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A Strategy for Highly Selective Detection and Imaging of Hypochlorite Using Selenoxide Elimination

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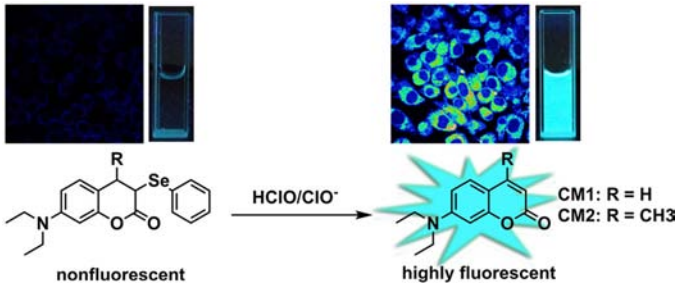
ABSTRACT



A new strategy for HOCl-specific fluorescent probes has been reported based on a selenoxide elimination reaction. Probes CM1 and CM2 were synthesized as the first fluorescent probes containing an arylseleno moiety for hypochlorite according to this strategy. Both probes displayed excellent properties, including high selectivity and sensitivity, fast response, and pH independency toward hypochlorite *in vitro* and *vivo*.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are well-known for their chemical reactivity and tight connection with various physiological processes and diseases.[1] Hypochlorous acid (HOCl), one of the most significant ROS, exerts a wide variety of physiological and pathological effects in living systems.[2] Endogenous HOCl, which is produced from the reaction of hydrogen peroxide with chloride catalyzed by myeloperoxidase (MPO), plays an important role in protecting the body against invasion of pathogens.[3] Nevertheless, excessive generation of this strong oxidant can also lead to many inflammation-related diseases, including rheumatoid arthritis,[4] cardiovascular diseases,[5] atherosclerosis,[6] renal disease,[7] lung injury,[8] and so on. Therefore, it is of vital importance to devise efficient ways to selectively detect HOCl in biological systems so as to understand its biological roles.

Fluorescence spectroscopy, which features real-time and real-space detection, and nondestruction, has been regarded as the most promising technique for detection of various ROS/RNS.[9] So far a number of small-molecule fluorescent probes for specific detection of HOCl/OCl^- have been

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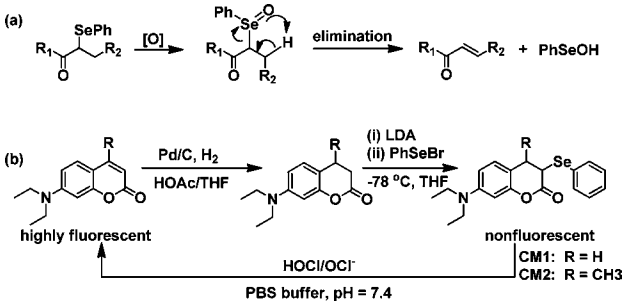
reported;[10] however, most of them still face some drawbacks, such as poor selectivity,[10a,b,h,o] low sensitivity,[10d,e,m,o] and pH dependency.[10c,e,g,k] Therefore, novel fluorescent probes that can overcome these limitations in detecting $HOCl/OCl^-$ are in urgent need.

Herein, we have developed a new strategy for HOCl-specific fluorescent probes based on a selenoxide elimination reaction that can well address the existing issues. The selenoxide elimination was synthetically used to prepare α, β-unsaturated esters and ketones, olefins and allyl alcohols in mild conditions.[11] As shown in Scheme 1a, the selenoxide elimination generally involves a two-step process. The phenylselenenyl group is first oxidized by selected oxidants, such as H_2O_2, leading to the formation of intermediate selenoxide, which is then eliminated via a spontaneous intramolecular *syn* elimination to generate a conjugated C=C bond.[11d] We reason that utilizing this mechanism to design fluorescent probes based on α-arylseleno carnonyl compounds will provide a new strategy for selective detection of ROS. To verify our hypothesis, the proof-of-concept fluorescent probes **CM1** and **CM2** (Scheme 1b) based on coumarin fluorophores were designed. We envision that very high specificity for hypochlorite could be achieved through a significant fluorescent turn-on signal if the conjugated structures of probes **CM1** and **CM2** are recovered by selenoxide elimination in the presence of the specific oxidant hypochlorite. Although several selenium-based fluorescent probes for hypochlorite or peroxynitrite have been reported recently,[12] all of them are based on a conventional PET (photoinduced electron transfer) mechanism and their responsive rate,[12a,c−e] pH dependency,[12b,e] and water solubility[12c] still need further refinement. In the present work, we reported the design, synthesis, and photophyscial evaluation of the probes **CM1** and **CM2** in aqueous buffer and their cellular applications.

Scheme 1. (a) General Process of Selenoxide Elimination; (b) Synthesis and Proposed Fluorescent Turn-On Strategy of the Probes



As shown in Scheme 1b, the probes can be easily synthesized by reduction of commercially available coumarin dyes and subsequent selenylation. The detailed synthetic procedures were described in the Supporting Information. To assess the success of our design concept, we first measured the photophysical properties of **CM1** and **CM2** in PBS buffer containing 10 mM phosphate, 8 g L^{-1} NaCl, 0.2 g L^{-1} KCl, pH = 7.4. Both **CM1** and **CM2** display no major absorption band and fluorescence emission ($\Phi < 0.001$) in the range of 300−600 nm, as the conjugated structures of probes are broken by selenylation. With the addition of excess NaOCl to the solutions, the absorption around 370−400 nm and fluorescence centered at 480 nm (**CM1**, $\Phi = 0.036$) and 468 nm (**CM2**, $\Phi = 0.047$) characteristic of corresponding coumarin dyes were observed (Figure S1).[13] The phenomena suggest that hypochlorite triggers the selenoxide elimination so as to restore the conjugated system of the highly fluorescent coumarins, which have been confirmed by HRMS analysis (Figures S2 and S3). In order to evaluate the sensing process of OCl^-, elaborate fluorimetric measurements were performed. As expected, the fluorescence of **CM1** increased gradually with the addition of NaOCl. Finally, the maximum fluorescence intensity was obtained while the concentration of NaOCl is up to 28 μM (Figure 1a).

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In particular, the fluorescence intensity showed good linearity with the concentration of NaOCl in the low level range (0−6.4 μM). The detection limit (LOD) is thus calculated to be as low as 10 nM (S/N = 3, Figures 1b and S4), indicating that with **CM1** it is feasible to quantitatively determine hypochlorite in biological environments. Similarly, **CM2** also responds to NaOCl but requires much larger amounts of NaOCl to complete the conversion (Figure S5). This can be well explained by the fact that the methyl group of **CM2** partially restricts the conformational preference for the intramolecular *syn* elimination (Scheme 1a). Moreover, it is worth noting that **CM1** can rapidly respond to NaOCl within seconds (Figure 1c), making it a good candidate for real-time detection of hypochlorite generated in situ.

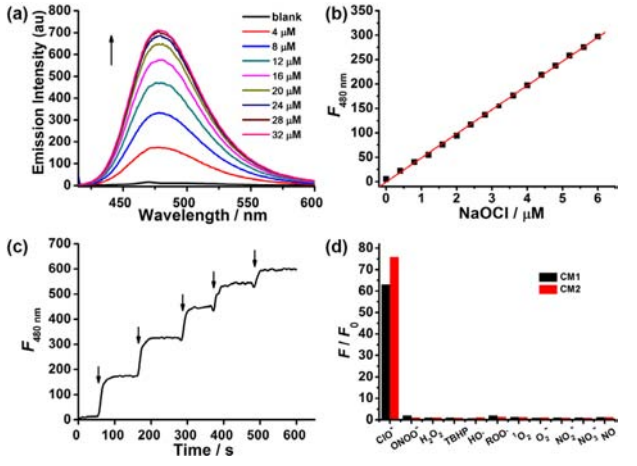

Figure 1. (a) Fluorescence spectra of **CM1** (4 μM) upon addition of NaOCl (0−32 μM). (b) Fluorescence intensities of **CM1** at 480 nm as a function of the concentrations of NaOCl in the range of 0−6.4 μM. (c) Fluorescence response of **CM1** (4 μM) to NaOCl; arrows represent addition of 4 μM NaOCl. (d) Fluorescence responses of **CM1** and **CM2** (4 μM) to NaOCl (100 μM) and other ROS/RNS (200 μM). Bars represent emission intensity ratios after (F) and before (F_0) addition of each ROS/RNS. Data were acquired at 25 °C in PBS buffer, λ_{ex} = 405 nm, λ_{em} = 480 nm for **CM1**, λ_{em} = 468 nm for **CM2**.

To evaluate whether the probes can selectively respond to OCl^- under simulated physiological conditions, the fluorescence responses of the probes to other potentially competing ROS/RNS, which were prepared according to the reported procedures (Supporting Information), were also performed. As shown in the selectivity profiles (Figure 1d), only OCl^- incurs a dramatic fluorescence enhancement for **CM1** and **CM2**, respectively. Other ROS or RNS, including $ONOO^-$, H_2O_2, *t*-BuOOH (TBHP), HO•, *t*-BuOO• (ROO•), $^1O_2$, O_2^-, NO_2^-, NO_3^-, and NO, exert no obvious spectral changes. Moreover, other biological thiols, which are abundant in living samples, have no effect as well under identical conditions (Figure S6).

Additionally, we also evaluated the effect of pH on the detection of hypochlorite. The variations of pH cause no or little influence on the fluorescence intensity and response of **CM1** and **CM2** to NaOCl over a wide range of 4−9 (Figure S7). Considering the pK_a of HOCl is 7.6,[10c,f] **CM1** and **CM2** are responsive to both HOCl and OCl^- at a wider pH range than the reported probes. Moreover, the probes are stable in PBS buffer despite being exposed to air for 10 h (Figure S8). These data clearly suggest that the probes are capable of selectively detecting hypochlorite without interference by other biological ROS and pH variations.

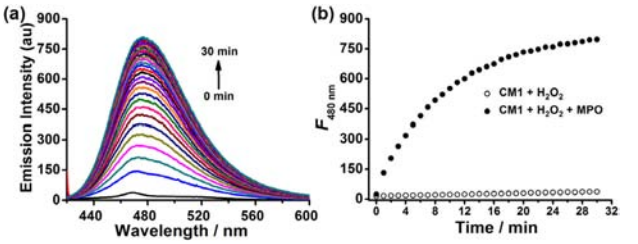

Figure 2. Application of **CM1** to the enzymatic MPO/H_2O_2/Cl^- system. (a) Fluorescence changes as a function of time for the reaction of 4 μM **CM1** in the enzyme-catalytic systems (PBS, 40 μM H_2O_2, 1 μg mL^{-1} MPO, pH = 7.4, 37 °C). (b) Fluorescence response of **CM1** in the absence (○) and presence (●) of MPO, λ_{ex} = 405 nm, λ_{em} = 480 nm.

Since hypochlorous acid is the myeloperoxidase (MPO)-derived oxidant in living organisms, we further evaluated the feasibility of **CM1** for monitoring HOCl/OCl^- in an MPO/H_2O_2/Cl^- enzymatic system. The fluorescence intensity periodically increased after adding H_2O_2 (40 μM) into **CM1** (4 μM) solution in PBS buffer (10 mM phosphate, 8 g L^{-1} NaCl, 0.2 g L^{-1} KCl, pH = 7.4) containing 1 μg mL^{-1} myeloperoxidase at 37 °C (Figure 2), whereas no obvious fluorescence response to H_2O_2 was observed in the absence of MPO. Thus, the observed fluorescence increase must be attributed to HOCl/OCl^- generated in the enzymatic reaction.

With these data in hand, we next applied **CM1** to living cells for fluorescence imaging of OCl^-. After incubation with 5 μM **CM1** at 37 °C for 20 min, NIH 3T3 cells exhibited faint fluorescence in the optical window 420−520 nm (Figure 3a). Treating the cells with 50 μM NaOCl for 30 s led to remarkable fluorescence enhancement (Figure 3b), indicating that **CM1** is extremely sensitive for detecting exogenous OCl^- in living cells.

Inspired by these results as demonstrated above, we further evaluated whether the probe **CM1** could be used to monitor endogenous hypochlorite in living cells. Because MPO is abundantly expressed in primary azurophilic granules of leukocytes including neutrophils[14] and macrophages,[15] RAW 264.7 macrophages and HL-60 human progranulocytic leukemia cell lines were chosen as bioassay models for endogenous OCl^-. After incubation with 5 μM **CM1** for

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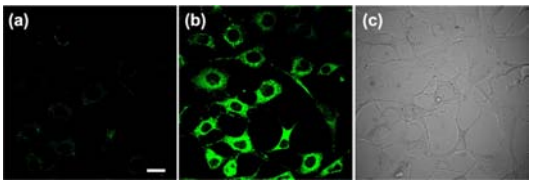

Figure 3. Fluorescent imaging of exogenous OCl^- in **CM1**-labeled NIH 3T3 cells. (a) Cells were stained with 5 μM **CM1** at 37 °C for 20 min. (b) **CM1**-labeled cells were treated with 50 μM NaOCl for 30 s at 25 °C. (c) Bright-field image of (a) and (b). Emission intensities were collected in an optical window 420−520 nm, λ_{ex} = 405 nm, Scale bar: 10 μm.

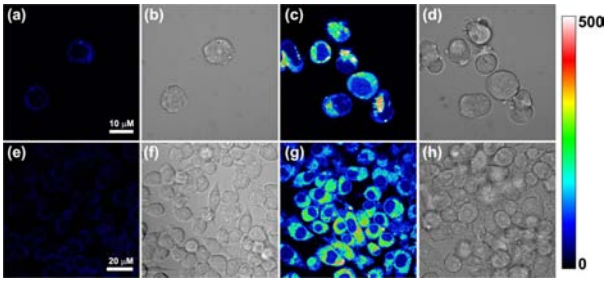

Figure 4. Fluorescent imaging of endogenous OCl^- in **CM1**-labeled HL-60 (top) and RAW 264.7 cells (bottom). (a) Fluorescence images of HL-60 cells loaded with 5 μM **CM1** at 37 °C for 30 min. (b) Bright-field images of cells in panel (a). (c) The cells after addition of 100 μM H_2O_2 at 25 °C for 20 min. (d) Bright-field images of cells in panel (c). (e) Fluorescence images of RAW 264.7 cells loaded with 5 μM **CM1** at 37 °C for 20 min. (f) Bright-field images of cells in panel (e). (g) After pretreatment with 3 μg mL^{-1} LPS for 12 h and additional 1 μg mL^{-1} LPS for 4 h, the cells loaded with 5 μM **CM1** at 37 °C for 20 min. (h) Bright-field images of cells in panel (g). Emission intensities were collected in an optical window 420−520 nm, λ_{ex} = 405 nm. Intensity bar: 0−500.

20 min at 37 °C, respectively, both kinds of cells exhibited very weak fluorescence (Figure 4a and e). When the **CM1**-loaded HL-60 cells were treated with 100 μM H_2O_2 for 20 min, much stronger fluorescence was detected (Figure 4c). Now that **CM1** is highly selective for hypochlorite over other ROS/RNS (Figure 1d), it is reasonable to believe that the exogenous H_2O_2 activated myeloperoxidase in HL-60 cells to transfer Cl^- to OCl^- and consequently caused the great enhancement of fluorescence.[10b] Lipopolysaccharide (LPS), an endotoxin, is commonly used for eliciting strong immune responses in mammalian cells and producing highly active ROS.[16] We further incubated the RAW 264.7 macrophage cells with LPS for 16 h (12 h first, and then additional 4 h) to produce endogenous OCl^- and then stained the cells with 5 μM **CM1** for 20 min.[10k] Under these conditions, the pseudocolor images also displayed a sharp contrast to that of the control (Figure 4e and g), suggesting that **CM1** had successfully detected OCl^- generated by the stimulation of LPS. Therefore, we can conclude that **CM1** is capable of monitoring and imaging both exogenous and endogenous HOCl/OCl^- with high sensitivity and selectivity in living cells.

In summary, on the basis of a specific intramolecular selenoxide elimination reaction, we have demonstrated a novel strategy in designing fluorescent probes for the highly selective detection of hypochlorite. The probe, **CM1**, which features high sensitivity, selectivity, fast response, and pH independency, was successfully utilized in detecting hypochlorite in aqueous media and living cells. Importantly, it can be used to visualize the generation of endogenous hypochlorite in progranulocytes and macrophages. We emphasize that this novel design strategy, which is easily adaptable, will provide a general method to construct HOCl-specific probes from various commercially available dyes with additional tunable photophysical properties. The development of ratiometric fluorescent probes based on this strategy is also a challenging project that is underway.

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Supporting Information Available. Synthetic procedures, characterization of compounds, and additional spectroscopic data. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

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