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SHORT COMMUNICATION

A Highly Sensitive Fluorescent Probe for HClO and Its Application in Live Cell Imaging

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Abstract A new rhodamine-based probe **1** was designed and synthesized as a new fluorescent molecular probe for HClO in PBS buffer at physiological condition. The free probe **1** almost nonfluorescence, however, a drastic enhancement of fluorescence intensity was observed in the presence of HClO. The new probe **1** exhibits good sensitivity and selectivity for HClO over other reactive oxygen and/or nitrogen species in PBS buffer, and the probe was successfully applied to image endogeneous HClO in the living cells.

Keywords Fluorescence · HClO · Sensing · Fluorescent probe · Rhodamine

Introduction

As a kind of reactive oxygen species (ROS), hypochlorous acid (HClO) play important roles in the immune defense against microorganisms and also in inflammation [1, 2]. In living organisms, hypochlorous acid is generated by the reaction of hydrogen peroxide with chloride ions under the catalysis of the heme enzyme myeloperoxidase (MPO), which is mainly localized in leukocytes including neutrophils, macrophages and monocytes [3–6]. Maintenance of appropriate concentrations of HClO is essential for numerous cellular functions, however, many evidences suggest that excessive formation of HClO can lead to tissue damage and a series of human diseases, such as atherosclerosis, arthritis and even cancers [7]. Therefore, a rapid, sensitive, and selective detection of HClO in biological samples is of significant interest.

Several techniques including electroanalysis [8], potentiometry [9], spectrophotometry [10], chemiluminescent [11] and fluorescent methods[12–24] have been reported for the analysis of HClO. Among these methods, fluorescent probe technique is particularly attractive due to its less cell-damaging, high time and spatial resolution capability to visualize analytes of biological interest in living cells. Most recently, some fluorescent probes for HClO have been reported [12–24], most of which are mainly based on the strong oxidation property of HClO.

Rhodamine [25, 26] is known as an extraordinary class of fluorescent dyes, which has been widely used in biological application because of its excellent photochemical properties, such as high fluorescence quantum yields, large molar extinction coefficient, longer excitation and emission wavelengths, and outstanding chemical, and photochemical stabilities. Recently, rhodamine derivatives have received increasing attention for constructing fluorescent turn-on probes, as the ring-closed forms of these dyes are generally nonfluorescent, however, the ring-opened states of these dyes are high fluorescence [25–31]. Up to now, a few fluorescent probes for HClO based on rhodamine scaffold

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have been reportd. For example, Nagano's group [17] reported a rhodamine-based fluorescent probes for HClO, but the fluorescent probes involved in complicated synthesis; Ma's group [15] also developed a fluorescent probes for HClO, which worked in organic co-solvent system at pH 12 and thus the biological use was limited. These results stimulated us to explore a new fluorescent probe which can be used in biological condition such as the imaging of HClO in living cells.

In order to address the above limitations, herein, we reported a novel fluorescent turn on probe for HClO in aqueous solution by employing a rhodamine derivative **1** (Fig. 1). The new probe **1** exhibits good sensitivity and selectivity for HClO over other reactive oxygen and/or nitrogen species in PBS buffer. Moreover, probe **1** is cell membrane permeable, and can be used to image of both exogenous and endogenous HClO within living cells

Experimental

Apparatus and Reagents

The fluorescence spectra and relative fluorescence intensity were measured on a F-4500 spectrofluorimeter (Hitachi, Japan) with a 1 cm×1 cm quartz cell. The slit widths of both the excitation and emission were set at 2.5 nm. The absorption spectra were measured with a Shimadzu UV-2450 spectrophotometer (Shimadzu, Japan). NMR spectra were measured with Varian unity INOVA-400 spectrometer (Varian, United States) with tetramethylsilane (TMS) as internal standard. Cells imaging was performed with a Olympic inverted microscope (IX50, Olympic, Japan). DEA/NONOate (diethylamineNONOate, NO donor), phorbol 12-myristate 13-acetate (PMA) and dehydroascorbic acid (DHA) were purchased from Sigma-Aldrich (St. Louis, Mo.) and Lipopolysaccharides (LPS) was purchased from Beyotime Institute of Biotechnology (China). Doubly distilled water was used throughout the experiments. NO was generated from DEA/NONOate (a commercially available NO donor) [32]. A stock solution of HClO was standardized at 290 nm using an extinction coefficient 350 M^{-1} cm^{-1} at pH 12 [33]. Superoxide was generated from KO_2 in aqueous solution [32]. Hydroxyl radical was generated by Fenton reaction in which ferrous chloride was added in the presence of H_2O_2 (5.0×10^{-5} M). Singlet oxygen was generated from 3,3′-(naphthalene-1,4-diyl)dipropionic acid, which was prepared according to the literature [34].

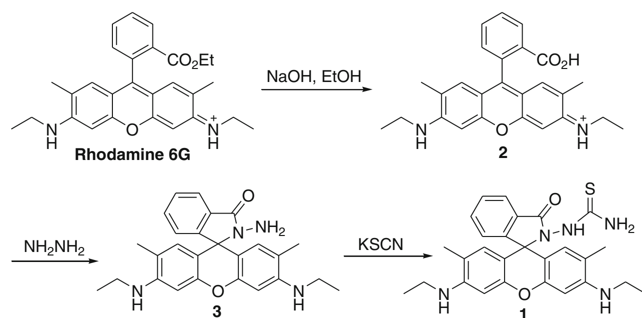

Fig. 1 The reaction mechanism of fluorescent turn-on probe **1**

Synthesis of Probe 1

The synthesis of the rhodamine derivative **1** is shown in Scheme 1. The Rhodamine **2** and **3** was prepared according to the known procedure [35]. Then, compound **1** was prepared by a simple one-step from compound **3** in acidic media (Fig. 1). Compound **3** (220.1 mg, 0.5 mmol) and KSCN (400.2 mg, 4.0 mmol) were dissolved in ethanol/water solution with 2 M HCl, the mixture was heated and refluxed for 8 h. The reaction mixture was then cooled to room temperature, and neutralized with saturated $NaHCO_3$ solution. The precipitates were filtered off and dried. The crude product was purified by silica-gel column chromatography with CH_2Cl_2 as eluent, affording desired product (82.9 g, 34 % yield) as a slightly purple solid. ^{1}H NMR ($CDCl_3$, 400 MHz), δ (ppm): 8.02(d, 1 H, *J*=7.2 Hz), 7.62

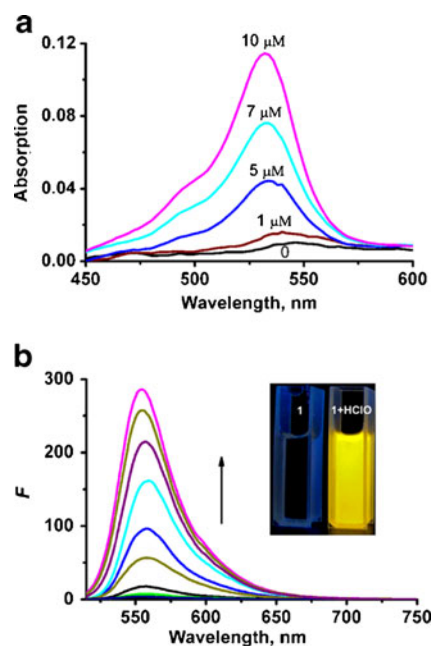

Fig. 2 **a** UV-vis spectra of probe **1** (3.0×10^{-6} M) and probe **1** upon addition of 1, 5, 7, or 10 μM of HClO in PBS (pH 7.0, containing 0.5 % DMF as cosolvent). **b** Fluorescence emission spectra of probe **1** (3.0×10^{-6} M) in PBS (pH 7.0, containing 0.5 % DMF as cosolvent) upon different concentrations of HClO (0–5.0×10^{-5} M) with an excitation wavelength of 500 nm. The insert shows the the photographs of probe **1** (3.0×10^{-6} M) in the absence of HClO or presence of HClO (1.0×10^{-5} M) under a handheld 365 nm UV lamp

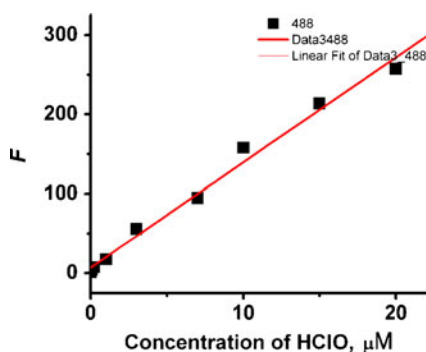

Fig. 3 Plot of the fluorescent intensity ratio at 556 nm as a function of the HClO concentration (R=0.995)

(m, 2 H), 7.18 (d, 1 H, *J*=7.2 Hz), 6.86 (s, 1 H), 6.39 (s, 2 H), 6.21 (s, 2 H), 5.75 (s, 2 H), 3.54 (q, 2 H), 3.123 (m, 4 H), 1.92 (s, 6 H), 1.35 (t, 6 H, *J*=6.8 Hz). ^{13}C NMR ($CDCl_3$, 100 MHz) δ (ppm): 184.50, 166.87, 152.65, 150.57, 148.09, 134.38, 129.05, 128.58, 126.92, 124.80, 123.72, 118.43, 104.35, 97.17, 67.00, 53.40, 38.38, 14.08. ESI-MS m/z=488.1 $[M+H]^+$.

General Procedure

Into a 5 mL volumetric flask, transfer 4.9 mL of potassium phosphate buffer (PBS buffer) solution (25 mM, pH 7.0) and a 0.02 mL of 7.5×10^{-4} M probe **1** stock solution. Then appropriate volume of HClO standard solutions (or other analytes samples) were added by a pipette. The mixture was diluted to 5 mL with PBS solution and mixed thoroughly. The resulting solution was shaken well and kept for 10 min at 25 °C and then recording the emission of the solutions excited at 500 nm.

Image Endogenous HClO in Living Cells Using Probe 1

Raw 264.7 murine macrophages were cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10 % (v/v) fetal bovine serum in an atmosphere of 5 % CO_2 in a humidified CO_2 incubator at 37 °C and the culture medium was replaced with fresh media every days. One day before the experiment, the cells was seeded on 6-well plates and allowed to adhere for 24 h.

Fig. 4 The MS (ESI^+) spectrum of compound **1** in the presence of HClO in PBS

Immediately before the experiments, the cells were washed with PBS buffer three times. Subsequently, the cells were incubated with probe **1** (3.0×10^{-6} M) for 30 min at 37 °C, and then washed with PBS three times. Subsequently, part of the treated cells was incubated with 1.0×10^{-5} M NaOCl for another 30 min at 37 °C. After the Raw 264.7 murine macrophage were washed again with PBS three times, and the fluorescence images were acquired through an Olympus inverted fluorescence microscopy equipped with a cooled CCD camera.

The living RAW 264.7 macrophages were first incubated with LPS (1 μg/mL) and IFN-γ (50 ng/mL) in culture medium for 8 h, and then further co-incubated with PMA (10 nM) and probe **1** (3×10^{-6} M) for 60 min. Prior to imaging, the cells were washed three times with PBS and the fluorescence images were acquired through an Olympus fluorescence microscopy equipped with a cooled CCD camera.

Results and Discussion

Absorption and Fluorescence Properties Studies

Probe **1** was evaluated for its ability to detect HClO under near physiological conditions (0.25 mM phosphate buffer, containing 0.5 %N,N-dimethylformamide (DMF) as a cosolvent, pH 7.0). The changes of the absorption and fluorescence emission spectra of probe **1** in the absence or presence of HClO in the PBS solution were displayed in Fig. 2. The probe **1** itself displayed almost no absorption at 530 nm and background fluorescence at around 556 nm (quantum yield : 0.002) [36], indicating that probe **1** are the spirocyclic form in the solution. However, addition of HClO triggered a dramatic increases fluorescence intensity (around 280-fold) with the maximum emission peak at 556 nm (Fig. 2b) and the fluorescence quantum yield increased up to 0.39. The results of the absorption titration studies are in good agreement with the turn-on fluorescence response (Fig. 2a). Furthermore, the fluorescence intensity of probe **1** solution was good linearly (R=0.995) proportional to the HClO concentration from 5.0×10^{-8} M to 2.0×10^{-5} M (Fig. 3) with detection limit of 1.5×10^{-8} M (based on S/N=3) in PBS solution. This shows that probe **1** has a high sensitivity for detecting HClO. In addition, the addition of HClO immediately turned the visual emission color of the probe **1** solution from dark to yellow (inset of Fig. 2b) when excited at a hand-held 365 nm UV lamp, which further supports the fluorescence turn-on response. The Ms-spectra indicated that HClO reaction with probe **1** formation of a ring-opened rhodamine 1,3,4-oxadiazole **4** (Fig. 4).

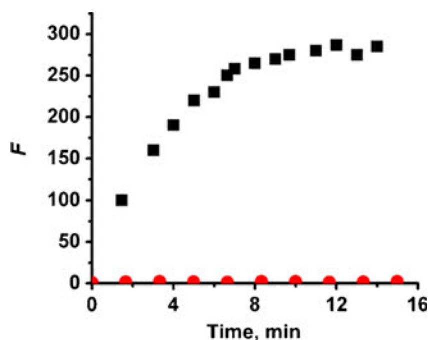

Fig. 5 Time course of the response of probe **1** (3.0×10^{-6} M) in the absence of HClO (●) and presence of 5.0×10^{-5} M of HClO (■) in PBS (pH 7.0, containing 0.5 % DMF as a cosolvent). The fluorescent intensities at 556 nm were continuously monitored at time intervals

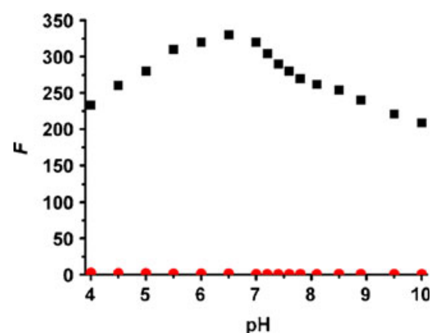

Fig. 6 Effect of pH on the fluorescence intensity of probe **1** (3.0×10^{-6} M) in the absence (●) and presence of HClO (■) (5.0×10^{-5} M) in PBS (pH 7.0, containing 0.5 % DMF as a cosolvent)

Effect of Reaction Time

The effect of the reaction time on the fluorescence intensity of the reaction system was studied and the results are shown in Fig. 5. Upon addition of HClO to the solution of probe **1** in PBS (pH 7.0, containing 0.5 % DMF as cosolvent), the fluorescence intensity of the detection system was recorded as a function of reaction time at the maximum emission peak at 556 nm. It can be seen that the fluorescence signal of the detection system increased rapidly with the reaction time

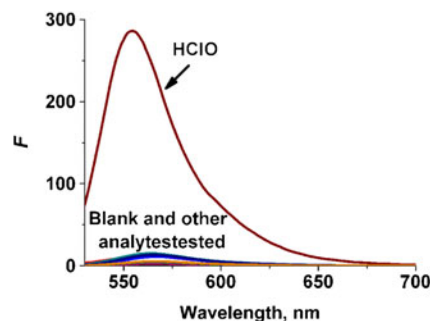

Fig. 7 Fluorescence response of probe **1** (3.0×10^{-6} M) to HClO (5.0×10^{-5} M) and other biologically relevant analytes (H_2O_2, TBHP, OH•, $O_2^{-\bullet}$, $^1O_2$, NO, CH_3COOOH, Na^+, K^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{3+}, Cl^-, PO_4^{3-}, NO_2^-, NO_3^-) in PBS (pH 7.0, containing 0.5 % DMF as cosolvent)

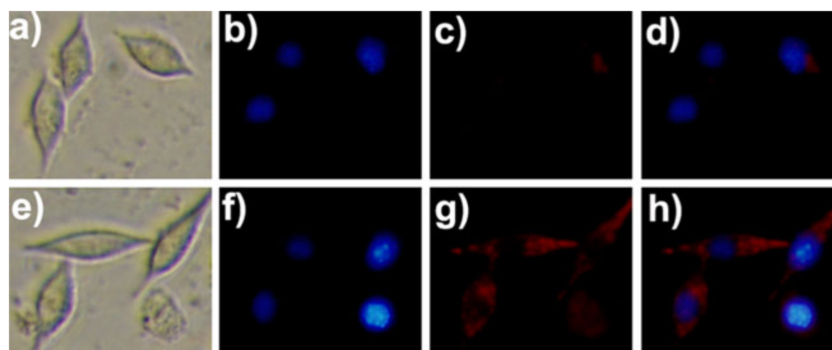

Fig. 8 Images of RAW 264.7 macrophages treated with probe **1**. **a** Brightfield image of RAW 264.7 macrophages incubated with probe **1** (3.0×10^{-6} M) only for 30 min; **b** Fluorescence image of (**a**) from blue channel; **c** Fluorescence image of (a) from red channel; **d** overlay of the blue, and red channels. **e** Brightfield image of RAW 264.7 macrophages incubated with probe **1** (3.0×10^{-6} M) for 30 min and then further treated with NaOCl (1.0×10^{-5} M); **f** Fluorescence image of (e) from blue channel; **g** Fluorescence image of (**e**) from red channel. **h** overlay of the blue, and red channels

prolonged, and the fluorescence signal reached maximum in less than 10 min. At the same time, it can be observed that the free probe **1** exhibited no noticeable changes in the emission intensity at 556 nm in the PBS buffer solution. Therefore, to obtain a high sensitive and reproducible result, a 10 min reaction time was selected in the following experiment.

Effect of pH

To study the practical applicability, the effects of pH on the fluorescence response to HClO of the new probe **1** were also investigated. The effect of pH on the fluorogenic reaction was studied in the range of 4–10 in PBS (pH 7.0, containing 0.5 % DMF as a cosolvent), and the results are shown in Fig. 6. As anticipated, probe **1** is stable within the pH range of 4–10, indicating that probe **1** are the spirocyclic form in this range. On the other hand, it readily reacts with HClO within the biologically relevant pH range (4–10) and shows a strong fluorescence. Such findings led us to consider that **1** could be used to detect the presence of cellular HClO without interference from pH effects.

Spectra Titration of Probe 1 with Biologically Relevant Analytes

For an excellent probe, high selectivity is a matter of necessity. To investigate the selectivity, the specificity of probe **1** toward HClO was determined by the fluorescence experiments. Probe **1** (3.0×10^{-6} M) was treated with various biologically relevant analytes (HClO, H_2O_2, TBHP, OH•, $O_2^{-\bullet}$, $^1O_2$, NO, CH_3COOOH, Na^+, K^+, Ca^{2+}, Mg^{2+}, Zn^{2+},

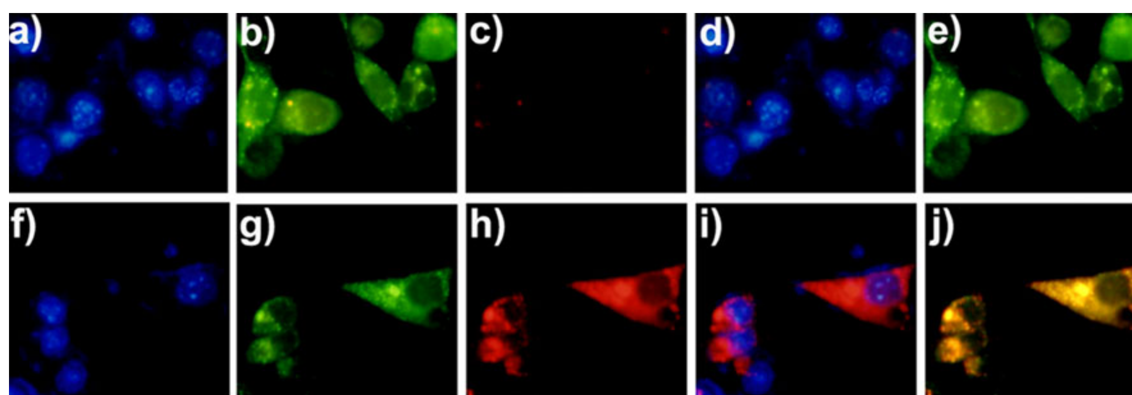

Fig. 9 Fluorescence imaging of endogenously HClO in RAW 264.7 macrophages cells. **a**–**e** fluorescence image of RAW 264.7 macrophages cells co-stained with probe **1** (3.0×10^{-6} M, containing 0.5 % dimethyl sulfoxide (DMSO)), Hoechst 33258, and MitoTracker Green for 60 min at 37 °C: **a** fluorescence image from blue channel (nuclear staining); **b** fluorescence image from green channel (mitochondria staining); **c** fluorescence image from red channel; **d** overlay of the blue and red channels; **e** overlay of the green and red channels. **f**–**j** Fluorescence images of RAW 264.7 macrophages cells stimulated with LPS (1 μg/mL) and IFN-γ (50 ng/mL) in culture medium for 8 h, and then further co-incubated with PMA (10 nM) and **1** (3.0×10^{-6} M), Hoechst 33258, and MitoTracker Green for 60 min. **f** fluorescence image from blue channel (nuclear staining); **g** fluorescence image from green channel (mitochondria staining); **h** fluorescence image from red channel; **i** overlay of the blue and red channels; **j** overlay of the green, and red channels

Fe^{3+}, Cl^-, PO_4^{3-}, NO_2^-, NO_3^-) in PBS (pH 7.0). As exhibited in Fig. 7, the fluorescence intensity of probe **1** was almost not influenced by the addition of H_2O_2, TBHP, OH•, $O_2^{-\bullet}$, $^1O_2$, NO, CH_3COOOH, Na^+, K^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, Fe^{3+}, Cl^-, PO_4^{3-}, NO_2^-, NO_3^-, respectively. However, under the same conditions, probe **1** exhibits a larger fluorescence response to HClO. These observations indicate that probe **1** is a highly selective fluorescence probe for HClO among various other biologically relevant species under physiological condition.

Fluorescent Imaging of HClO in the Living Cells

The desirable features of probe **1** such as fast response, high sensitivity, working well at physiological pH, and high selectivity encouraged us to further evaluate the potential of the probe **1** for imaging HClO in living cells. The pictures of fluorescence images were taken by inverted fluorescence microscope. The RAW 264.7 macrophages cells incubated with only probe **1** (3.0×10^{-6} M) exhibited almost no fluorescence (Fig. 8c) in red channel. By contrast, the cells stained with both the probe **1** and HClO showed bright fluorescence (Fig. 8g) in red channel, in good agreement with the fluorescence turn-on profile of the probe **1** in the presence of HClO in the solution. These results indicated that probe **1** is cell membrane permeable and capable of sensing HClO in the living cells. Notably, the brightfield imageing (Fig. 8a and e) and nuclear staining with Hoechst 33258 (Fig. 8b, d, f and h) suggests that the probe has no obvious cytotoxicity to the cells.

The new probe **1** was further applied to detect endogenously produced HClO in living macrophage cells (Fig. 9). It have been reported that macrophage cells may produce endogenous HClO when macrophage cells stimulated by phorbol myristate acetate (PMA) [37, 38]. RAW 264.7 macrophages cells co-incubated with probe **1**, Hoechst 33258, and MitoTracker Green for 60 min at 37 °C display almost no fluorescence in red channel (Fig. 9c). However, the macrophage cells first incubated with LPS (1 μg/mL) and IFN-γ (50 ng/mL) in culture medium for 8 h, and then further co-incubated with PMA (10 nM) and probe **1** (3.0×10^{-6} M), Hoechst 33258, and MitoTracker Green for 60 min at 37 °C exhibit a bright fluorescence in the red channel (Fig. 9h). Therefore, this method provides a facile way to visualize the endogenously produced HClO in living macrophage cells. Notably, the nuclear staining with Hoechst 33258 (Fig. 9a, d, f, and i) suggests that the sensor has no obvious cytotoxicity to the cells. In addition, the mitochondria staining (Fig. 9b, e, g, and j) reveals that probe **1** associates with the mitochondria of RAW264.7 macrophage cells.

Conclusions

In conclusion, the current study successfully developed a rhodamine-based HClO-reactive fluorescent probe in an aqueous environment. Probe **1** exhibits a pH insensitive, fast and 'off-on' fluorescence response to HClO over other reactive species and most of the common metal ions. The probe quick fluorescent response was linearly proportional to the amount of HClO under near physiological conditions. Furthermore, the probe was cell membrane permeable and could respond to the changes of HClO concentrations in living cells without causing cellular damage. All these unique features demonstrates the feasibility of the probe for HClO level monitoring in biological samples.

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