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# A Rhodamine−Hydroxamic Acid-Based Fluorescent Probe for Hypochlorous Acid and Its Applications to Biological Imagings

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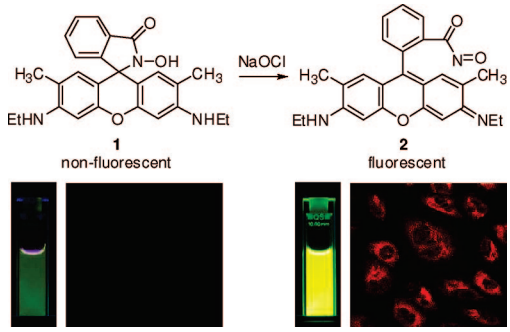
## ABSTRACT




**A new rhodamine−hydroxamic acid-based fluorescent chemosensor for the rapid detection of HOCl in aqueous media was developed. The system, which utilizes an irreversible HOCl-promoted oxidation reaction, responds instantaneously at room temperature with linear proportionality to the amount of HOCl. This system is highly selective for HOCl over other reactive oxygen species (ROS) and highly sensitive in aqueous solutions. Biological imaging studies using living cells and organisms (A549 cells and zebrafish) to detect HOCl are successfully demonstrated.**

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are known to be essential to several biological functions.[1] Hypochlorous acid (HOCl), one of the biologically important ROS, is weakly acidic and partially dissociates into the hypochlorite ion ($OCl^-$) in the physiological pH solutions.[2] Biologically, the hypochlorite ion is synthesized from hydrogen peroxide and chloride ions in activated neurophils catalyzed by myeloperoxidase (MPO).[3] Hypochlorous acid reacts with various biomolecules including DNA, RNA, fatty acids, cholesterol, and proteins. While hypochlorous acid has strong antibacterial properties, excess amounts of HOCl can also cause serious damage to the biological systems.[4] Therefore, a lot of effort has been given to the studies of the various biological effects of HOCl.[5]

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(2) In the physiological pH solutions, HOCl rather than $OCl^-$ is the actual species. See refs 6a, c, and d for the discussion.

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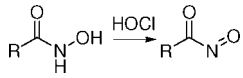
Recently, fluorescent chemosensor-based detections of HOCl have been given great attention.[6] Known fluorescent chemosensors for HOCl are based on the strong oxidation property of HOCl. Oxidation reactions of *p*-methoxyphenol to benzoquinone,[6a] dibenzoylhydrazine to dibenzoyl diimide,[6b] thiol to sulfonate derivative,[6c] and *p*-alkoxyaniline[6d] have been employed as the key reacting components in the design of HOCl-selective fluorescent probes. Although these reported chemosensors have demonstrated reasonable selectivity for HOCl over other ROS, fluorescent probes of better sensitivity and reactivity for HOCl are still required for the biological imaging applications. Herein, we report a highly sensitive fluorescent probe for hypochlorous acid and its applications in biological imaging studies.

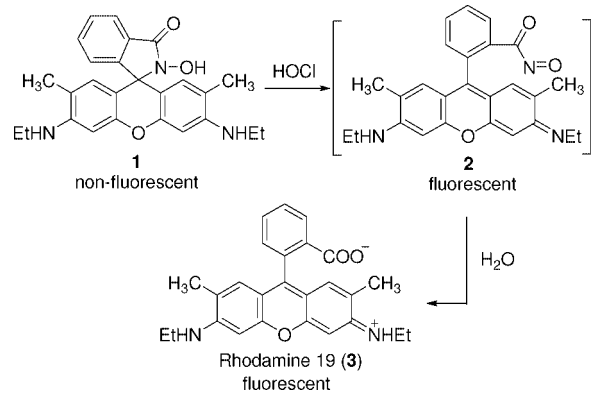
The oxidation reactions of thiols and amines by HOCl involve chlorination reactions where −S-Cl[7] and −N-Cl[8] species are generated. Similarly, the HOCl-mediated oxidation reaction of hydroxamic acid is expected to follow the same chlorination pathway to give the acyl nitroso compound after elimination of HCl (Scheme 1).

**Scheme 1.** Oxidation of Hydroxamic Acid by HOCl




We envisioned that this irreversible reaction could be incorporated into the rhodamine amide system to convert the nonfluorescent spirocyclic form to the fluorescent ring-opened one.[9,10] Therefore, the rhodamine derivative **1** was prepared from rhodamine 6G in three steps (1, NaOH, $H_2O$−EtOH; 2, $POCl_3$, $CH_2Cl_2$; 3, $NH_2OH$, $Et_3N$, $CH_2Cl_2$).[11] Fluorescent probe **1** forms a colorless solution in PBS buffer−DMF (0.1%) at pH 7.4, indicating that it exists in the spirocyclic form predominantly. Addition of aqueous NaOCl (10 equiv) to **1** developed instantaneous color and strong fluorescence intensity changes. Although probe **1** showed no fluorescence at pH 5−13, it showed strong fluorescence intensity changes in the presence of NaOCl at pH 5−10 (see Supporting Information). This observation indicates that the HOCl-induced oxidation of **1** takes place rapidly at room temperature. The reaction responsible for the change completes within 20 s to yield the ring-opened acyl nitroso compound **2** (Scheme 2). Elimination of HCl from the proposed chlorination intermediate **A** or **B** (Figure 1) is expected to be facile under the reaction conditions.

**Scheme 2.** HOCl-Induced Oxidation of Fluorescent Probe **1**



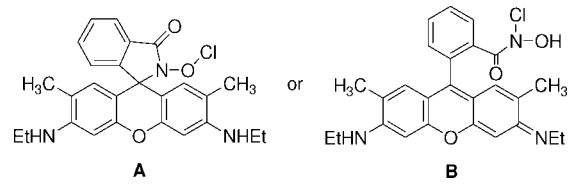




**Figure 1.** Proposed chlorination intermediates.

Interestingly, the hydroxamic acid derivatives of rhodamine B and fluorescein showed only slight fluorescence intensity changes (see Supporting Information).[12] Although we were not able to detect the highly unstable acyl nitroso[13] compound **2**, we could confirm the formation of rhodamine 19 (**3**) from the reaction mixture using $^1$H NMR and ESI-MS experiments.[14]

Fluorescence intensity changes of **1** (1 $\mu$M) upon additions of HOCl (5.0 equiv) and other ROS[15] (100 equiv of $H_2O_2$, NO•, •OH, ROO•, $•O_2^-$) in PBS buffer−DMF (0.1%) at pH 7.4 are shown in Figure 2. The selectivity profile proved to

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(11) For the synthesis and characterization of fluorescent probe **1**, see Supporting Information.

(12) It seems that the hydroxamic acid derivatives of rhodamine B and fluorescein are less likely to promote ring opening of the chlorinated spirocyclic intermediates to give the corresponding acyl nitroso compounds. From our previous studies, we found that the irreversible spirocyclic ring-opening reaction is generally favored by NHEt over $NEt_2$ or OH at the xantene ring (see ref 10a).

(13) Cohen, A. D.; Zeng, B.-B.; King, S. B.; Toscano, J. P. *J. Am. Chem. Soc.* **2003**, *125*, 1444–1445.

(14) We could detect rhodamine 19 (**3**) from the $^1$H NMR spectrum obtained from the reaction mixture run in an NMR tube. Furthermore, rhodamine 19 is isolated as the major product after flash column chromatography of the reaction mixture (see Supporting Information).

(15) Additions of 5∼100 equiv of ROS other than NaOCl showed no fluorescence intensity changes at all (see Supporting Information).

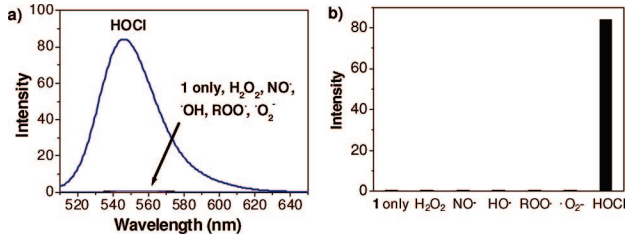


**Figure 2.** (a) Fluorescence intensity changes of **1** (1 $\mu$M) upon addition of NaOCl (5.0 equiv) and other ROS (100 equiv of $H_2O_2$, NO•, •OH, ROO•, $\cdot O_2^-$) in PBS buffer−DMF (0.1%) at pH 7.4 (25 °C, excitation at 500 nm). (b) Fluorescence intensity changes at 546 nm.

be excellent in aqueous solutions. Only HOCl enhanced the fluorescence intensity dramatically, while other ROS are silent to **1**. In addition to the fluorescent changes, the colorless to pink color changes associated with the reaction of **1** with HOCl are readily detectable visually, but no significant color changes were promoted by other ROS.

The fluorescence titration profile of **1** (0.1 $\mu$M) with HOCl demonstrates that the detection of HOCl is possible at the 25 nM level (Figure 3). Under these conditions, the

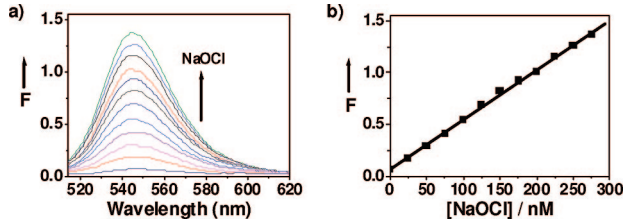


**Figure 3.** (a) Fluorescence response of **1** (0.1 $\mu$M) upon addition of NaOCl (by 25 nM) in PBS buffer−DMF (0.1%) at pH 7.4 (25 °C, excitation at 500 nm). (b) The fluorescence intensities at 547 nm.

fluorescence intensity of the solution of **1** is linearly proportional to the amounts of HOCl added (Figure 3b). Because the fluorescent probe **1** is highly reactive and highly sensitive toward HOCl, it could be ideal for the biological imaging applications.[16]

Next, we studied bioimaging applications of **1** for HOCl detection in biological systems. A549 (lung cancer) cells were incubated with and without 10−50 $\mu$M NaOCl for 20 min at 37 °C and washed with PBS buffer (pH 7.4) to remove the remaining NaOCl, and then the treated cells were incubated with **1** (5 $\mu$M) in culture medium for 10 min at 37 °C. After incubation under these conditions, they were imaged using a confocal fluorescence microscope. The cells untreated with NaOCl showed no fluorescence (Figure 4c),

(16) It is not easy to directly compare properties of **1** with those of the known probes for HOCl. But we believe that the readily available rhodamine amide derivative **1** could find broad biological applications.

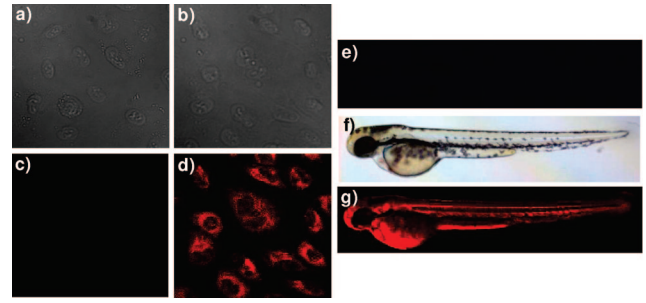


**Figure 4.** Images of A549 (lung cancer) cells and zebrafish. (a) Microscopic image of A549 cells treated with **1** (5 $\mu$M) in the absence of NaOCl (control). (b) Microscopic image of A549 cells treated with both NaOCl (10 $\mu$M) and **1** (5 $\mu$M). (c) Fluorescence image of A549 cells treated with **1** (5 $\mu$M) in the absence of NaOCl (control). (d) Fluorescence image of A549 cells treated with both NaOCl (10 $\mu$M) and **1** (5 $\mu$M). (e) Fluorescence images of zebrafish treated with **1** (20 $\mu$M) in the absence of NaOCl (control). (f) Microscopic image of zebrafish treated with **1** (20 $\mu$M) and NaOCl (100 $\mu$M). (g) Fluorescence image of zebrafish treated with **1** (20 $\mu$M) and NaOCl (100 $\mu$M).

but the cells treated with NaOCl displayed strong red fluorescence (Figure 4d). The fluorescence intensities increased as the concentration of NaOCl increased (see Supporting Information). Similarly, the imaging of 5-day old zebrafish treated with 100 $\mu$M NaOCl and **1** (20 $\mu$M) was also conducted using a dissecting microscope and a fluorescence microscope. The zebrafish treated with probe **1** only exhibited no fluorescence (Figure 4e). However, strong red fluorescence was observed from the zebrafish treated with both NaOCl and **1** (Figure 4g).

In conclusion, we have described a highly selective and sensitive fluorescent chemosensor for the detection of HOCl in aqueous media. The hydroxamic acid unit of the fluorescent probe is oxidized by HOCl to produce a highly fluorescent acyl nitroso compound. The chemical reaction is fast at room temperature and irreversible. The probe can detect ~25 nM concentration of HOCl in aqueous media. Fluorescent imagings of A549 cells and zebrafish are also successfully demonstrated to detect HOCl in living cells and organisms. We expect that this imaging technique will serve as a practical tool for hypochlorous acid-related biological studies.

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**Supporting Information Available:** Experimental procedures for the synthesis, spectral data, copies of $^1$H NMR and $^{13}$C NMR of **1** and **3**, and data for fluorescence titrations of **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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