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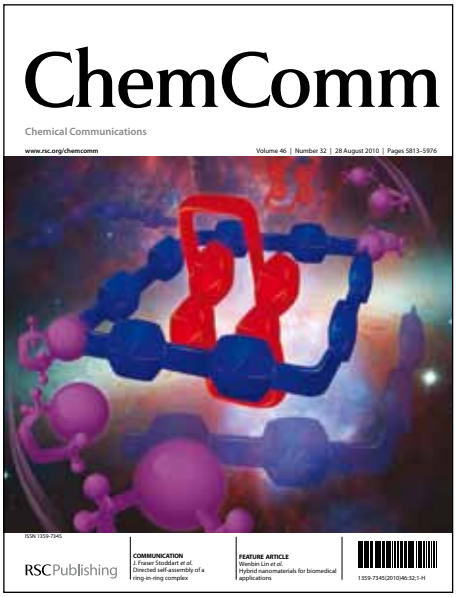

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ARTICLE TYPE

Dual-Emission and Large Stokes Shift Fluorescence Probe for Real-time Discrimination of ROS/RNS and Its Imaging in Living Cells

Ting Guo, Lei Cui, Jiaoning Shen, Rui Wang, Weiping Zhu, Yufang Xu* and Xuhong Qian*

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A novel dual-emission fluorescence probe has been developed for specific and sensitive detection of hypochlorite (ClO^-). Upon addition of ClO^-, significant changes of fluorescence emission intensity at two discrete wavelengths were observed. Meanwhile $OONO^-$ only led to a single-channel fluorescence enhancement. This feature makes it a clear advantage in distinguishing ClO^-, RNS between other ROS.

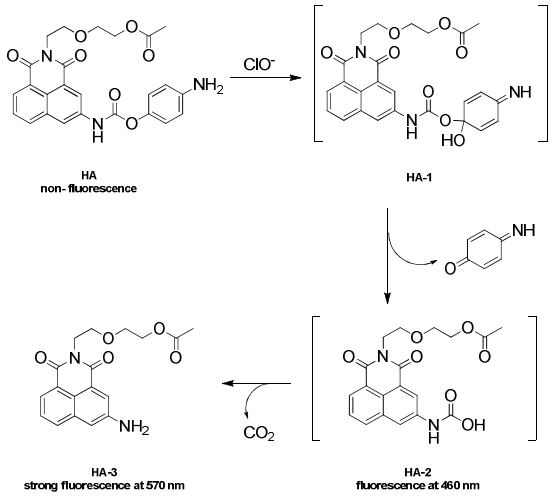
Reactive oxygen species (ROS) are produced mainly through electron transfer reaction from oxygen, which play crucial roles in many physiological processes and are also implicated in many human diseases, including cancer, neurodegenerative disorders, and inflammatory processes.[1] Reactive nitrogen species (RNS) are another group of reactive chemical species, which also play key roles in cell signalling during many physiological processes.[2] The more powerful oxidant peroxynitrite ($ONOO^-$), formed by nitric oxide (NO) and superoxide ($O_2^{\bullet-}$) in cells, is implicated with various human diseases, such as inflammatory, cardiovascular disease, neurodegeneration and cancer.[3]

Hypochlorite anion (ClO^-) is an important ROS converted from H_2O_2 and chloride catalyzed by the enzyme myeloperoxidase (MPO) in phagosomes.[4] The highly active hypochlorite can mediate the chemical modification of various biomolecules such as proteins, lipids, DNA and RNA.[5] Hypochlorite plays critical roles in hosting innate immunity during microbial invasion.[6] However, disorder of MPO or other reasons could cause uncontrollable production of hypochlorite, which in turn leads to a variety of diseases including cardiovascular diseases, neurondegeneration and osteoarthritis.[7] Therefore, it is of great importance to develop an effective method to detect ClO^- for understanding the role of hypochlorite in immunization and diseases.

Fluorescence probes with high sensitivity and spatial and temporal resolutions have become a powerful tool for noninvasive and real-time monitoring of bio-relevant species in living systems.[8] Recently, several small fluorescence probes have been reported for ClO^-. These probes showed excellent selectivity for ClO^- over other ROS/RNS, whereas most of them displayed fluorescent intensity fluctuation at a single channel.[9,10,11] Dual emission fluorescence probes enable a self-calibration for other actors, including unstable excitation source, photobleaching of fluorophores or uneven probe loading. Thus, this kind of fluorescence probes has potential application in quantitative measurement.[12] Several ratiometric fluorescence probes have been developed for ClO^-. However, the poor water solubility of the probe may limit their use in some biological applications.[13] In this work, we described a novel dual channel fluorescence probe **HA,** which exhibits a good aqueous solubility, quantitative detection of ClO^- from other ROS/RNS and the potentials for living cell imaging.

HA is constructed by tethering 4-aminophenoxyl to 1,8-naphthalimide via carbamate linkage (Scheme 1). This is because 4-aminophenyl ether has been proved to be a viable substrate for oxidant detection and 1,8-naphthalimide is a bright and photostable fluorophore with a large Stokes shift.[14, 15] The probe **HA** was easily prepared from 3-amino-1,8-naphthalimide **HA-3** in one-pot. **HA-3** was first stirred with 4-nitrophenyl chloroformate in anhydrous acetonitrile for 12 hours prior to Pd/C catalyzed hydrogenation to yield **HA**. The structure was unambiguously confirmed by NMR and HRMS (Scheme S1, ESI†). The probe **HA** exhibits good aqueous solubility. This is a direct benefit of the incorporation of an ethylene glycol moiety.

Probe **HA** displays a maximum absorption at 340 nm and is essentially non-fluorescent (Fig.S1, ESI†). This is expected since 4-aminophenol moiety is electron-rich and can quench the fluorescence via the photoinduced electron transfer (PeT) mechanism. The fluorescence response of **HA** to different pH was investigated (Fig.S2, ESI†). HCl was slowly introduced into a solution of **HA** at pH 11.0 and the fluorescence of the solution was monitored with excitation at 340 nm. The fluorescence of the



Scheme 1 Proposed detection mechanism of **HA**

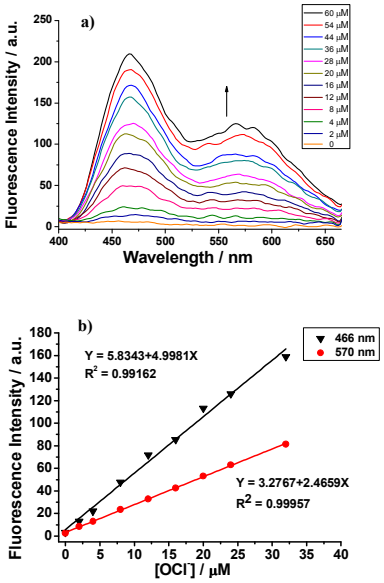


Fig. 1 (a) The emission spectra of probe **HA** (20 μM) upon addition of hypochlorite anion (0 to 3 equiv) in 0.1 M phosphate buffer (pH 7.4, with 1% DMF). (b) A liner calibration graph of the response to ClO^- concentration. Excitation wavelength was 340 nm.

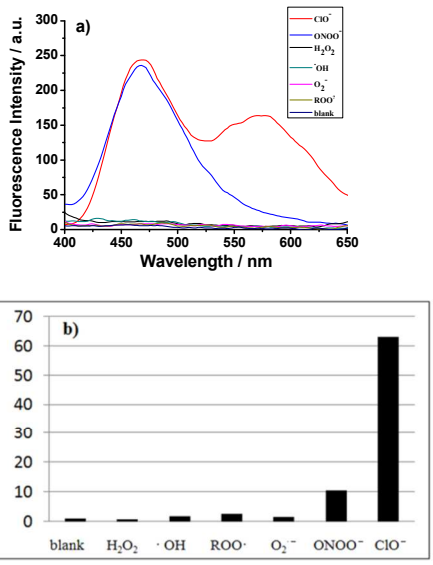


Fig. 2 (a) Fluorescence changes of probe **HA** (20 μM) in response of various ROS. ClO^- (60 μM), $ONOO^-$ (60 μM), H_2O_2 (1 mM), •OH (300 μM Ferrous perchlorate and 1 mM H_2O_2), $O2^{\bullet-}$ (KO_2, 200 μM), ROO• (200 μM tert-Butyl hydroperoxide). The fluorescent assays of probe **HA** for various ROS were carried out in phosphate buffer (0.1 M, pH 7.4, with 1% DMF) at room temperature. (b) Emission intensity ratio (I_{570}/I_0) of probe **HA** with various ROS, measured at 570 nm with excitation at 340 nm.

solution remained stable until the pH reaches 6.5, from which point the emission intensity at 460 nm started to rise steadily. When the solution went near or below the pKa of amine, more and more NH_2 got protonated. This prohibits the PET process and restores the fluorescence. The pKa of **HA** was determined to be 5.38, which is 2.0 pH unit lower than the physiological pH. This verifies that the probe can be used in biological system.

To investigate the reactivity and spectral properties of probe **HA** with ClO^-, spectroscopic titration was carried out at pH 7.4 (0.1 M sodium phosphate buffer, with 1% DMF) at room temperature. Upon oxidation by ClO^-, two emission bands at 460 nm and 570 nm appeared from the dark background in a dose dependant manner with excitation at 340 nm (Fig.1a) and levelled off in a few seconds (Fig. S3, ESI†). The absorption spectra of the solution displayed a characteristic red-shift to 410 nm (Fig. S4, ESI†). This suggested that the probe **HA** has potentials for real-time detection of ClO^-. The emission intensity was enhanced by 71-fold at around 460 nm and 63-fold at around 570 nm with 3 equiv of ClO^-. A linear response to the ClO^- concentration was found from 0 μM to 32 μM (Fig.1b) and the detection limit was estimated to be 0.7 μM.

We studied the oxidation mechanism of the probe **HA**. Compound **HA-3** (3-amino-1,8-naphthalimide) was confirmed to be generated from the solution upon oxidation and responsible for the emission at 570 nm, by comparison of spectral properties and HPLC retention time (Fig.S5, ESI†) with independently synthesized **HA-3**. However, we could not unambiguously determine the identity of the species with an emission at 460 nm. Attempts of isolation by HPLC or direct detection by LC-MS were not successful. We suppose it is an analog of **HA-3** with the amino group acylated based on the following facts. First, amino group of 4-aminophenoxyl moiety in probe **HA** was protonated in acidic media (pH < 6.5) in which the PET process was inhibited, and **HA** showed an emission maximum at 460 nm when excited at 340 nm. Compared with the fluorophore **HA-3**, the acylation of 3-amino group makes it a weaker electron donating group, which explains the hypochromatic shift of emission maximum to 460 nm. Second, variously acylated 3-aminonaphthalimides display emission maximum at 460 nm in the literatures.[15] Compound **CA** from acetylation of the fluorophore **HA-3** was synthesized and also displayed an emission band at 460 nm when excited at 340 nm (Scheme S2, Fig.S1 ESI†). Two such acylated derivatives from **HA** oxidation would be **HA-1** and **HA-2**.[16] We propose that the species is likely **HA-1** since carbamic acids like **HA-2** are known to be unstable.[17]

The selectivity of probe **HA** was also studied. The probe **HA** (20 μM) was treated with various ROS/RNS in sodium phosphate buffer (0.1 M, pH 7.4, 1% DMF) at room temperature. As shown in Fig. 2, when **HA** reacted with ClO^-, the two characteristic fluorescence peaks at 460 nm and 570 nm were observed. In contrast, other ROS species, including superoxide ($O_2^{\bullet-}$, 10 equiv), hydrogen peroxide (H_2O_2, 50 equiv), lipid peroxides (ROO•, 10 equiv) and hydroxyl radical (•OH, 15 equiv), induced nearly no fluorescence responses even after 30 min at 37 °C. HPLC studies verified that the probe was not oxidized (Fig. S6, ESI†). Whereas upon addition of peroxynitrite ($ONOO^-$, 3 equiv), a large and immediate increase of fluorescence intensity at 460 nm (ca. 68-fold) was observed (Fig. S7, S8, ESI†). Single-channel response only at 460 nm for $OONO^-$ makes it non-interfering to ClO^-, which induces a dual-channel signal at 460 nm and 570 nm, respectively. The mechanism of the probe oxidation with $OONO^-$ is currently under investigation.

The bioimage application of the probe **HA** was investigated. HeLa cells were first incubated with probe **HA** (50 μM) for 20 min at 37 °C and washed with phosphate buffer (pH 7.4). Then, the cells were treated with (or without, as the control) ClO^- (250

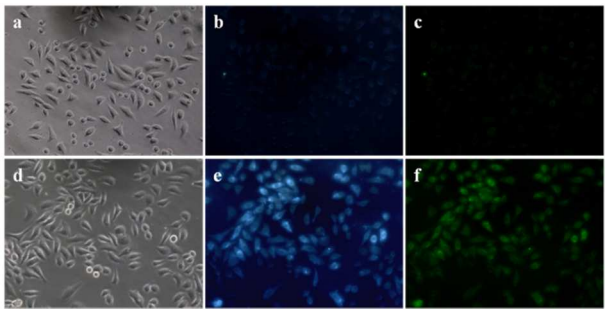


Fig. 3 Fluorescence and bright-field images of HeLa cells. (a) Bright-field image of HeLa cells incubated with **HA** (50 μM) for 20 min. (b) (c) Fluorescence image of HeLa cells incubated with **HA** (50 μM) for 20 min. (d) Bright-field image of HeLa cells pre-treated with **HA** (50 μM) for 20 min and then incubated with ClO^- (5 eq) for another 30min. (e) (f) Fluorescence image of pre-treated cells with **HA** (50 μM) for 20 min and then incubated with ClO^- (5 eq) for another 30min. The middle and right columns were collected at 430-495 nm (blue channel) and 535-600 nm (green channel), respectively.

μM) for another 30 min at 37 °C and washed three times with phosphate buffer (pH 7.4). As shown in Fig. 3b and 3c, HeLa cells without ClO^- showed nearly no fluorescence, whereas the cells treated with ClO^- displayed strong fluorescence in both blue and green channels (Fig. 3e and 3f). Fluorescence enhancements at both channels are dose-dependent with the added ClO^- (Fig. S9). 3-morpholinosydnonimine (SIN-1, an $OONO^-$ donor) induced moderate signal only at the blue channel (Fig. S10, ESI†), while other ROS did not yield any noticeable signals in both channels (Fig. S11, ESI†). Above results demonstrated the potentials of the probe **HA** to selectively and quantitatively detect ClO^- in living cells.

In summary, we have designed and synthesized a novel fluorescence probe **HA**. Good water solubility and inertness of its fluorescence to pH from 6.5 to 11.0 made the probe suitable for applications under physiological conditions. With the addition of ClO^-, probe **HA** showed a dual-channel emission at 460 nm and 570 nm. $OONO^-$ led to a single-channel enhancement at 460 nm only and other ROS/RNS induced no spectral changes. These mean that the probe **HA** could distinguish ClO^- from ROS/RNS by this dual-channel signal. Fluorescence imaging of HeLa cells also showed that the probe **HA** can be used as hypochlorite probe in living cell imaging.

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Notes and references

State key Laboratory of Bioreactor Engineering, Shanghai Key Laboratory of Chemical Biology, School of Pharmacy, East China University of Science and Technology, 130 Meilong Road, Shanghai, 200237, China. Fax: (+86) 21-64252603; E-mail: xhqian@ecsut.edu.cn.: yfxu@ecust.edu.cn;

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