- to form HOCl.[26-28] Therefore, developing HOCl fluorescence probes which can be used in acid condition is of significance for the detection of HOCl in living cells. In this work, we employed rhodamine thiohydrazide (Scheme 1) as the detection group which could make excellent response toward HOCl under acid condition. Although rhodamine thiohydrazide was reported as HOCl fluorescence probe, the reaction product of rhodamine thiohydrazide with HOCl was not given.[29] In this work, we confirmed the reaction product using MS (Fig. S1, ESI†). Thus, a possible detection mechanism was recommended as Scheme 1.



Scheme 1 Structures of CRSH, Donor and Acceptor, and proposed detection mechanism of rhodamine thiohydrazide for HOCl.

As is well known, ratiometric fluorescent probes possess advantages over single emission probes in resisting interference from environment, probe concentration and excitation intensity. Such probes are more favorable for intracellular imaging than other fluorescent probes, because they not only possess larger pseudo-Stokes shifts, but also have recorded ratio signal of two emission intensities at different wavelength, which could afford a built-in correction.[16,30-33] At present, ratiometric fluorescent probes are designed primarily based on several strategies including internal charge transfer (ICT), through bond energy transfer (TBET) and fluorescence resonance energy transfer (FRET).[34-45] For a FRET system, a substantial spectral overlap which is necessary between the donor emission band and the acceptor absorption band is closely related to the energy transfer

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efficency.[16] Coumarin and rhodamine can meet this requirement that is a substantial spectral overlap (Fig. 1), so coumarin-rhodamine platform was broadly used in FRET fluorescent probes.[32,46,47] In addition, coumarin as energy donor was high favorable because of its excellent photophysical properties including good photostability, high quantum yields, and excellent extinction coefficient.

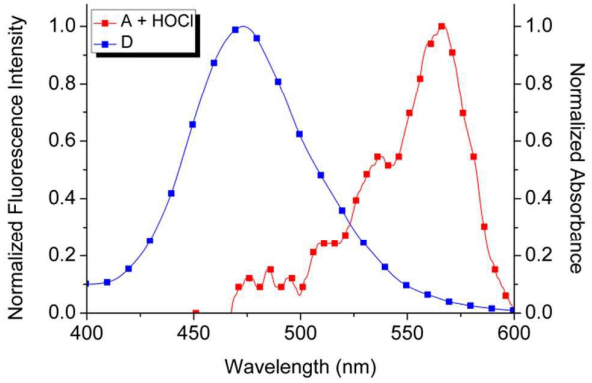


Fig. 1 Normalized emission spectra of donor (blue line) and normalized absorption spectra of acceptor (red line) after the addition of HOCl (4 μM) Condition: [donor] = 1 μM, [acceptor] = 1 μM, NaH_2PO_4 (0.05 M, pH = 5) : EtOH (5 : 5, v/v), λ_{ex}: 350 nm (slit widths: 10 nm/10 nm).

In this work, we designed a ratiometric fluorescence probe CRSH based on coumarin-rhodamine platform for detecting HOCl under acid condition. Probe CRSH which was suitable for imaging endogenous HOCl in the living cells showed excellent sensitivity, high selectivity, rapid response, little cytotoxicity, and good cell-membrane permeability. The FRET energy transfer efficiency (E) was calculated to be 67.0% as $E=1-F_{DA}/F_D$. Where, F_{DA} and F_D denote the donor fluorescence intensity with and without an acceptor, respectively (Fig. S2, ESI†). [31,39,48]

2. Results and Discussion

We first evaluated the pH effect on the fluorescence of CRSH toward NaClO. It can be seen from Fig. S3 (ESI†) that variations of pH did not affect the fluorescence intensity ratios I_{580}/I_{470} for CRSH in the absence of NaOCl. After the addition of NaOCl, the fluorescence intensity ratios for the probe became significantly higher at pH 4-6. The result indicated that probe CRSH exhibited high sensitivity toward HOCl rather than $^-$OCl because pKa of HOCl is 7.6.

To test the selectivity of the probe toward HOCl, we measured the absorption and emission spectral of CRSH by adding independently various ROS/RNS including $^1O_2$, H_2O_2, HO·, NO, $ONOO^-$, *t*-BuOOH, *t*-BuO·, $^-O_2$. As shown in Fig. 2a, treatment of CRSH with 4 μM HOCl resulted in significant fluorescence changes under pH 5 condition. Emission intensities of donor decreased dramatically at 470 nm and acceptor emission appeared at 580 nm. Although $^1O_2$ (20 μM) also affected slightly emission intensities of donor, it can be seen from the fluorescence ratio (I_{580}/I_{470}, Fig. 2b) that this affection is insignificant comparing with HOCl. Other ROS/RNS (20 μM) could not bring about fluorescence changes of CRSH. We examined interference from other ROS/RNS on monitoring HOCl (Fig. S4a, ESI†). The result indicated that CRSH possessed high selectivity toward HOCl in the presence of ROS/RNS. Moreover, the absorbance spectral showed that absorbance of donor changed little and that of acceptor enhanced obviously after addition of HOCl (Fig. S4b, ESI†). These results indicated that HOCl reacted with acceptor rather than donor. When other ROS/RNS were added in the solution of CRSH, respectively, absorbance of donor and acceptor change little. Therefore, it can be concluded from both the spectra of absorption and emission that both probe CRSH showed excellent selectivity toward HOCl over other ROS/RNS.

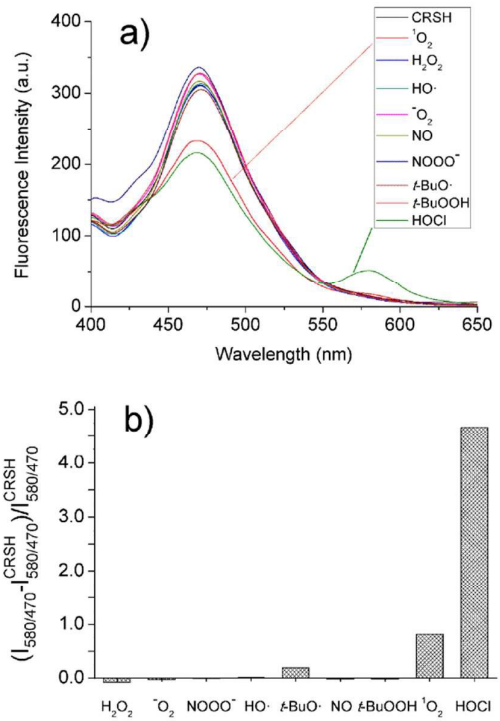


Fig. 2 Fluorescence spectrum changes of CRSH (a) toward HOCl (4 μM) and other ROS/RNS (20 μM, HO·, $ONOO^-$, NO, H_2O_2, *t*-BuOOH, *t*-BuO·, $^1O_2$, $^-O_2$). The relative ratio (b) of fluorescence intensity ratio changes, Condition: [CRSH] = 1 μM, NaH_2PO_4 (0.05 M, pH = 5) : EtOH (5 : 5, v/v), λ_{ex}: 350 nm (slit widths: 10 nm/10 nm).

To evaluate quantitative detection capacity and detection limits, the titration experiments were implemented with the addition of NaOCl into 1 μM probe solution at pH 5.0. Only donor fluorescence emission was observed at 470 nm for CRSH in absence of NaOCl. When NaOCl was titrated from 0-4 μM, the donor fluorescence intensity at 470 nm diminished significantly, and a new emission of the acceptor at 580 nm appeared and increased gradually (Fig. 3a). The results can be attributed to the reaction of rhodamine thiohydrazide (acceptor) with HOCl inducing thiohydrazide spiro-ring opening. At the same time, the FRET system was formed between the

donor and the acceptor (Scheme 2). The corresponding ratio (I_{580}/I_{470}) increased gradually with addition of NaOCl (Fig. 3b). It is interesting that the mechanism was confirmed by the reaction of the Acceptor with HOCl (Scheme 1). Probe CRSH showed good linearity between the intensity ratio and the corresponding concentration of HOCl. The detection limit was calculated to be 0.21 μM according to the formula 3σ/k (σ is the standard deviation of ten blank solutions of probe without HOCl and k was the slope of linearity between the ratio and the concentration of HOCl). Fig. 3 also showed that CRSH could make response to low concentration (0.2 μM) of HOCl in real sample. The results indicated that CRSH showed high sensitivity toward HOCl. The absorption peak of CRSH was observed at around 410 nm (donor) for CRSH. Upon addition of HOCl, new absorption peak at 560 nm (acceptor) appeared and increased gradually, and absorption peak of donor changed little (Fig. S5, ESI†). These results also indicated that acceptor in probe can react with HOCl efficiently and the donors were immune to HOCl.

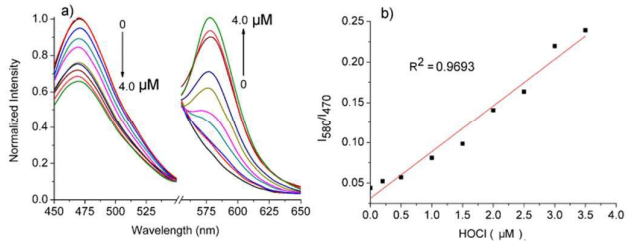
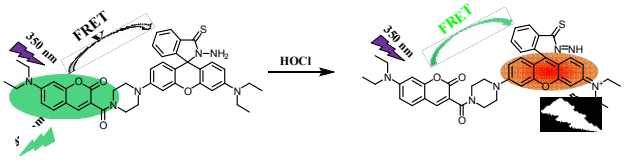


Fig. 3 (a) Fluorescence spectra and (b) Fluorescence intensity ratio (I_{580}/I_{470}) changes with the addition of HOCl (0–4 μM). Condition: [CRSH] = 1 μM, NaH_2PO_4 (0.05 M, pH = 5) : EtOH (5: 5, v/v), λ_{ex}: 350 nm (slit widths: 10 nm/10 nm).



Scheme 2 The proposed detection mechanism of CRSH toward HOCl.

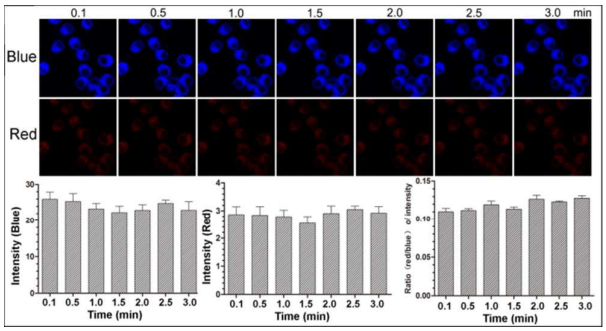


Fig. 4 Confocal fluorescence images of living RAW264.7 cells on incubation with 1 μM CRSH for different times (0.1, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0) min. excitation: 405 nm, emission: blue 405–550 nm, red 550–700 nm).

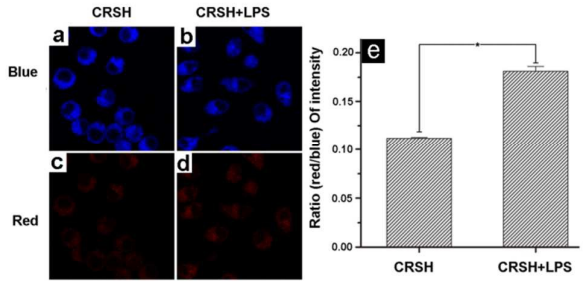


Fig. 5 Fluorescence images of RAW264.7 cells incubated with 1 μM CRSH for 1 h, (a) blue channel and (c) red channel. Fluorescence images of RAW264.7 cells pretreated with 0.1 μg mL^{-1} lipopolysaccharide (LPS) for 12 h, then incubated with 1 μM probe for 1 h , (b) blue channel and (d) red channel. Ratio (red to blue) of fluorescence intensity (e). The results are presented as means ± SE with replicates n=3 (*p<0.05).

In addition, to evaluate the response time of the probe toward HOCl, the experiments were implemented with addition of HOCl (2 equiv.) into the solution of CRSH (1 μM) at pH 5. Fig. S6 (ESI†) exhibited that fluorescence intensity ratio reached a plateau after 1 min (operation time). This indicated that the probe could rapidly respond to HOCl and could be used for real time imaging of HOCl *in situ*.

To evaluate its capacity of imaging HOCl in living cells, we first tested their cell-membrane permeability which is a critical parameter for the use of probe in intracellular detection. RAW264.7 cells were incubated with CRSH (1 μM) for 3 min. Bright blue fluorescence and faint red fluorescence were observed, and the fluorescence intensity changed little over time (Fig. 4). The result demonstrated that the probe possessed excellent membrane permeability and stability. Then, we studied the cytotoxicity of CRSH in RAW264.74 cells. Fig. S7 (ESI†) showed little toxicity affected cell viability after incubation with CRSH (1, 5, 10 μM) for 6 h or 12h. These indicated that the probe could be applied in living organisms. Murine RAW264.7 cells were employed to investigate probe CRSH for imaging endogenous HOCl in living cells, because RAW264.7 cells can produce HOCl after the stimulation with lipopolysaccharide (LPS).[49-53] In the control group, strong blue fluorescence (Fig. 5a) and faint red fluorescence (Fig. 5b) were observed after RAW264.7 cells were incubated with CRSH (1 μM) for 1 h. After stimulating with LPS for 12 h, RAW264.7 cells were incubated with probe CRSH for 1 h, the strong blue fluorescence darkened (Fig. 5c) and the faint red fluorescence brightened (Fig. 5d). The corresponding ratio (red to blue) of fluorescence intensity increased (Fig. 5e). Therefore, CRSH was suitable for imaging endogenous HOCl in the living cells.

3. Experimental Section

3.1. Apparatus and chemicals

^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz or 100 MHz) spectra were acquired on a Bruker Avance 300 spectrometer or Bruker Avance 400 spectrometer, with *d6*-DMSO 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. Fluorescent 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 used without further purification from merchants. Twice-distilled water was used throughout all experiments.

3.2. Preparation of test solutions

Probe was dissolved in EtOH for a stock solution (1 mM). Test solutions were prepared by displacing 10 μL the stock solution into a 10 mL volumetric flask. The solution was diluted to 10 mL in a mixture of phosphate buffer (0.05 M, pH = 5) and EtOH (5 : 5, 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.

3.3. 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. Cells underwent imaging measurement with a confocal microscope (Zeiss LSM780, Carl Zeiss Canada) at excitation 405 nm. The emission of the blue channel was 405-550 nm and red channel 550-650 nm.

3.4. Synthesis of CRSH

Compound 1 and 3 were prepared according to papers (Scheme S1).[47,54]

Synthesis of compound 2

Compound **1** (1.00 g, 2.13 mmol) and Lawessons reagent (0.98 g, 2.2 mmol) were dissolved in 40 mL dry toluene. The reaction solution was heated and kept reflux for 8 h under N_2 atmosphere, and then toluene was removed under reduced pressure to give violet solid. The crude product was used for next step without purification.

Synthesis of rhodamine thiohydrazide (acceptor)

Rhodamine B hydrazide (1.0 g, 2.2 mmol) and Lawessons reagent (890 mg, 2.2 mmol) were dissolved in dry toluene (40 mL). The reaction solution was heated and kept reflux for 8 h under N_2 atmosphere. Toluene was removed under reduced pressure and the crude compound was purified by column chromatography (petroleum ether : ethyl acetate = 4 : 1) to give white solid (263 mg); Yield: 25.3%. ^{1}H NMR (300 MHz, *d6*-DMSO): δ 7.94 – 7.83 (m, 1H), 7.62 – 7.46 (m, 2H), 7.05 (dd, *J* = 5.6, 2.7 Hz, 1H), 6.40 (d, *J* = 2.4 Hz, 2H), 6.37 (d, *J* = 2.5 Hz, 1H), 6.34 (d, *J* = 2.5 Hz, 1H), 6.17 (d, *J* = 8.8 Hz, 2H), 5.36 (s, 2H), 3.38 – 3.27 (m, 8H), 1.09 (t, *J* = 6.9 Hz, 12H) ppm; ^{13}C NMR (100 MHz, *d6*-DMSO): δ 181.02, 153.33, 149.71, 149.13, 136.35, 132.43, 129.12, 128.20, 124.11, 123.40, 108.49, 103.76, 97.88, 73.13, 44.17, 12.89 ppm. HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{28}H_{33}N_4OS$: 473.2375, found: 473.2402.

Synthesis of CRSH

Compound **2** (243 mg, 0.50 mmol) was dissolved in dry dichloromethane (5 mL) and Et_3N (0.5 mL). The solution was cooled with stirring under nitrogen atmosphere to 0 ℃. Subsequently, Compound **3** (167 mg, 0.60 mmol) was added into the solution in batches. After addition, the reaction was stirred at 0 ℃ for 30 min and then at room temperature for 3 h. The solvent was removed under reduced pressure and the crude product was purified by column chromatography (dichloromethane : ethyl acetate = 4:1) to give yellow solid CRSH (188 mg). Yield: 51.6%. ^{1}H NMR (300 MHz, *d6*-DMSO) δ 8.01 (s, 1H), 7.90 (dd, *J* = 5.7, 2.6, 1H), 7.57 – 7.45 (m, 3H), 7.05 (dd, *J* = 5.7, 2.4, 1H), 6.75 (d, *J* = 2.4, 2H), 6.67 (dd, *J* = 8.9, 2.4, 1H), 6.57 (d, *J* = 2.1, 1H), 6.41 – 6.34 (m, 2H), 6.27 – 6.16 (m, 2H), 5.37 (s, 2H), 3.70 (s, 2H), 3.46 (d, *J* = 6.9, 6H), 3.35 (s, 1H), 3.30 – 3.22 (m, 7H), 1.20 – 1.06 (m, 12H). ^{13}C NMR (75 MHz, *d6*-DMSO) δ 181.89, 164.57, 158.89, 157.12, 153.18, 152.89, 152.17, 151.78, 149.58, 149.24, 144.38, 136.30, 132.56, 130.59, 129.26, 128.20, 128.01, 124.22, 123.39, 116.32, 112.20, 109.89, 108.76, 108.03, 107.63, 103.56, 102.45, 97.87, 96.83, 72.89, 63.94, 60.19, 44.65, 44.17, 12.87, 12.77. HRMS: m/z $[M+H]^+$ calcd for $C_{42}H_{45}N_6O_4S$: 729.3223, found: 729.3212.

Synthesis of donor

The synthesis method for donor 1 and donor 2 is the same to that for CRSH. Yield 52.1%, ^{1}H NMR (300 MHz, *d6*-DMSO): δ 7.90 (s, 1H), 7.50 (d, *J* = 8.7 Hz, 1H), 6.73 (dd, *J* = 9.0, 2.4 Hz, 1H), 6.56 (d, *J* = 2.4 Hz, 1H), 3.48 – 3.33 (m, 6H), 3.26 (t, *J* = 6.9 Hz, 2H), 1.15 – 1.08 (m, 12H) ppm. ^{13}C NMR (75 MHz, *d6*-DMSO): δ 164.74, 158.52, 156.27, 150.89, 141.86, 129.81, 117.33, 109.21, 106.98, 96.30, 44.07, 42.57, 13.98, 12.69, 12.21 ppm. HRMS: m/z $[M+H]^+$ calcd for $C_{18}H_{25}N_2O_3$: 317.1865, found: 317.1864.

Conclusions

In this work, we employed coumarin-rhodamine platform to designed a ratiometric fluorescence probe CRSH based on FRET for detecting HOCl under acid condition, and evauated its capacities of detecting HOCl. The results indicated that probe CRSH could make response to low concentration of HOCl in real sample, and exhibited high selectivity, rapid response, little cytotoxicity, and good cell-membrane permeability. The probe was suitable for imaging endogenous HOCl in the living cells.

Acknowledgements

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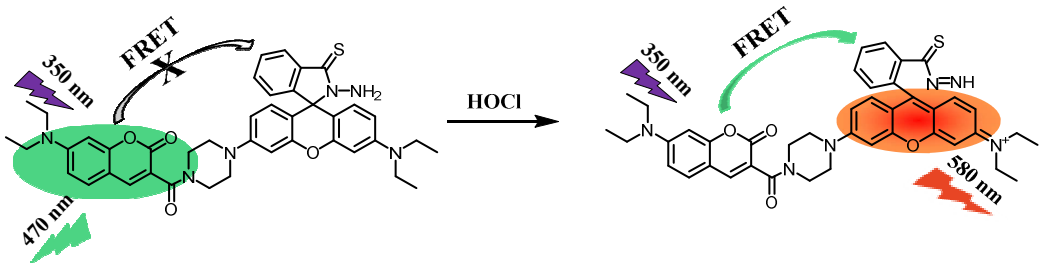

A ratiometric fluorescent probe CRSH based on FRET platform for detecting HOCl. CRSH showed high selectivity, excellent sensitivity and fast response toward HOCl.