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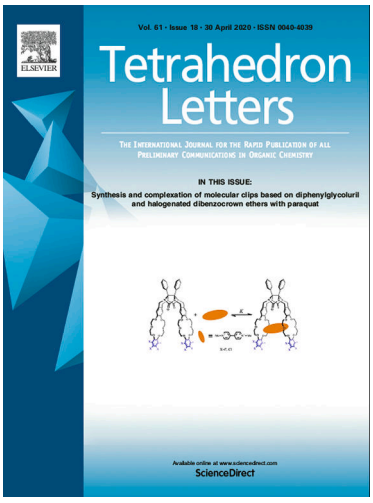

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Endoplasmic reticulum-targetable fluorescent probe for visualizing HClO in EC1 cells

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ABSTRACT

The endoplasmic reticulum is the largest organelle in the cell. Studies have shown that the concentration of HClO is related to many diseases. A new type of probe R1 has positioned to the endoplasmic reticulum. The probe shows good positioning effect, excellent selectivity and high sensitivity, excellent anti-interference, and can quickly reach the response platform. The obvious color change can be used for naked eye observation. Due to its low cytotoxicity, probe R1 was successfully used for EC1 cells imaging under a laser scanning confocal microscope.

1. Introduction

Reactive oxygen species and reactive nitrogen species play important roles in biological pathways and stress processes [1-3]. Hypochlorous acid (HClO) is one of the most important reactive oxygen species produced by peroxides and chloride ions catalyzed by bone marrow oxidase in active organisms [4,5]. It is an extremely important germicidal oxidant in the immune system and also widely used as a bleach and disinfectant for drinking water in daily life [6,7]. In the physiological environment, the normal concentration of HClO is beneficial to the human body and is necessary to maintain the normal function of living cells [8]. However, excessive production of HClO may cause tissue damage and many diseases such as arthritis, kidney disease, lung injury, cardiovascular disease, asthma and even cancer [9-13]. Most of the developed HClO detection methods such as iodometric method, colorimetric and polarographic methods have limitations on organisms [14,15]. Therefore, new fluorescent probes need to be developed for the selective and sensitive detection of HClO [16-20]. The fluorescent probes can be used for biological analysis and labeling [21-24]. And they have become the basis for sensing biological molecules in living systems due to their low cost, simplicity, excellent selectivity, high sensitivity, and real-time analysis [25,26].

The endoplasmic reticulum (ER) is the largest organelle in cell and plays an important role in the synthesis of biological proteins. ER homeostasis can be disturbed by misfolded and unfolded protein accumulation in the ER cavity. This condition is called ER stress. When misregulated, the accumulation of HClO may cause oxidative damage to cellular proteins. It can even trigger apoptosis by cleaving caspase-12 in the ER [27-30], leading to apoptotic neurodegenerative and cardiovascular diseases, metabolic diseases and cancers [31]. Therefore, it is of great significance to develop an effective HClO analysis method in ER.

Although some fluorescent probes for HClO were reported[16-18], they suffered from co-organic solvent or slow response speed. To address this, here, we developed a simple and effective probe **R1**, which consisted of a fluorophore, *p*-toluenesulfonamide and a HClO-responsible site dimethylthiocarbamate (DMTC). *p*-Toluenesulfonamide is typical ER targetable group[32-34]. The targeting mechanism maybe due to its good binding to lots of protein in ER. In the presence of HClO, the DMTC part of **R1** was oxidized, and intramolecular charge transfer (ICT) was turned on, then fluorescence emission appeared. As expected, this probe can target ER and sensitively measure HClO in PBS buffer. After adding HClO, it will show a very rapid response in 30 S. When reaching the response platform, the fluorescence intensity increased by 12 times. And co-localization experiments also showed that **R1** has a good positioning ability for endoplasmic reticulum and good biocompatibility.

2. EXPERIMENTAL SECTION

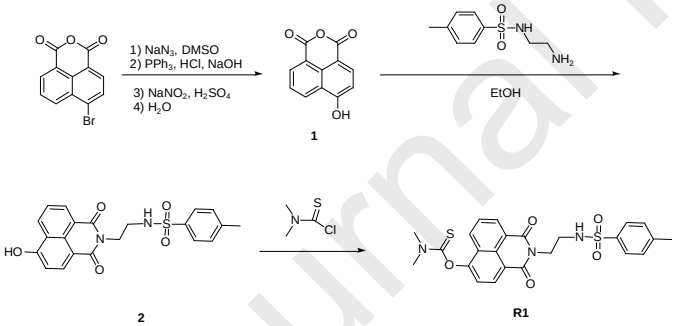
Apparatus, chemical and reagents. The absorption spectra were precisely gauged employing the UV-2102 dual beam UV/Vis spectrophotometer. We used a F-4500 FL spectrophotometer with a 10 mm quartz cuvette for fluorescent spectroscopy record. Its excitation and emission wavelength band pass was set to 5.0 nm. The pH was surveyed using a DAPU PHS-3C instrument. Employing TMS as an inner standard, nuclear magnetic resonance spectra were recorded on a Bruker DTX-400 spectrometer. Mass spectrometry was performed with high-performance liquid chromatography Q-T HR-MS. Reagents and materials gained from commercial suppliers were used directly. The water used in the experiment was fresh double distilled water.

The synthesis of probe R1. *Compound **1*** was synthesized by reported methods [35]. ^{1}H NMR (400 MHz, DMSO-d_6): δ 12.35 (s,

1H 8.42 (d, *J* = 8.5 Hz, 1H), 7.81 (dd, *J* = 8.3, 7.3 Hz, 1H), 7.22 (d, *J* = 8.1 Hz, 1H); ^{13}C NMR (100 MHz, DMSO-d_6): 161.94, 161.88, 160.81, 136.18, 133.55, 132.37, 130.66, 126.53, 123.03, 119.13, 110.96, 110.96. (Figures S1−S2).

*Synthesis of Compound **2***: Compound 1 (1.070 g, 5 mmol) and *N*-(2-amino-ethyl)-4-methyl-benzenesulfonamide (1.605 g, 7.5 mmol) were dissolved in 60 mL of ethanol under nitrogen, and heated at 80 °C overnight. After the reaction was completed, the solvent was removed under reduced pressure. The residue was purified by column chromatography (dichloromethane : methanol = 30 : 1). Compound **2** was obtained as a tan solid in 51% yield. ^{1}H NMR (400 MHz, DMSO-d_6): δ 11.83 (s, 1H), 8.55 (d, *J* = 8.4 Hz, 1H), 8.54 (d, *J* = 7.2 Hz, 1H), 8.45 (d, *J* = 8.1 Hz, 1H), 7.79 (dt, *J* = 12.5, 7.0 Hz, 2H), 7.74 (d, *J* = 7.2 Hz, 2H), 7.70 (d, *J* = 7.4 Hz, 2H), 7.60 (d, *J* = 8.3 Hz, 1H), 4.09 (t, *J* = 6.4 Hz, 2H), 3.06 (q, *J* = 6.1 Hz, 2H), 2.26 (s, 3H); ^{13}C NMR (100 MHz, DMSO-d_6): δ 164.17, 163.45, 160.67, 142.86, 138.06, 133.86, 131.42, 129.89, 129.67, 129.23, 126.80, 125.88, 122.74, 122.20, 113.02, 110.27, 40.63, 39.46, 21.30. (Figures S3−S4).

*Synthesis of **R1***: Compound **2** (410 mg, 1 mmol) and dimethylthiocarbamoyl chloride (618 mg, 5.0 mmol) were dissolved in 10 mL of anhydrous dichloromethane, followed by the addition of 400 uL of *N*, *N*-diisopropyl ethylamine. The mixture was stirred at room temperature for 4 h, and the dichloromethane was removed under reduced pressure. Purification by column chromatography (dichloromethane) gave a pale-yellow solid **R1**, Yield 43%. ^{1}H NMR (400 MHz, DMSO-d_6): 8.47 (dd, 2H, *J*=8.0 Hz, 4.8 Hz), 8.28 (d, 1H, *J*=8.4 Hz), 7.88 (t, 1H, *J*=8.0 Hz), 7.78 (t, 1H, *J*=6.4 Hz), 7.59 (dd, 3H, *J*=8.0 Hz, 5.6 Hz), 7.25 (d, 2H, *J*=8.0 Hz), 4.12 (t, 2H, *J*=6.4 Hz), 3.51 (s, 3H), 3.44 (s, 3H), 3.09 (d, 2H, *J*=6.4 Hz), 2.26 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6): 190.6, 168.6, 168.1, 159.6, 147.7, 142.8, 136.2, 136.2, 134.7, 133.8, 133.6, 132.7, 130.8, 127.7, 126.6, 125.1, 44.1, 48.3, 26.1. HR-MS: *m/z* calcd for $C_{24}H_{24}N_3O_5S_2^+$ $[M]^+$: 498.1152, found: 498.1150.



Scheme 1. Synthesis of probe **R1**

Optical studies. A stock solution of **R1** (1 mM) was dissolved with DMSO. Stock solutions (1-10 mM) of other analytes, containing amino acids (for instance, Hcy, GSH, Cys), and corresponding salts (such as KI, KBr, NaCl, NaF, $NaHSO_3$,Na_2SO_4, $Na_2S_2O_5$, $Na_2S_2O_8$, Na_2CO_3, $NaHCO_3$, K_3PO_4, $NaNO_3$, $NaNO_2$, Ac^-, NaHS, $ONOO^-$, K^+, Na^+, H_2O_2, O^{2-}, NO., HClO) were prepared in ultra-pure H_2O. For optical studies, 3.0 mL of **R1** (10 μM) in PBS (pH=7.4, 10 mM) buffer solution was decanted in a quartz cuvette. The UV or fluorescent spectra was measured after adjunction of analytes. The excitation voltage is set to 700V. For the reaction kinetics of **R1** with HClO, 10 mM of HClO was incubated with 1 mM of **R1** in PBS at 37 °C. Next, fluorescent intensity was recorded at a different time point.

and four seasons serum. 1% streptomycin, 10% fetal bovine serum (FBS), and 1% penicillin were added to the Petri dish. The cells were then placed in a cell incubator containing 5% CO_2 and cultured at 37 °C. One day before imaging, the cells were digested into confocal dishes for subsequent experiments.

Cytotoxicity test. EC1 cells were prepared into a density of 10^6 cell / mL with Petri dish , and then the cells were seeded into 96-well plates. 100 μL of medium was added to each well and placed in a cell culture incubator (5% CO_2 / 95% Air). Medium culture, after attachment is completed, adding a series of probe solutions (0 μM, 2.5 μM, 4 μM, 6 μM, 8 μM, 10 μM) to 96-well plates, and placing them in the cell culture incubator (5% CO_2 / 95% Air) continuely incubating for 24 h, aspirating the culture medium from the 96-well plate, adding 100 μL of culture medium, and then adding 10 μL of CCK-8 solution to each well, and continuely in the cell incubator (5 % CO_2 / 95% Air) for 1-4 h, and then taking out culture medium under the absorbance at 450 nm on a microplate reader.

Cells imaging. EC1 cells were plated on Petri dish for 24 hours. Cells were washed using PBS before the experiments. In order to know the subcellular position of **R1**, the cells were stained with endoplasmic reticulum sensor, mitochondrial staining sensor, and lysosomal staining sensor for 30 minutes. Cells imaging was obtained by Leica TCS SP8 confocal microscope.

3. CONSEQUENCE AND DISCUSSION

The synthetic route of **R1** was shown in Scheme 1. The title compound was prepared through three steps and was well characterized by ^{1}H NMR, ^{13}C NMR and ESI-HRMS (Fig. S5–S7).

First, the **R1** kinetics was measured (Fig. 1a). After adding 10 equivalents of HClO, the response was very quick within 30 seconds. We analyzed the selectivity of fluorescent probe **R1** to HClO, other amino acids and inorganic salts (Fig. 1b). It can be seen that the strong fluorescence appeared at 550 nm, after adding 10 equivalents of HClO. The fluorescence intensity was increased by 11.6 times. After adding other amino acids and inorganic salts, the fluorescence intensity hardly changed. Therefore, the fluorescent probe **R1** has high selectivity. In addition, a competitive experiment (Fig.1c) was carried out. After adding 10 eq. of HClO in the presence of other amino acids and inorganic salts, it was gratifyed that the fluorescence intensity of R1 was significantly enhanced. Therefore, the fluorescent probe **R1** has excellent anti-interference with other amino acids and inorganic salts. A comparison of the reported probes with the probe R1 was list in Table. S1. It can be found that the performance of the synthesized probe was good.

In order to study the sensitivity of fluorescent probe **R1** to HClO in PBS buffer solution, we measured the fluorescence titration experiment (Fig.2a). As can be seen from the figure, when the content of HClO was gradually increased, the fluorescence intensity at 550 nm was gradually enhanced. As shown in Fig.2b, the fluorescence intensity of the probe had a good linear relationship with HClO (0.1-0.35 eq.), and the correlation coefficient was as high as 0.9971 and with a detection limit of 12.59 nM (S/N=3), which was better than other methods for detecting HClO in endoplasmic reticulum. The quantum yield (Φ) of Y1 and the probe R1 are 0.12 and 0.014 respectively using coumarin 6 as a reference.

The UV-selective spectrum of probe **R1** (Fig.S8) showed that absorption of **R1** at 450 nm is only 0.03 after adding other amino acids and inorganic salts. The absorption of **R1** at 450 nm was enhanced after the addition of HClO. It indicated that probe **R1** can specifically recognize HClO. We also tested the UV titration spectrum of probe **R1** (Fig. S9). As the concentration of HClO increased, the UV absorbance also increased.

3, the probe was insensitive to pH in the range of pH 3-6. In the range of 7-13, when 10 eq. of HClO was added, the fluorescence intensity at 550 nm was obviously enhanced, indicating that **R1** was widely tolerated to pH. Since the normal physiological environment of the human was neutral, the probe **R1** can be well applied to the endoplasmic reticulum of the cell, which was also useful for further cell imaging.

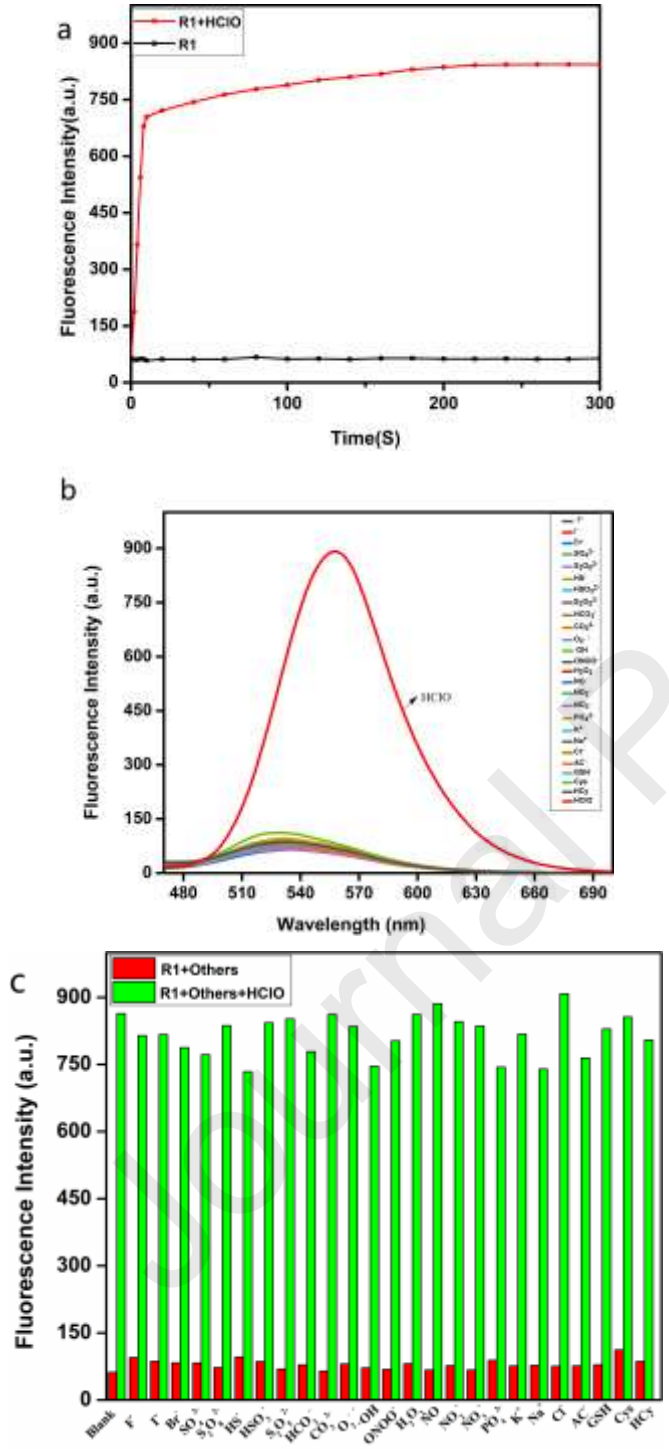


Fig. 1. (a) Fluorescent kinetics of **R1** (10 μM). Fluorescence was calendared at 550 nm. (b) The fluorescent intensity of **R1** (10 μM) with HClO (10 equiv.) and other various analytes (10 equiv.). (c) The fluorescent intensity of **R1** (10 μM) at 550 nm changes upon the addition of various analytes (100 μM) in the presence of ClO^- (100 μM).

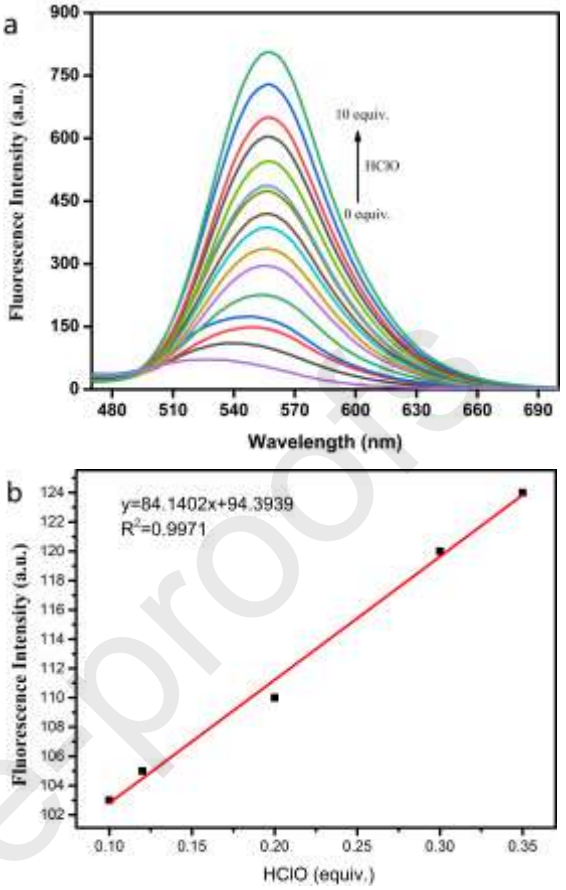


Fig. 2. (a) Fluorescent emission spectra of **R1** (10.0 μM) in the presence of ClO^- (0–10 equiv.). (b) Linear plot of the fluorescence intensity at 550 nm against NaClO concentrations in the PBS buffer (10 mM, pH = 7.4 λ_{ex}= 450 nm, slit: 5 nm).

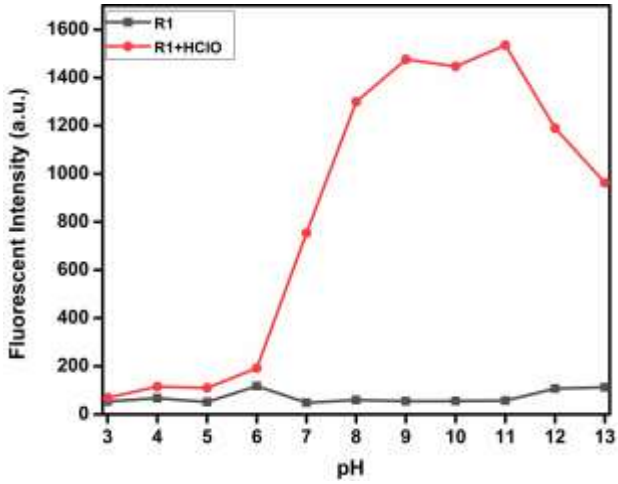
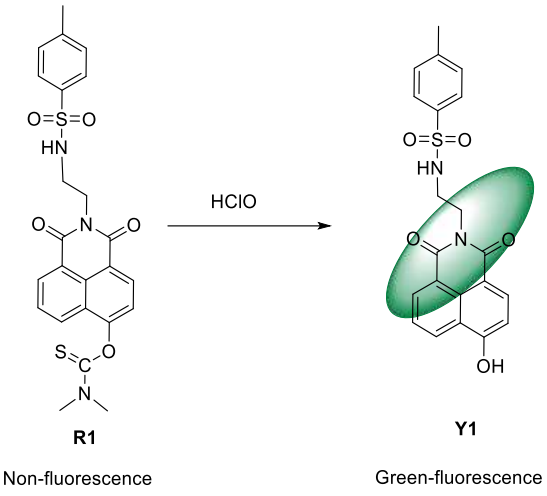


Fig. 3. The fluorescent intensity (at 550 nm) of **R1** (10 μM) in the PBS buffer under different pH (3–13) in the absence and presence of NaOCl (10 equiv.) (PBS 10 mM, pH = 7.4 λ_{ex}=450 nm, slit: 5 nm).

According to the reported literature [36,37], we gave a possible response mechanism (shown in Scheme 2). Under the addition of HClO, the thiourethane moiety in the probe **R1** underwent S-oxidation to release SO_2, then compound R1 was hydrolyzed to the compound Y1, and the ICT was turned on. So the fluorescence

recognition mechanism, the HR-MS of **R1** with HClO was measured. After adding HClO to the test system of probe **R1**, the mixture was tested by high resolution mass spectrometry (Fig. S10). New peak $[M + H]^+$ 411.1010 was found, indicating that compound Y1 (theoretical value $[M + H]^+$ = 411.1009) was formed after **R1** responding to HClO.



Scheme 2. The proposed sensing mechanism of **R1** for detecting ClO^- based on ICT.

Before imaging application of **R1** in cells, the cytotoxicity assay of probe **R1** was determined (Fig.4). After incubating EC1 cells with different concentrations of probe **R1** for 24 h, it can be seen that the cells survival rate was as high as 86% as the concentration of the 10 μM of the probe. The probe **R1** showed very low toxicity to cells in the range of 0-10 μM.

In order to determine whether **R1** can be localized to the endoplasmic reticulum, a cell co-localization experiment was performed (Fig.5). From Fig.5, it can be seen that the probe showed green fluorescence after aligning with HClO. Commercially positioned endoplasmic reticulum dyes (Fig. 5c), mitochondrial dyes (Fig. 5k), and the localized lysosomal dyes (Fig. 5g) showed red fluorescence. The overlap coefficient between probe **R1** and the endoplasmic reticulum dye was as high as 0.8052, while the overlap coefficient between probe **R1** and mitochondrial dye was 0.2091, and the lysosomal dye overlap coefficient was 0.0280, indicating that the probe **R1** can locate the endoplasmic reticulum well. It may provides a good tool for us to further reveal the relationship between the endoplasmic reticulum and active oxygen.

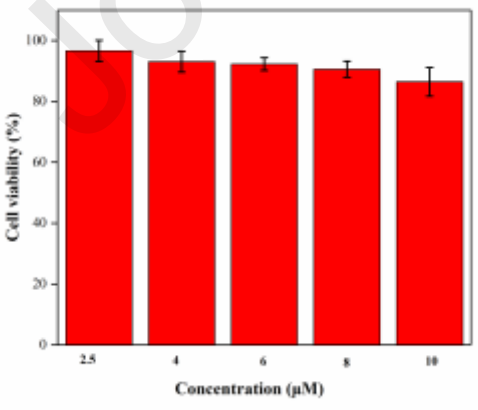


Fig. 4. Different **R1** concentrations (2.5 μM, 4 μM, 6 μM, 8 μM, and 10 μM) were

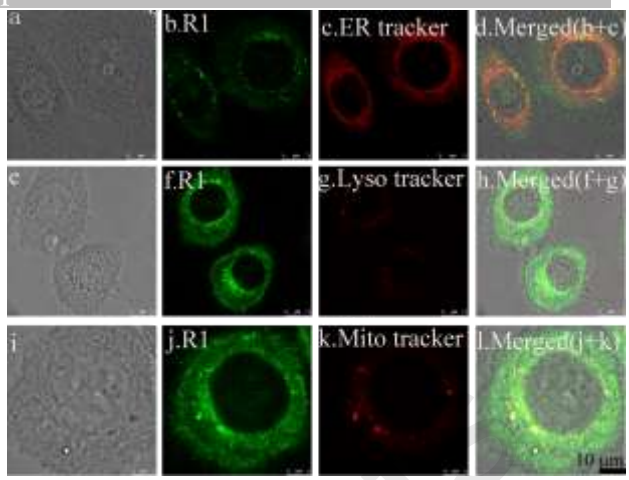


Fig. 5. Co-localization experiment of **R1** (10.0 μM) with various organelle specific markers in EC1 cells. The panels (b, f and j) showed the fluorescence imaging of **R1** (λ_{ex}=488 nm, λ_{em}=510-570 nm). The panels (c, g and k) showed the images of ER Tracker (1.0 μM) (λ_{ex}=552 nm,λ_{em}=538-640 nm), Lyso-Tracker (500.0 nM) (λ_{ex}=552nm, λ_{em}=538-620 nm) and Mito-Tracker (500.0 nM) (λ_{ex}=638 nm, λ_{em}=608-670nm), respectively. The panels (d, h and l) showed the images of b + c, f + g, and j + k merging, respectively.Scale bar = 10 μ m

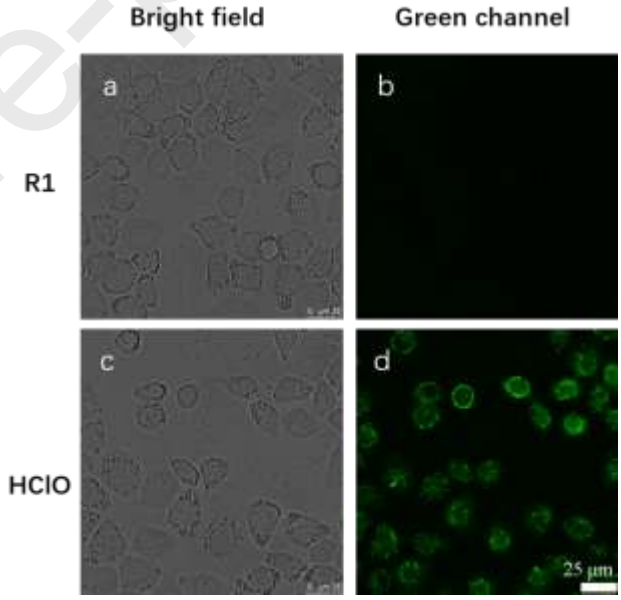


Fig.6. Confocal fluorescence images of ClO^- with **R1** in EC1 cells. (b) EC1 cells pretreated **R1** (10 μM) for 30 min. (d) EC1 cells were pretreated with probe **R1**(10 μM) and then incubated with HClO (100 μM) for 30 min. (a) and (c): bright field images of (b) and (d), correspondingly.Scale bar =25 μ m

Next, we further performed **R1** cell bioimaging experiments, and allowing the cells to incubate with 10 μM **R1** for 30 min. It can be seen from Fig. 6b that no obvious fluorescence appeared in the green channel. And after adding 10 eq. of ClO^-, obvious green fluorescence appeared (Fig. 6d). Thus, the cell membrane permeability of **R1** was confirmed, and biofilming of HClO in a biological sample was possible.

4. Conclusion

In conclusion, we synthesized a probe that located the

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probe can quickly reach the response platform. The probe has good selectivity, excellent anti-interference, and good cell penetration. Furthermore, it can be well imaged in cells, and positioned to the endoplasmic reticulum.

Declaration of competing interest

There are no conflicts to declare.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://...

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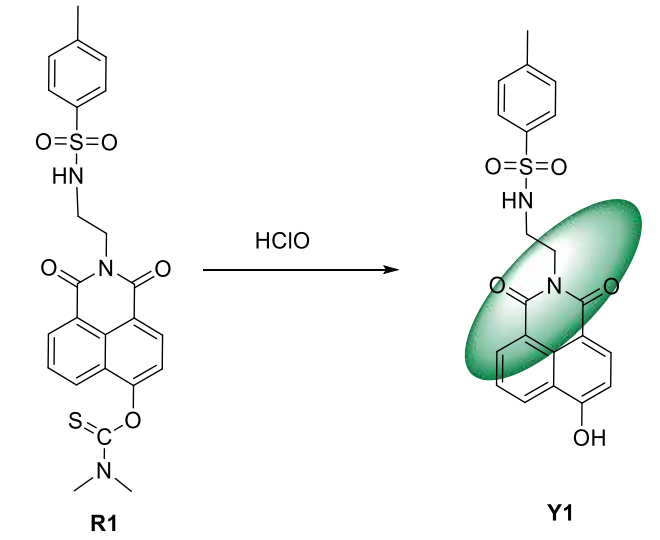

A ER-targetable probe R1 for HOCl was synthesized. R1 shows excellent selectivity and high sensitivity, excellent anti-interference, and can quickly reach the response platform.

1. probe **R1** for HClO was developed.
2. Probe have rapid response (30 s), big Stock's shift and a wide range of pH(7-13).
3. The imaging in EC1 cells demonstrated its value of practical application.

Declaration of Interests

We submit this paper which title is " Endoplasmic reticulum-targetable fluorescent probe for visualizing HClO in EC1 cells " and declare that we have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.