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A BODIPY aldoxime-based chemodosimeter for highly selective and rapid detection of hypochlorous acid†

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A BODIPY-based fluorescent probe integrated with an aldoxime unit shows a remarkable fluorescence "turn-on" response to hypochlorous acid (HOCl). The oxidative dehydrogenation of BODIPY aldoxime by HOCl results in a distinct fluorescence enhancement as well as a change in color from red to orange.

Hypochlorous acid (HOCl) is a biologically important reactive oxygen species (ROS) produced in living organisms from hydrogen peroxide and chloride ions in a reaction catalyzed by the heme-enzyme myeloperoxidase (MPO).[1] HOCl has a critical role as an antimicrobial agent in the natural defense system.[2] It is endogenously generated in cellular environments to kill invading bacterial pathogens. Excessive production of HOCl, however, leads to the oxidation of biomolecules which results in tissue damage and various diseases such as atherosclerosis,[3] arthritis,[4] and cancer.[5]

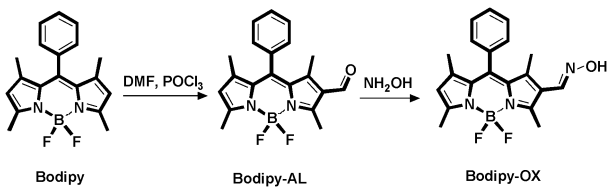
Scientists are performing extensive research to elucidate the mechanism by which HOCl kills bacteria and destroys human tissue. Nevertheless, the detailed understanding of HOCl formation during pathogenic biological events still remains a challenge due to the lack of methods for monitoring HOCl in living organisms. Sensitive and accurate detection methods for hypochlorous acid that function in living samples are thus highly demanded for the research in biology and medicine.

Fluorescence sensing methods in this context are highly promising as they allow the possibility of real-time visual detection of analytes both in solution and living organism. In recent years, a variety of fluorescent probes have been developed for detection of HOCl, most of which exploit the strong oxidation properties of HOCl.[6] Functional groups, sensitive to hypochlorite oxidation, such as *p*-methoxyphenol,[7] dibenzoylhydrazine,[6f] thiol,[6d] selenide,[6a–c] oxime[8] and *p*-alkoxyaniline[9] have been extensively utilized in the probe design. Some of these probes, however, show pH dependency and function only under basic conditions (pH > 9.0), which is an undesired feature for cell imaging studies. In addition to this, there are common shortcomings that undermine their general applicability, including cytotoxicity, low sensitivity, slow response time, selectivity and cross-sensitivity towards other species. Thus, it is of considerable interest to design and develop new probes able to overcome these limitations.

In this work, we have designed a novel BODIPY (boron-dipyrromethene) based fluorescent probe integrated with an aldoxime unit (**Bodipy-OX**) for selective detection of HOCl in various environments. The oxime functionality has been well established as a sensitive group to hypochlorous acid and thus provides a suitable signalling platform in the design of fluorescent probes.[8] The BODIPY fluorophore was chosen as the signal reporter because of its exceptional photophysical properties such as long excitation/emission wavelengths, high molar absorption coefficients and fluorescence quantum yield.[10]

Bodipy-OX was prepared using the synthetic route outlined in Scheme 1. A Vilsmeier–Haack formylation reaction of Bodipy gave **Bodipy-AL** which was further treated with $NH_2OH{\cdot}HCl$ in EtOH at reflux temperature to yield **Bodipy-OX**. After chromatographic purification, the structure of **Bodipy-OX** was confirmed using NMR spectroscopy and mass spectrometry.[11]

We commenced our investigation by first examining the parameters of the sensing media. Among various solvent systems tested, a combination of H_2O–DMF (4 : 1, v/v) proved to be highly efficient for the sensing process.[11] The spectral properties of the probe towards the addition of HOCl were examined under simulated physiological conditions phosphate buffer–DMF (pH 7.2, v/v, 4 : 1). The plot of the fluorescence intensity of **Bodipy-OX** against the increase in HOCl concentration is displayed in Fig. 1a. As shown, **Bodipy-OX** in solution is almost non-emissive (Φ_F = 0.03) possibly due to a non-radiative deactivation through rapid isomerization of the C=N–OH group.



Scheme 1 Synthesis of **Bodipy-OX**.

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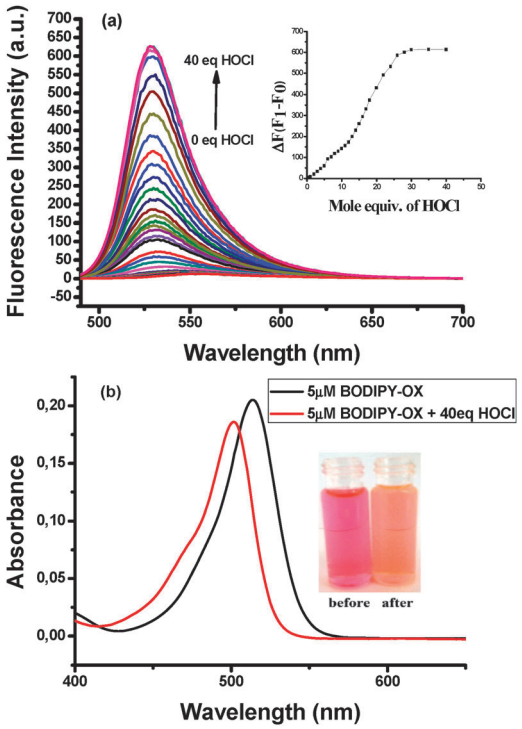


Fig. 1 (a) Fluorescence spectra of **Bodipy-OX** (5.0 μM) + HOCl (0.5 to 200 μM, 0.1 to 40 equiv.) in pH 7.2 buffer–DMF (v/v, 4 : 1) (λ_{ex}: 470 nm). Inset: fluorescence intensity changes of **Bodipy-OX** *vs.* equivalents of HOCl. (b) Absorption spectra of **Bodipy-OX** (5.0 μM) and **Bodipy-OX** + HOCl (200 μM, 40 equiv.) Inset: the colorimetric detection of HOCl.

However, the addition of HOCl to the solution of **Bodipy-OX** resulted in a significant enhancement of the emission intensity positioned at 529 nm. The emission intensity reached its maximum when 40 equiv. of HOCl was added, with an enhancement factor over 48-fold. Notably, the spectroscopic response of the probe was extremely fast and complete saturation of the signal intensity was observed within seconds (Fig. S10, ESI†). There was a linear dependence of the fluorescence intensity on the HOCl concentration (0.5 to 200 μM, 0.1 to 40 equiv.) and the minimum amount of HOCl that can be detected has been evaluated to be 0.5 μM (LOD). Meanwhile, upon addition of HOCl to the solution of **Bodipy-OX**, a new absorption band centered at 502 nm which was shifted to a slightly shorter wavelength (blue shift) as compared with the absorbance of **Bodipy-OX** (λ = 514 nm), (see Fig. 1b and Fig. S2b, ESI†). All spectroscopic changes were observed immediately upon the addition of HOCl, indicating that the oxidation reaction occurred rapidly at room temperature. The addition of HOCl to the probe solution developed an instantaneous color and fluorescence change which was easily detectable by the naked eye under light and UV irradiation.

The pH-dependence of **Bodipy-OX** in the detection of HOCl was investigated carefully. **Bodipy-OX** was stable and weakly fluorescent (Φ_F = 0.03) over a wide pH range (pH 3.0 to 11.0). On the other hand, the fluorescence response of the probe towards the addition of HOCl was indeed pH dependent. **Bodipy-OX** displayed an efficient fluorescence response to HOCl in the pH range of 4.0–9.0. However, the fluorescence response decreased sharply at pH > 9.0, which was an indication that the active species detected was the hypochlorous acid (HOCl) rather than the hypochlorite ion (OCl^-). Eventually, it turned out that **Bodipy-OX** can be efficiently used for the detection of HOCl in a wide pH range (pH 4–9). Notably, the fluorescence enhancement was significantly greater at physiological pH (pH = 7.2), which suggests that **Bodipy-OX** is highly suitable for biological applications.

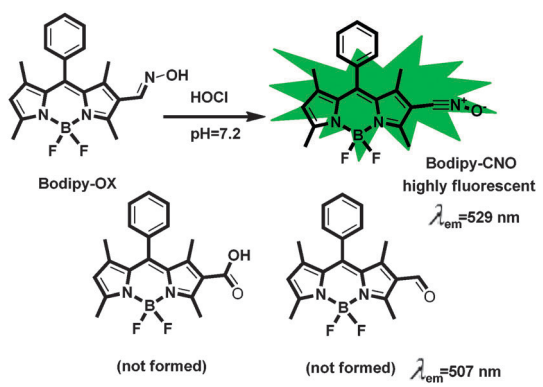
It was important to rule out the possible involvement of other reactive species in the sensing process. Therefore, the titration of **Bodipy-OX** with various other ROS, metal ions and also anions was carried under the same sensing conditions. To our delight, related ROS such as H_2O_2, $^{\bullet}OH$, $ROO^{\bullet}$ and ions including Mg^{2+}, Zn^{2+}, Cu^{2+}, CO_3^{2-}, F^-, I^-, AcO^-, NO_3^-, NO_2^-, SO_4^{2-}, ClO_3^- did not mediate any chemical reaction to result in a spectroscopic response. As displayed in Fig. S5 (ESI†), only the addition of HOCl resulted in an enhancement of fluorescence at 529 nm, which implied the high selectivity of **Bodipy-OX** to HOCl.

In order to assess any possible interference by other analytes we explored the fluorescence response of **Bodipy-OX** towards HOCl (200 μM, 40 equiv.) in the presence of the other ROS and metal ions (200 μM, 40 equiv.). As shown in Fig. S6 (ESI†), the tested analytes displayed no interference with the detection of HOCl.

In order to get insight into the sensing mechanism we focused on the reaction products formed by treating the probe with HOCl under the sensing conditions (DMF–H_2O (v/v, 1 : 4)). The reaction quantitatively afforded one major product in a short reaction time (5 min) as identified using TLC. The crude product was successfully purified using flash column chromatography and the structure of the isolated product was confirmed using ^{1}H-NMR, ^{13}C-NMR and mass analysis (Maldi-TOF-TOF).

Aldoximes are reported in the literature to give the corresponding aldehyde[8a–c] or the carboxylic acid[8d] upon oxidation with HOCl depending on the reaction medium. Surprisingly, the only product isolated was a nitrile oxide derivative of the BODIPY core (Scheme 2). Mass analysis confirmed the formation of a BODIPY-nitrile oxide (**Bodipy-CNO**, *m*/*z* = 365.173 found, 365.151 calc.) in the solution.

The majority of nitrile oxides are, in general, unstable and thus difficult to isolate and purify.[11] Surprisingly, the BODIPY based nitrile oxide (**Bodipy-CNO**) showed an unexpected stability presumably due to the extended conjugation and steric shielding of the nitrile oxide group. To our delight, **Bodipy-CNO** was successfully isolated (rapid chromotography through a short pad of silica) and characterized using NMR spectroscopy. As shown in the ^{1}H-NMR spectrum of the isolated product (Fig. 2), the aldoxime and the hydroxyl proton signal at 10.47 ppm and 8.07 ppm disappeared upon treatment with HOCl.



Scheme 2 Oxidative dehydrogenation of **Bodipy-OX**.

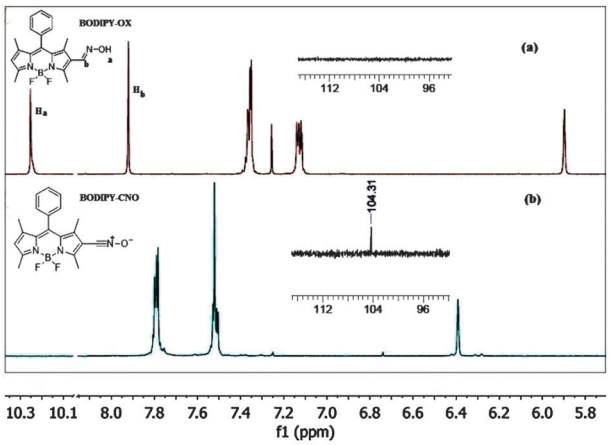


Fig. 2 (a) Partial ^{1}H NMR spectra of **Bodipy-OX**, (b) **Bodipy-CNO**; inset: partial ^{13}C NMR spectra of (a) **Bodipy-OX**, (b) **Bodipy-CNO**.

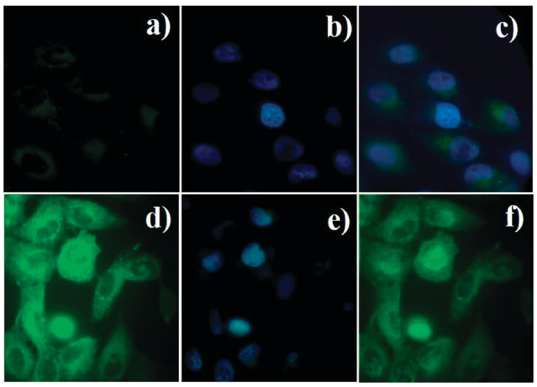


Fig. 3 (a) Fluorescence image of cells treated with only probe (1 μM) for 10 min; (b) fluorescence image of cells treated with Hoechst-34580 (1 μM); (c) merged image of frames a and b; (d) fluorescence image of cells treated with HOCl (10 μM), (λ_{ex}: 470 nm); (e) fluorescence image of cells treated with HOCl, (λ_{ex}: 350 nm); (f) merged image of frames d and e.

Moreover, the ^{13}C-NMR spectrum displays a characteristic carbon peak at 104.31 ppm that belongs to the nitrile oxide carbon atom.

Based on the structure of the isolated product, fully characterized using NMR spectroscopy and mass spectrometry, there was no doubt that the sensing event proceeds through an oxidative dehydrogenation of the oxime (**Bodipy-OX**) generating a highly fluorescent and sensitive nitrile oxide (Φ_F = 0.86).

It is worth noting that in the solution **Bodipy-CNO** decomposes into unidentifiable products. The decomposition of **Bodipy-CNO** was also confirmed using fluorescence spectrometry. As displayed in Fig. S8 (ESI†), the fluorescence intensity of the *in situ* formed nitrile oxide (**Bodipy-CNO**) was stable only for a very short period of time (5 min), and decreased gradually in the course of time.

Relying on the promising properties of **Bodipy-OX**, we next questioned the potential of the probe for tracking HOCl in living cells. Human Mammary Epithelial Cells (MCF10A) were incubated with **Bodipy-OX** (1.0 μM) for 10 min, and then stained with Hoechst-34580 (1.0 μM). MCF10A cells incubated with **Bodipy-OX** exhibited a very weak intracellular fluorescence whereas a significant fluorescence increase was monitored in the cells after treatment with HOCl (Fig. 3d). This preliminary cell imaging study suggested that the probe is cell membrane permeable and can be efficiently used for *in vitro* imaging of HOCl in living cells.

In summary, we have developed a "turn-on" type fluorescent probe that shows remarkable fluorescence emission enhancement in response to hypochlorous acid (HOCl) with high sensitivity and selectivity over other ROS and metal ions. This novel probe operates through an irreversible chemical reaction and thus can be classified as a chemodosimeter. The probe has a low detection limit and operates efficiently under physiological conditions, which is of crucial importance for biological imaging studies. Besides the rapid and specific response to HOCl, this probe can also be applied for *in vitro* imaging of HOCl in living cells.

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