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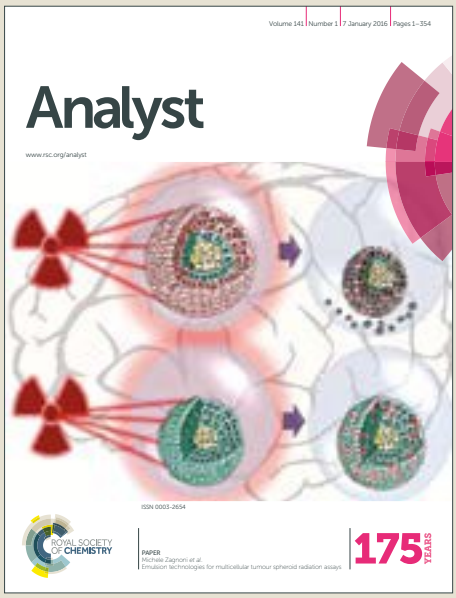

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A S-Alkyl Thiocarbamate-Based Biosensor for Highly Sensitive and Selective Detection of Hypochlorous Acid

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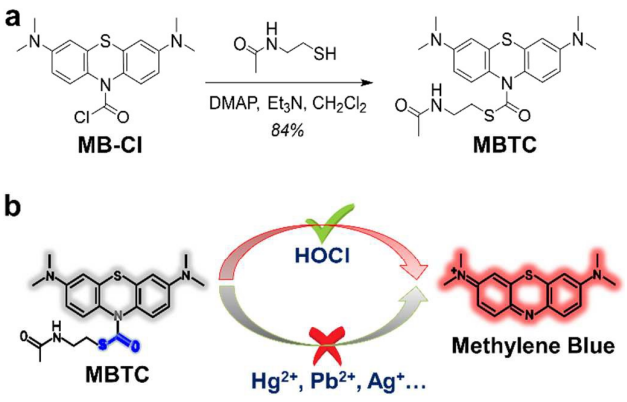
We reported a near infrared biosensor that features a dihydromethylene blue and a S-alkyl thiocarbamate linker to detect hypochlorous acid in a drastic fluorescence turn-on response. We achieved high sensitivity at a nanomolar level and high selectivity that resolves the interference issue with mercury ion and other transition metal ions. We demonstrated the response mechanism by analysing the released segments, and investigated imaging capability to detect both exogenous and endogenous hypochlorous acid in living cells.

As one of endogenous reactive oxygen species (ROS), hypochlorous acid (HOCl) plays a protective role in the immune defense due to its strong bactericidal effect.[1] However, excessive amounts of HOCl mainly generated by activated neutrophils can damage proteins, induce cell lysis, and cause various diseases such as Alzheimer disease, inflammatory bowel disease, and rheumatoid arthritis.[2-4] Recently, increasing efforts have been made into exploitation of detection methods sensitive and specific for HOCl by taking advantage of chemiluminescence[5], bioluminescence[6], colorimetric analysis[7], electrochemical analysis[8], fluorescence[9], etc. Varieties of fluorescent HOCl probes have been developed on a basis of the reactivity of HOCl toward amines, oximes, hydrazines, or S/Se/Te groups.[10] For example, thiospirolactone is exclusively reactive to HOCl rather than other ROS via hydrolysis and thus frequently installed as a lock in fluorophores including fluorescein and rhodamine for HOCl detection.[11-13] However, potential interference from metals such as Hg^{2+} and Pb^{2+} has to be carefully considered, since thiospirolactone is also a well-known trigger for these transition metal ions.[14, 15] Hence, besides excellent sensitivity, high specificity over a broad spectrum of reactive species and transition metals urges design of new HOCl probes that are compatible with biological systems.

A few studies demonstrate that O-alkyl thiocarbamate could be readily recognized by HOCl, however, with complicated proposed mechanism regarding the production of fluorescently detectable aryloxy species.[16, 17] In this work, we designed a methylene blue (MB) derivative possessing a S-alkyl thiocarbamate linkage that is expected to be HOCl reactive and release an arylamine product (Scheme 1). As a model fluorophore, although with a relatively low fluorescence quantum yield, MB is FDA-approved for methemoglobinemia use and has absorption and emission in the infrared region that is crucial for in vivo imaging studies.[18, 19] The probe molecule MBTC was synthesized in a high yield (84%) from a thiol esterification reaction (Scheme 1a). The lack of π-conjugation in the backbone makes MBTC nonfluorescent, and the recognition with HOCl can be indicated by the released MB as an arylamine product in a fluorescence "turn on" response (Scheme 1b).



Scheme 1 (a) The synthesis of MBTC. (b) The chemical conversion of MBTC to methylene blue, mediated by HOCl rather than transition metal ions like Hg^{2+}, Pb^{2+}, and Ag^{+}.

MBTC was obtained as a colorless solid, characterized by NMR and HRMS (Fig. S1a, S2a&S3). Lacking π-conjugation and

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electronic pull-push effect, MBTC has minimal absorption in the visible and infrared regions. Upon titration of NaOCl, as a HOCl donor, into the solution of MBTC (5 μM) in PBS buffer (20 mM, pH 7.4), characteristic absorption of MB showed up rapidly with a peak at 666 nm (Fig. 1a). The optical intensity increased with the gradual addition of NaOCl, showing a linear dependence on NaOCl concentration (0-1.0 μM) (Fig. 1b). The colorless reaction mixture turned blue in the course of NaOCl titration (Fig. S4). Meanwhile, MBTC has extremely low background signal in fluorescence, and dramatic fluorescence enhancement at 690 nm was detected upon the addition of NaOCl (Fig. 1c). A large fluorescence enhancement up to 1700-fold was achieved in the presence of 10 μM NaOCl, comparable to the deformylation-based probe.[20] Consistent to the spectrophotometric analysis, the fluorescence response was linear with increasing concentration of added NaOCl at 0-1.0 μM ranges (Fig. 1d). The limit of detection (LOD) was evaluated according to the equation LOD = 3σ/k, and obtained as 4.6 nM, which was lower than most of reported HOCl probes.[10] The low LOD illustrates the excellent sensitivity of MBTC in HOCl detection. It is of note that the response was very rapid and complete in only one minute (Fig. S5). The recognition of MBTC toward HOCl was slightly affected by pH over a broad range of pH 4-8.6 (Fig. 2), well compatible with the physiological conditions.

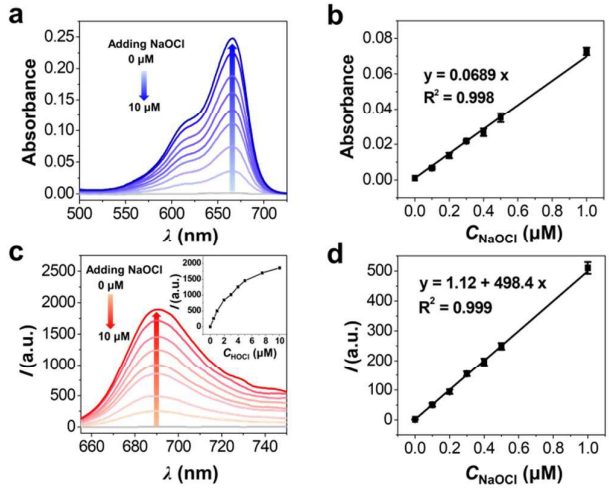

Fig. 1 The absorption (a) and fluorescence spectra (c) of the solution of probe MBTC (5 μM) in 20 mM PBS, pH 7.4) upon titration of NaOCl (0-10 μM). Linear C_{NaOCl}-dependence absorption intensity at 666 nm (b) and fluorescence intensity at 690 nm (d) by titration of NaOCl (0-1.0 μM). Inset in (c): Plot of fluorescence intensity at 690 nm as a function of C_{NaOCl} (0-10 μM).

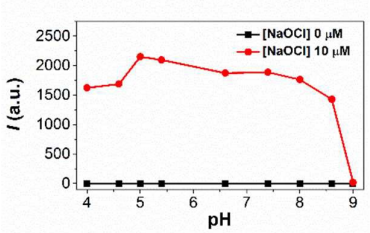

Fig. 2 The effect of pH on the fluorescence response of MBTC (5 μM) in the absence and presence of NaOCl (10 μM). $\lambda_{ex}/\lambda_{em}$ = 620 nm/690 nm.

To look close into the reaction HOCl mediated, we employed ultrahigh performance liquid chromatography (UPLC) that was equipped with PDA-FL dual detectors to detect the product at 620 nm in PDA channel and 690 nm in fluorescence channel (λ_{ex} 620 nm), respectively. As shown in Fig. 3a, as NaOCl concentration increased, increasing amount of sole product was eluted at 1.47 min, exactly the same retention time with the reference compound MB. HRMS analysis of reaction mixture containing MBTC and NaOCl also evidenced the generation of MB (Fig. S6). The detection in fluorescence channel provided the similar results (Fig. 3b). In the broad range of NaOCl concentration (0-10 μM, or 0-2 eq.), byproducts were undetected in both channels. These data confirm the conversion of MBTC to MB mediated by HOCl in an efficient and clean manner.

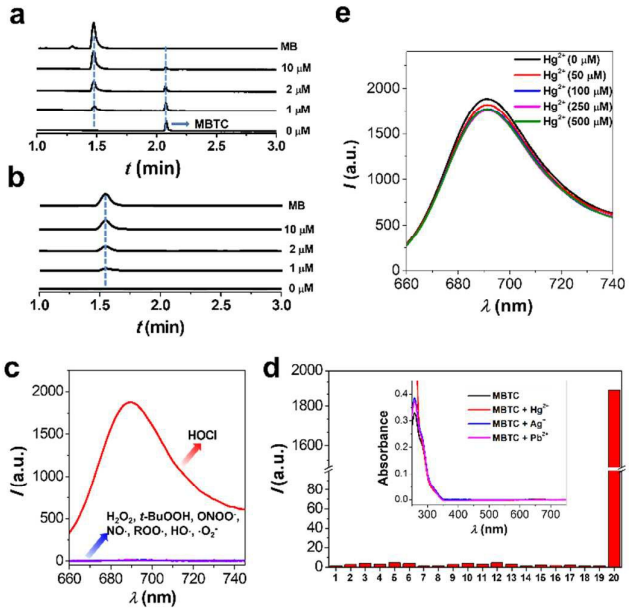

Fig. 3 (a-b) UPLC analysis of reaction mixture containing MBTC (5 μM) and varied concentration of NaOCl (0, 1, 2, 10 μM) in (a) PDA channel (620 nm) and (b) fluorescence channel (690 nm). (c) Fluorescence spectra of the mixture containing MBTC (5 μM) and various ROS/RNS (λ_{ex} 620 nm). (d) Fluorescence intensity at 690 nm of the mixture containing MBTC (5 μM) and various analytes (500 μM, unless otherwise stated) labelled by numbers 1-20. 1: control; 2-10: Hg^{2+}, Pd^{2+}, Ag^{+}, Zn^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+}, Al^{3+}; 11-12: Cys; GSH (5 mM); 13-15: Gly, Arg, Glu; 16-19: ClO_4^-, CO_3^{2-}, $S_2O_3^{2-}$, NO_2^-; 20: NaOCl (10 μM). Inset: absorption spectra of MBTC (5 μM) upon addition of Hg^{2+}, Pd^{2+}, and Ag^{+} at 500 μM. (e) Fluorescence spectra of the mixture 1 min post addition of NaOCl (10 μM) to the solution containing MBTC (5 μM) and varied concentrations of Hg^{2+} (0-500 μM) in PBS. The excitation was at 620 nm.

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To assess the detection selectivity against other oxidants, we performed fluorescence analysis under the same assay condition except that NaOCl was replaced by physiologically related ROS or reactive nitrogen species (RNS) including H_2O_2, *t*-BuOOH, $ONOO^-$, NO·, ROO·, HO·, and $\cdot O_2^-$. As shown in Fig. 3c, MBTC was resistant to each of the tested ROS in fluorescence response, sharp contrast to the extreme reactivity toward HOCl. This means the S-alkyl thiocarbamate bond is not the target of common ROS/RNS, but selective to HOCl cleavage. We also tested the fluorescence response of MBTC to the biologically abundant thiols including GSH (5 mM) and Cys (500 μM) and found undetectable fluorescence enhancement at all (Fig. 3d), in good agreement with the UPLC analysis (Fig. S7), confirming that nucleophilic attack on the S-alkyl thiocarbamate bond by –SH and $–NH_2$ groups did not take place. Similar results were observed with other species such as amino acids (Gly, Arg, and Glu) and anions (ClO_4^-, CO_3^{2-}, $S_2O_3^{2-}$, and NO_2^-). Importantly, no fluorescence response was observed upon addition of metal ions, including Pb^{2+}, Ag^+, Zn^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Ni^{2+}, and Al^{3+}, into MBTC solution (Fig. 3d).

It is of note that Hg^{2+} is a special metal ion with strong thiophilicity on a basis of hard soft acid base (HSAB) principle and reacts with a variety of thiocarbonyl-based functionalities like thiourea, thioamide, thiolactone, thioester and thiocarbamate, which frequently serve as key components in Hg^{2+} detection.[21, 22] Concerning the risk of interference from Hg^{2+}, this arises a concern about the use of thiocarbonyl substituents as a HOCl-responsive trigger. Interestingly, the addition of Hg^{2+} (500 μM) did not change either fluorescence or absorption of probe MBTC which lacks a thiocarbonyl moiety (Fig. 3d). As shown in Fig. S7, the UPLC analysis data indicates that the retention time of MBTC was unaffected by the presence of Hg^{2+} (500 μM), which suggests that MBTC remains in an intact structure and the interaction between the S-alkyl thiocarbamate in MBTC and Hg^{2+} is negligible. The treatment of MBTC with Hg^{2+} (500 μM) at elevated temperature (60 °C) did not induce detectable changes of absorption spectra (Fig. S8), illustrating the high stability of MBTC against Hg^{2+}. Hence, the interference issue associated with transition metal ions, not limited to Hg^{2+} as Ag^+ and Pb^{2+} are also susceptible, is successfully overcome (Fig. 3d). To the best of our knowledge, MBTC exhibits superior selectivity to HOCl among the reported fluorescent HOCl sensors.

The coordination between Hg^{2+} and MBTC was revealed by the ^1H NMR spectra of Hg^{2+} and MBTC in CD_3CN/D_2O (9:1, v/v). As shown in Fig. S9, in comparison to MBTC alone, the presence of Hg^{2+} caused disappearance of the -CO*NH*- signal at 7.0 ppm, and also changed -CH_2-CH_2- signals in the upfield region as well as benzyl proton resonances in the downfield region, which evidences the occurrence of mixed coordination interactions between Hg^{2+} and MBTC presumably at the sites of -CONH-CH_2-CH_2-S- and diphenyl thioether. In the mass spectra, however, the Hg^{2+}-coordinated complex or MB was not detected. Only MBTC was detected at m/z 431.8 ($MBTC+H^+$) (Fig. S10), which implies that the coordination between Hg^{2+}-MBTC is not strong. In PBS, this coordination is weakened by the anions and water, and only MBTC was detected by the mass spectrometry (Fig. S11) and UPLC analysis of Hg^{2+}/MBTC mixture (Fig. S7). Different from the recent report that oxidants such as H_2O_2 may mediate hydrolysis of thiocarbonate derivatives in the presence of Hg^{2+},[23] the hydrolysis of probe MBTC mediated by Hg^{2+}/H_2O_2 was not observed.

On a basis of the above evidence, Hg^{2+} weakly interacts with MBTC in the aqueous buffer and is not expected to disrupt the detection of HOCl. In fact, the addition of NaOCl at a final concentration of 10 μM into the solution of MBTC (5 μM) and Hg^{2+} (0-500 μM) in PBS caused a Hg^{2+} concentration-independent fluorescence enhancement that indicates the rapid formation of MB even in the presence of high concentration of Hg^{2+} (Fig. 3e). Hence, the disturbance of Hg^{2+} in detection of HOCl by MBTC is negligible.

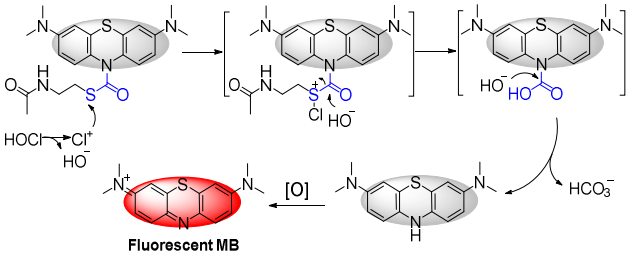
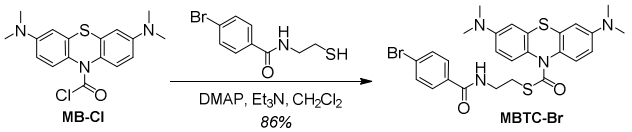


Fig. 4 The proposed mechanism of HOCl-mediated formation of MB from MBTC.



Scheme 2. The synthetic route of MBTC-Br.

We proposed a multiple oxidation mechanism for the produce of MB mediated by HOCl. As shown in Fig. 4, the reaction is initiated with the oxidation on the sulfur of thiocarbamate substituent by the highly reactive Cl^+ that is available from HOCl decomposition.[16] The unstable carbamic acid is formed via rapid hydrolysis and followed by release of HCO_3^-. In the end, the resulted intermediate dihydromethylene blue is quickly oxidized to MB as the final product.[20] It is known that the carbamate counterpart, replacing the sulfur atom of thiocarbamate with oxygen atom, has very low reactivity toward HOCl, which emphasizes the necessity of the use of S-alkyl thiocarbamate substituent.

To confirm the form of released moiety upon oxidation on the S-alkyl thiocarbamate linker, we synthesized a MBTC analogue, MBTC-Br (Scheme 2, Fig. S1b-2b) that possesses a bromine to show a characteristic isotope pattern in the mass spectra of released segment. Similar to MBTC, the MBTC-Br was converted to fluorescent MB by NaOCl in PBS (Fig. S12-13), although the reaction became slower. The reaction mixture was immediately analysed by UPLC-MS to avoid the possible severe decomposition or hydrolysis of the unstable intermediates. The product MB showed up at 1.19 min in both

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620 and 254 nm PDA channels (Fig. S14a-b). In particular, several species were detected at longer retention times (1.65-1.82 min), assumed to be the released intermediates less polar than MB. The most dominant species at 1.82 min was distinguished as a brominated compound with a m/z (ES+) detected at 362.6 (Fig. S14c). It can not be anionic species like sulfonate and sulfinate, but more likely to be a polychlorinated species with a reasonable structure shown in Fig. S14c. We inferred from the UPLC-MS spectra that the highly reactive Cl^+ is responsible for the oxidation event and results in polychlorination on the sulfur of the released intermediate. This inference is well consistent to the proposed Cl^+-mediated oxidation mechanism (Fig. 4).

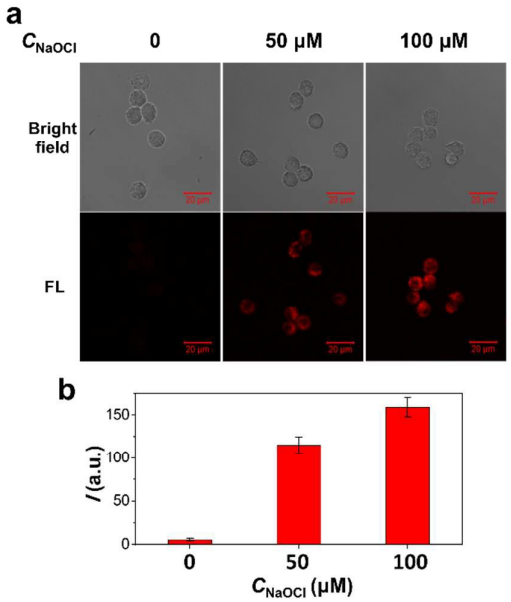

Fig. 5 (a) CLSM images of RAW 264.7 cells treated by MBTC (5 μM) and varied concentrations of exogenous NaOCl (0, 50 and 100 μM). (b) The median fluorescence intensities of RAW 264.7 cells derived from the images in (a).

To evaluate the capability of MBTC to detect HOCl in the living cells, we first examined the cytotoxicity of MBTC in macrophage RAW 264.7 cells by MTT assay. As shown in Fig. S15, viabilities for MBTC (0-5 μM) treated cells over 24 h were higher than 95%, illustrating good biocompatibility of MBTC. Next, we introduced exogenous HOCl in a form of NaOCl at varied concentrations (0-100 μM) to RAW264.7 cells pretreated with MBTC at a low concentration (5 μM), and detected MB fluorescence under confocal laser scanning microscopy (CLSM) (λ_{ex} 633 nm; λ_{em} 670-750 nm). As shown in Fig. 5a, as a control, MBTC-treated cells were nearly invisible in the red channel, thanks to the extremely low background signal. In contrast, upon addition of 50 μM NaOCl, MBTC-treated cells were imaged with bright red fluorescence. Increasing NaOCl concentration to 100 μM resulted in stronger imaging intensity (27 fold relative to the control, Fig. 5b) without causing significant cytotoxicity. The results demonstrate that intracellular components did not activate the probe which selectively responded to HOCl. The high signal to noise ratio benefits the sensitivity of probe in the cells.

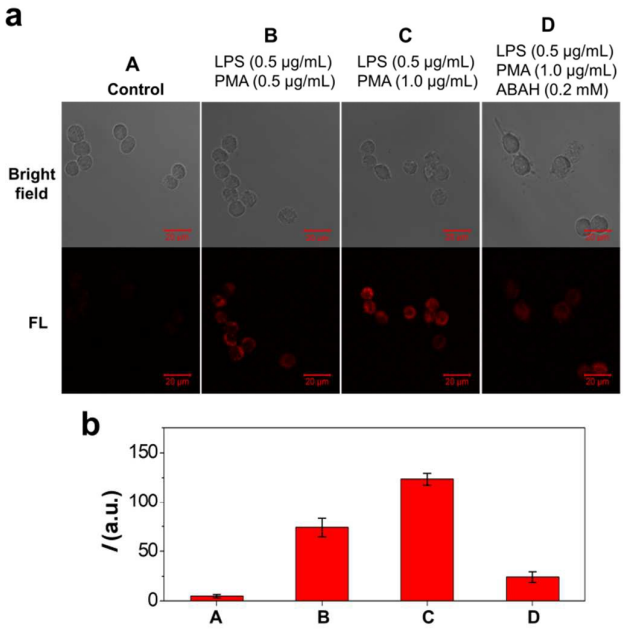

Fig. 6 (a) CLSM images of RAW 264.7 cells treated by MBTC (5 μM) and endogenous HOCl generated by conditions labelled as A-D. (b) The median fluorescence intensities of RAW 264.7 cells derived from the images in (a). A-D denote the conditions indicated in (a).

To detect endogenous HOCl by MBTC, we stimulated RAW 264.7 cells with lipopolysaccharides (LPS) for 12 h and subsequent phorobol myristate acetate (PMA, a ROS stimulant to induce HOCl) for 30 min, followed by treating the cells with MBTC (5 μM) for 1 h before CLSM imaging. As a control, lacking stimulation step to generate HOCl, the probe was inactivated, resulting in minimal MB fluorescence (Fig. 6a). In contrast, the fluorescence was enhanced by a factor of 15-fold in the cells upon stimulation with LPS (0.5 μg/mL) and PMA (0.5 μg/mL) (Fig. 6b). The increase of PMA concentration (1.0 μg/mL) gave rise to greater fluorescence enhancement (25-fold relative to the control), due to the more efficient generation of HOCl (Fig. 6b). To further confirm whether HOCl is the responsible cause of fluorescence enhancement, we applied ABAH, a specific MPO inhibitor to suppress HOCl generation, before LPS/PMA stimulation. As expected, the addition of ABAH (200 μM) led to much weaker fluorescence, with the intensity reduced by 80% (Fig. 6b). These results indicate that endogenous HOCl was fluorescently detectable by MBTC, and the fluorescence enhanced was correlated with the level of HOCl in the living cells.

In summary, we developed a S-alkyl thiocarbamate locked dihydromethylene blue structure for HOCl sensing and imaging in aqueous solutions and living cells, and demonstrated the Cl^+-based oxidation mechanism supported by the UPLC-MS evidence. The probe could complete a quick fluorescence turn

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on response to HOCl within no more than one minute, and sensitively detect HOCl with a LOD of 4.6 nM. The excellent selectivity against reactive oxidants, reductants, and particularly the transition metal ions such as Hg^{2+} that did not disturb HOCl detection attributed to the weak coordination at the binding sites in buffer solutions, would allow the NIR probe to find broad applications in the complicated physiological environments. Additional advantage to introduce the S-alkyl thiocarbamate linker as the HOCl-responsive trigger is that it affords availability for versatile functionalization in the S-alkyl substituent with any desired ligands.

Conflicts of interest

There are no conflicts to declare.

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Table of content

A S-alkyl thiocarbamate bond in a caged fluorophore is selective to target hypochlorous acid but stable against Hg^{2+}.