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A Highly Specific BODIPY-Based Fluorescent Probe for the Detection of Hypochlorous Acid

Zhen-Ning Sun,[†] Feng-Qin Liu,[‡] Yan Chen,[‡] Paul Kwong Hang Tam,[‡] and Dan Yang*,[†]

Department of Chemistry and Department of Surgery, The University of Hong Kong, Pokfulam Road, Hong Kong, P.R. China

yangdan@hku.hk

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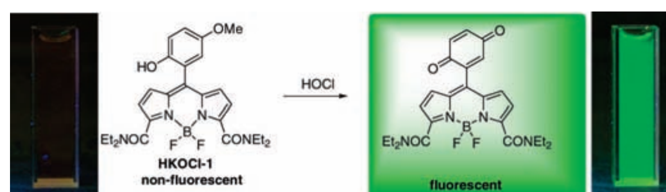
ABSTRACT



A fluorescent probe, HKOCl-1, has been successfully developed for the detection of hypochlorous acid on the basis of a specific reaction with *p*-methoxyphenol. The formation of HOCl has been successfully detected not only in an abiotic system but also in an enzymatic system (myeloperoxidase/H_2O_2/Cl^- system) and in living macrophage cells upon stimulation. This new probe might be used as an efficient tool for probing the roles HOCl plays in biological systems.

Unlike most other reactive oxygen species (ROS) and reactive nitrogen species (RNS), hypochlorite (OCl^-) and its protonated form hypochlorous acid (HOCl) are encountered widely in our daily lives. For example, sodium hypochlorite is frequently used as a disinfectant and a bleaching agent (common "bleach"). Hypochlorite is a key microbicidal agent that is used for natural defense because it behaves as a strong nucleophilic nonradical oxidant; its efficacy lies in the fact that neither bacteria nor mammalian cells can neutralize its toxic effects—they lack the enzymes required for its catalytic detoxification.[1] In living organisms, hypochlorite is synthesized from hydrogen peroxide and chloride ions in a chemical reaction catalyzed by the enzyme myeloperoxidase (MPO), which is localized mainly in leukocytes, including neutrophils, macrophages, and monocytes.[2] MPO is released into the phagolysosomal compartment following stimulation by inflammatory mediators such as cytokines, antibodies, and the endotoxic bacterial membrane component lipopolysaccharide (LPS). Although hypochlorite functions mainly in the prevention of microorganism invasion, increasing evidence suggests that it is also involved in several human diseases, including neuron degeneration,[2] cardiovascular diseases,[3] and osteoarthritis[4] caused by the presence of abnormal levels of MPO.[5] Nevertheless, the mechanism of action of hypochlorite in these diseases is much less clear than that of other ROS and RNS because of the lack of sensitive and specific probes for detecting hypochlorite.[6] Recently, two red fluorescent probes for hypochlorous acid were reported by Nagano's group[6c] and Libby's group,[6d] but the sensitivity of those fluorescent probes still needs to be improved. On the other hand, for

[†] Department of Chemistry.
[‡] Department of Surgery.

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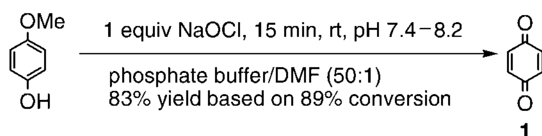
cell imaging applications, it is important to develop green fluorescent probes for hypochlorous acid. Here, we report a green fluorescent probe for highly specific and sensitive detection of hypochlorous acid and its application in cell imaging.

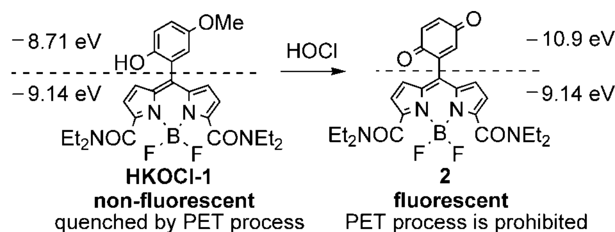
Our approach is to find a specific reaction for hypochlorite—one that does not proceed in the presence of other ROS, such as peroxynitrite, hydroxyl radical, and H_2O_2. Because hypochlorite is a strong oxidant in biological systems, we investigated its reactivity toward *p*-methoxyphenol. We found that 1 equiv of NaOCl oxidized *p*-methoxyphenol to benzoquinone (Scheme 1). Gratifyingly, we found that *p*-methoxyphenol was stable toward most other common ROS and RNS. For $ONOO^-$, the nitration product was formed, but the conversion was very low (<5%). Thus, the oxidation of *p*-methoxyphenol to **1** is specific for hypochlorite.

Scheme 1. Formation of Benzoquinone from *p*-Methoxyphenol



With this specific reaction for hypochlorite in hand, we designed a probe, **HKOCl-1**, that would function based on a photoinduced electron transfer (PET) mechanism.[7] Scheme 2 displays a proposed mechanism for the reaction between **HKOCl-1** and hypochlorite. Before the reaction with hypochlorite, the HOMO energy level (−8.71 eV) of the *p*-methoxyphenol moiety is higher than that of the BODIPY unit (−9.14 eV);[8] hence, the fluorescence of **HKOCl-1** is quenched through a PET process (Φ_{fl} < 0.01). After oxidation, the HOMO energy levels of the benzoquinone moiety (−10.9 eV) is lower than that of the BODIPY unit.[8] Therefore, the PET process is prohibited and product **2** should be fluorescent.

Scheme 2. Reaction of Probe **HKOCl-1** with HOCl



We first investigated the reactivity of **HKOCl-1** toward hypochlorite in an abiotic chemical system. As indicated in Figure 1a, the probe was nonfluorescent prior to its reaction with hypochlorite; the fluorescence signal appeared and increased dramatically upon increasing the hypochlorite concentration. A linear correlation existed between the emission intensity and the concentration of hypochlorite within the range from 3 to 8 μM (Figure 1a, inset). After treatment with 1 equiv of hypochlorite, the solution of **HKOCl-1** exhibited a 1079-fold increase in its fluorescence intensity at pH 7.5. This result suggested that **HKOCl-1** is by far the most sensitive probe for the detection of hypochlorite in abiotic systems.[6c,d] Moreover, we confirmed the formation of compound **2** through an ESI-MS measurement.[8]

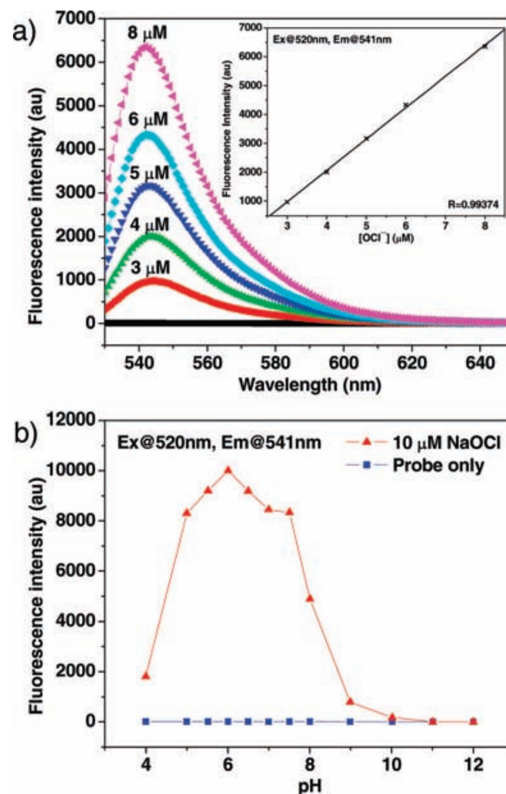


Figure 1. (a) Fluorescence spectra of **HKOCl-1** (final concentration: 10 μM) in 0.1 M potassium phosphate buffer (pH 7.5) recorded 2 min after the addition of NaOCl (ranging from 0 to 8 μM). The fluorescence intensity was determined with excitation at 520 nm. Inset: Fluorescence intensity (Ex@520 nm, Em@541 nm) plotted against the concentration of NaOCl. (b) Fluorescence spectra of **HKOCl-1** (final concentration: 10 μM) with the addition of NaOCl (final concentration: 10 μM) in 0.10 M sodium phosphate buffer at various pH values (Ex@520 nm, Em@541 nm).

We noticed that **HKOCl-1** showed pH-dependence in the detection of hypochlorite. As shown in Figure 1b, the fluorescence increase was significantly higher at pH 5−7.5

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(8) See the Supporting Information.

than at other pH values. Considering the pK_a of HOCl is 7.6, we concluded that **HKOCl-1** detects HOCl rather than OCl^-, similar to the behavior of fluorescent probes reported by other groups.[6c,d] Because our probe **HKOCl-1** exhibits high sensitivity toward HOCl, it can be used for the detection of HOCl at a wider pH range (pH 4−9) than previously reported probes.

Next we compared the reactivity of **HKOCl-1** toward various ROS and RNS, including H_2O_2, $^1O_2$, NO, $O_2^{\bullet -}$, $^{\bullet}OH$, HOCl, $ONOO^-$, and alkylperoxyl radicals ($ROO^{\bullet}$), added independently to the **HKOCl-1** solution. The changes in fluorescence intensity before and after the addition of ROS and RNS (Figure 2a) indicated that fluorescence augmentation occurred only upon reaction with HOCl; i.e., **HKOCl-1** exhibits excellent selectivity toward HOCl among the various ROS and RNS in abiotic systems. In fact, this high selectivity toward HOCl was also observed over a wide pH range (pH 4−9).[8]

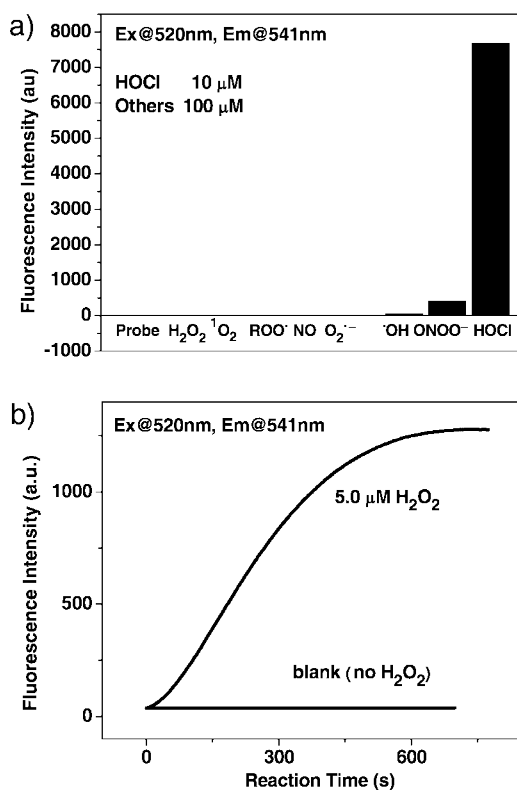

Figure 2. (a) Fluorescence intensity of **HKOCl-1** (final concentration: 10 μM) in various ROS- and RNS-generating systems. (see Supporting Information for details). (b) Application of **HKOCl-1** to an $MPO/H_2O_2/Cl^-$ system. **HKOCl-1** (final concentration: 10 μM) was added to sodium phosphate buffer (0.1 M, pH 7.4) containing MPO (1 U/100 mL) and NaCl (150 mM) at 37 °C. Lower line: Background fluorescence in the absence of H_2O_2 (blank). Upper line: H_2O_2 added (final concentration: 5 μM).

Because hypochlorite in living organisms is synthesized predominantly from hydrogen peroxide and chloride ions in a reaction catalyzed by the enzyme MPO,[9] we applied **HKOCl-1** to an $MPO/H_2O_2/Cl^-$ system (Figure 2b). The fluorescence intensity increased dramatically upon the addition of **HKOCl-1** whereas almost no fluorescence was detected in the control experiment (in the absence of H_2O_2). Because **HKOCl-1** does not respond to H_2O_2, the observed fluorescence increase must be related to the formation of HOCl; i.e., this system allows the detection of the production of HOCl in the enzymatic system.

A cytotoxicity assay exhibited that **HKOCl-1** was nontoxic to macrophages when its concentration was below 80 μM.[8] Therefore, we applied our **HKOCl-1** probe to macrophages for fluorescence imaging of HOCl in cells.

The murine macrophage cell line RAW264.7 produces MPO upon stimulation.[10] In addition, exposure of macrophages to stimuli such as LPS/IFN-γ (interferon-γ)[11] and phorbol myristate acetate (PMA)[12] will also activate the generation of other ROS and RNS. In this experiment, we used RAW264.7 as a model to test whether we could use **HKOCl-1** (20 μM) to detect HOCl generated by an MPO/H_2O_2/Cl^- system under stimulation. We observed no obvious fluorescence in the cells prior to treatment with the stimulants (Figure 3a). It is noteworthy that fluorescent cells appeared after the stimulation with LPS/IFN-γ followed by PMA (Figure 3b). However, after the cells were pretreated with 2,2,6,6-tetramethylpiperidinooxy (TEMPO), a radical that can scavenge superoxide (the precursor to HOCl),[11] much weaker fluorescence was observed upon stimulation. Our results

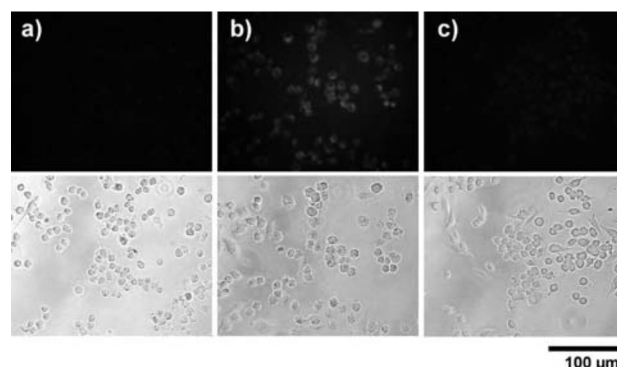

Figure 3. Images of RAW 264.7 macrophages (lower: phase-contrast images; upper: fluorescence imaging) treated with various stimulants and then incubated with **HKOCl-1** (20 μM) for 1 h: (a) control; (b) LPS (1 μg/mL) and IFN-γ (50 ng/mL) for 4 h, then PMA (10 nM) for 0.5 h; (c) TEMPO (100 μM), LPS (1 μg/mL), and IFN-γ (50 ng/mL) for 4 h, then PMA (10 nM) for 0.5 h.

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confirm for the first time that macrophages do indeed produce HOCl upon stimulation-and that this process can be visualized using **HKOCl-1**.

In conclusion, on the basis of a specific reaction for hypochlorite and exploiting a PET mechanism, we have successfully developed a new BODIPY-type green fluorescent probe, **HKOCl-1**, which is highly sensitive and specific for the detection of HOCl in buffer-based and enzyme-containing systems. Most importantly, **HKOCl-1** can be employed to image the production of HOCl selectively in living macrophage cells; use of this efficient probe might allow an evaluation of the roles HOCl plays in biological systems.

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Supporting Information Available: Synthesis, experimental details, and characterization of **HKOCl-1**. This material is available free of charge via the Internet at http://pubs.acs.org.

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