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A fluorescein semicarbazide-based fluorescent probe for highly selective and rapid detection of hypochlorite in aqueous solution†

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Fluorescein semicarbazide (1) was synthesized and developed as a novel fluorescent probe for the highly selective and rapid detection of hypochlorite (ClO^-) in aqueous solution. The signaling mechanism was proposed based on ESI-MS identification of the oxidation product and captured chlorinated intermediate species.

Introduction

Hypochlorite (ClO^-) is one of the most important reactive oxygen species (ROS), known to be essential for life but harmful when excessively produced.[1] Although the quantitative determination of ClO^- is very significant to understand its biological functions, the development of suitable methods for selective detection of reactive hypochlorite in physiological environments is still challenging. Owing to their high sensitivity and selectivity, fluorescence techniques have been widely used for rapid detection of various analytes.[2] In this regard, the design and development of fluorescent probes for ClO^- detection based on its inherent oxidation ability has attracted much current attention.[3,4] In these reported probes, they either showed poor selectivity, poor water-solubility (addition of more than 10% organic co-solvent),[3b-f] or prolonged oxidation reaction time (a few hours are required).[3a,b] The development of water-soluble and rapid-response fluorescent probes for ClO^--selective detection in aqueous solution is highly preferable for practical bioanalytical applications.

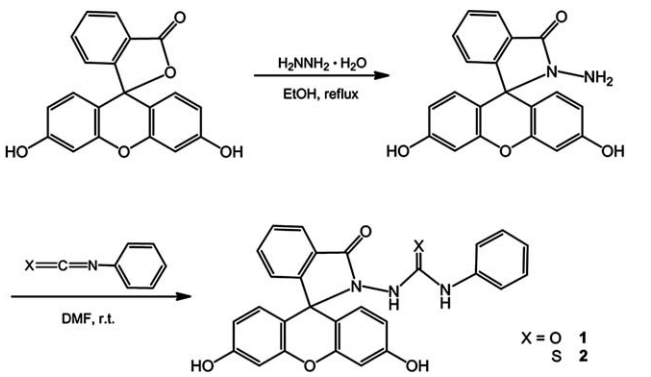
Fluorescein derivatives exhibit excellent photophysical properties, such as visible absorption and fluorescence emission, high fluorescence quantum yield, good photostability and water-solubility, leading to their broad applications in fluorescence sensing and bioanalytical labeling.[2b] It is known that the fluorescein molecule employs spirocyclic and open-cycle structural forms, in which the former is nonfluorescent and the latter is fluorescent.[5] Recently, fluorescein-based turn-on fluorescent probes for various analytes, such as metal cations and anions, have been proposed based on the analyte-induced change in structure between the spirocyclic and open-cycle forms.[5,6] However, fluorescein-based fluorescent probes for ClO^- detection in aqueous solution are rare. To the best of our knowledge, only two reports have been presented,[4] but one requires 10% DMSO as a co-solvent,[4a] and the other requires prolonged reaction times of more than 60 minutes, even if it does only require 1% DMSO as a co-solvent.[4b]

Herein, we report that a fluorescein semicarbazide derivative (**1**, Scheme 1) behaves as a novel fluorescent probe for rapid and selective detection of ClO^- in aqueous solution. It was found that **1** is nonfluorescent in buffer solution at physiological pH 7.4, but ClO^- selectively and rapidly transforms it into a fluorescent product. Its thiosemicarbazide counterpart **2** (Scheme 1), showed a fluorescence enhancement in the presence of both ClO^- and Hg^{2+}. The signaling mechanism was proposed by ESI-MS identification of oxidation products and captured chlorinated intermediate species.

Experimental

Materials and methods

Fluorescein, phenyl isocyanate, phenyl isothiocyanate and hydrazine monohydrate were purchased from J&K and used as



Scheme 1 Synthesis of probes **1** and **2**.

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received. Hypochlorite, hydrogen peroxide (30%), other reagents and all organic solvents were analytical grade and obtained from Sinopharm Chemical Reagents Co. (Shanghai).

The stock solution of probe (1 mM) was prepared in EtOH and the working solution was obtained by dilution with 10 mM PBS buffer solution (pH 7.4).

ESI-MS spectra were obtained on a Varian 310 mass spectrometer. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AVANCE III 400 MHz spectrometer with TMS as standard. Fluorescence spectra were recorded on a Hitachi F-7000 fluorescence spectrometer (Ex/Em slit widths: 2.5 nm). Absorption spectra were measured on a Persee TU-1901 spectrophotometer. The measurements were carried out with a 1 cm path length quartz cell.

Synthesis of probes 1 and 2

SYNTHESIS OF **1**">1. Fluorescein hydrazide (346 mg, 1.0 mmol) and phenyl isocyanate (0.3 mL, 2.8 mmol) were dissolved in 5 mL dried DMF, and the mixture was stirred at room temperature overnight. Water was added into the above solution and extracted with CH_2Cl_2 (3 × 20 mL). The combined organic phase was dried over anhydrous Na_2SO_4 and the solvent was evaporated in vacuum to dryness. Purification by column chromatography (silica gel, CH_2Cl_2/MeOH = 10/1, v/v) and crystallization from acetone give **1** as a pale-yellow powder (290 mg, 62.2%). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm) = 9.85 (s, 2H), 8.65 (s, 1H), 8.40 (s, 1H), 8.08 (s, 1H), 7.88 (d, 1H, J = 8.0 Hz), 7.62–7.55 (m, 2H), 7.30–7.17 (m, 5H), 7.06 (d, 1H, J = 8.0 Hz), 6.91 (t, 1H, J = 6.0 Hz), 6.57 (s, 2H), 6.48 (d, 2H, J = 8.0 Hz); ^{13}C NMR (100 MHz, DMSO-d_6) δ (ppm) = 165.45, 158.92, 154.00, 152.87, 151.91, 140.16, 139.67, 134.00, 130.00, 129.23, 129.07, 128.94, 124.34, 123.33, 122.34, 122.28, 118.67, 118.52, 112.46, 109.29, 102.56, 65.28. ESI-MS (M + H$^+$): m/z calcd 466.1; found 465.9.

The fluorescein hydrazide and **2** were synthesized according to a reported procedure[6h] and identified by ESI-MS (M + H$^+$): fluorescein hydrazide m/z calcd 347.1; found 347.0; **2** m/z calcd 482.1; found 481.9.

Results and discussion

Fig. 1 shows fluorescence emission spectra (λ_{ex} = 480 nm) of **1** and **2** (10 μM) measured in pH = 7.4 PBS buffer solution in the absence and presence of various species (3 equiv. NaClO and 10 equiv. others), where the NaClO solution was freshly prepared and the ClO^- concentrations were determined using its absorbance at 292 nm in basic conditions (pH = 12.0, ε_{292nm} = 350 M^{-1} cm^{-1} for ClO^-).[7] It can be seen that **1** only is nonfluorescent, but is NaClO-selective, leading to an appearance of green fluorescence at 528 nm with an enhancement factor of 156 (Fig. 1a). In contrast, other species such as NaCl, KCl, $CaCl_2$, $Mg(ClO_4)_2$, $Fe(ClO_4)_2$, $Zn(ClO_4)_2$, $Cu(ClO_4)_2$, $Hg(ClO_4)_2$, H_2O_2 and $^{\cdot}OH$ did not induce any fluorescence enhancement. On the other hand, the thiosemicarbazide counterpart **2** only is weakly fluorescent, whereas the addition of NaClO leads to an appearance of green fluorescence at 527 nm with an

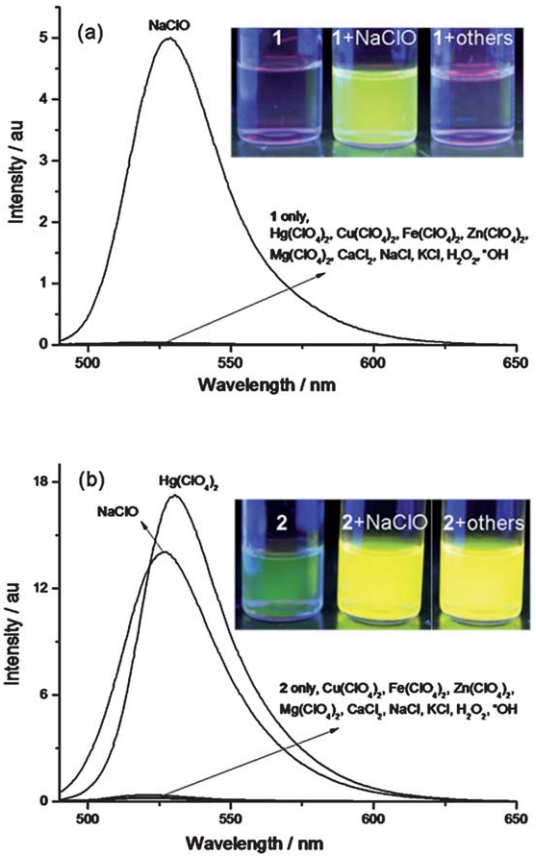


Fig. 1 Fluorescence spectra of 10 μM **1** (a) and **2** (b) in the absence and presence of 3 equiv. of ClO^- and 10 equiv. of other species in 10 mM PBS solution (pH = 7.4).

enhancement factor of 70 (Fig. 1b). However, a fluorescence enhancement (80-fold) at 530 nm was also observed in the presence of Hg^{2+} (Fig. 1b), while other species did not cause a noticeable fluorescence change. These indicate that the probe **1** can serve as a ClO^--selective fluorescent probe in PBS buffer solution at physiological pH of 7.4.

Fig. 2 shows the fluorescence titration of **1** (10 μM) with NaClO. It was found that the green fluorescence emission steadily increased with increasing NaClO concentration up to 3 equiv., along with a 13 nm red-shift from 515 to 528 nm (Fig. 2a). The spectral red-shift may result from the oxidation-induced, more delocalized conjugated structure of **1**. Upon further NaClO addition, however, a slight decrease of fluorescence intensity was observed (Fig. S1, ESI†) that could be explained by a bleaching effect of excess NaClO on **1**. A good linear relationship between the fluorescence intensity at 525 nm (FI_{525nm}) and the NaClO concentration was found in the range of 6.7 × 10^{-7} to 1.8 × 10^{-6} M (Fig. 2b) with a detection limit of 3.3 × 10^{-7} M (S/N = 3). The fluorescence response of **1** to NaClO was found to be complete within 2 minutes (Fig. S2, ESI†), indicating a capability of rapid detection of ClO^-. The titration of **2** with NaClO showed a fluorescence enhancement and saturation up to 3 equiv. of NaClO (Fig. 3). Further addition of NaClO led to a substantial decrease in fluorescence (Fig. S3,

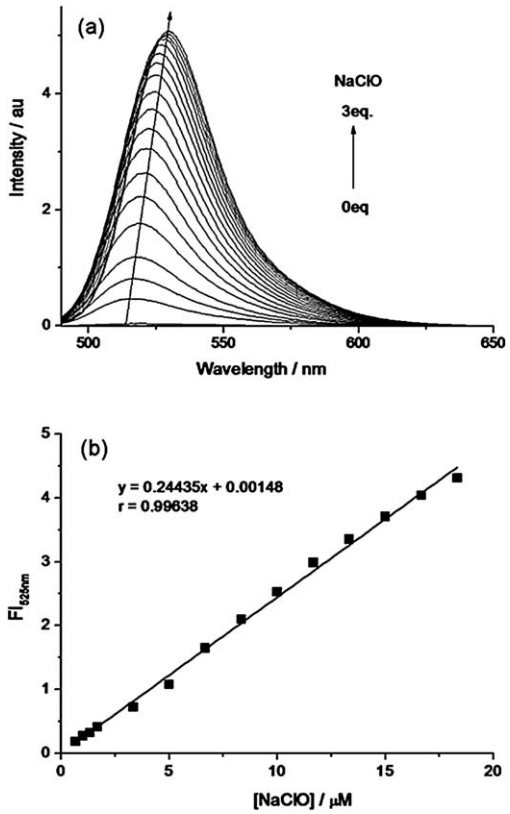


Fig. 2 Fluorescence titration spectra of **1** (10 μM) with NaClO (a) in 10 mM PBS solution (pH = 7.4) and the linear relationship between fluorescence intensity at 525 nm and NaClO concentration (b).

ESI†), indicating a strong bleaching effect of excess NaClO on **2**. The fluorescein-urea type probe **1**, therefore, showing a higher stability to oxidant than its thiourea counterpart **2**, may serve as a novel fluorescent probe for the highly selective and rapid detection of ClO^- in aqueous solution.

The spirocyclic form of probe **1** was confirmed by the existence of the spiro-carbon signal at 65.28 ppm in the ^{13}C NMR spectrum. Thus the switch-on of fluorescence emission of **1** must involve a molecular structure change between spirocyclic and open-cycle forms, as is usually observed in fluorescein-based probes.[5] Without NaClO, **1** showed no absorption in the visible region, but an absorption appeared at 505 nm in the presence of NaClO and shifted to 510 nm with increasing NaClO concentration (Fig. S4, ESI†), clearly indicating that the fluorescence turn-on response of **1** to ClO^- can be similarly explained by the opening of the spirocycle. To elucidate the detailed signal mechanism, an ESI-MS analysis of the reaction mixture of **1** with ClO^- was performed (Fig. 4 and S5, ESI†). It was noted that without ClO^-, the mass spectral signal of probe **1** was observed at m/z = 465.9 $(M + H)^+$, where M denotes the molecular weight of **1**; however, with the addition of 3 equiv. of ClO^-, three distinct signals at m/z = 463.2, 487.8, and 521.8 appeared in the mass spectrum, corresponding to $(M - 2)$, $(M + 23)$, and $(M + 57)$. By considering the differences in the mass values, we tentatively ascribed the peaks at m/z = 463.2, 487.8, and 521.8 to oxidation product (**P**), **1** and chlorinated intermediate product (**IP**), corresponding to $\mathbf{P}^+$, $(\mathbf{1} + Na)^+$ and $(\mathbf{IP} + Na)^+$, respectively, as illustrated in Fig. 4. The observation of $(\mathbf{IP} + H)^+$ signal at m/z = 500.0 further supports the above ascription (Fig. 4). The signaling mechanism was thus proposed as depicted in Scheme 2, namely, the NaClO oxidation of the spirocyclic nonfluorescent **1** produces a chlorinated intermediate product **IP**, and further elimination of HCl from **IP** will give the final product **P**, where both **IP** and **P** are open-cycle forms and fluorescent. We further examined the mixtures of probe **2** with NaClO and Hg^{2+} by ESI-MS. It was found that both showed a distinct peak at m/z = 448 (Fig. 5 and S6, ESI†), which has been attributed to fluorescein-1,3,4-oxadiazole formed by a Hg^{2+}-promoted desulfurization and cyclization reaction as described previously.[3i,6h,8] Therefore, the signalling mechanism of **2** to ClO^- can be similarly described as in Scheme 3. It is obvious that the water-soluble probe **1** developed in this work employed a novel signaling mechanism, that may contribute to the development of new bioanalytical methods and the design of a

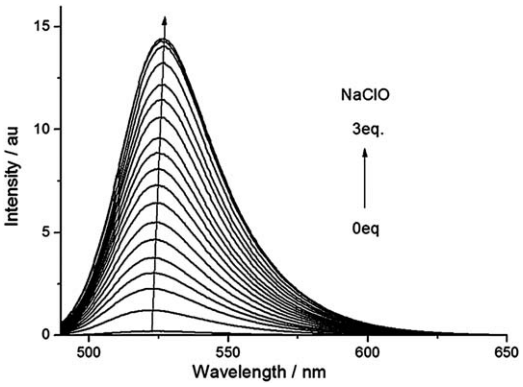


Fig. 3 Fluorescence titration spectra of **2** (10 μM) with NaClO in 10 mM PBS solution (pH = 7.4).

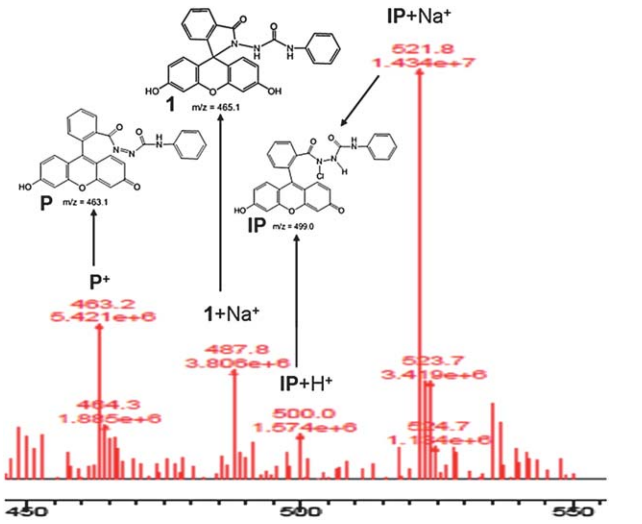
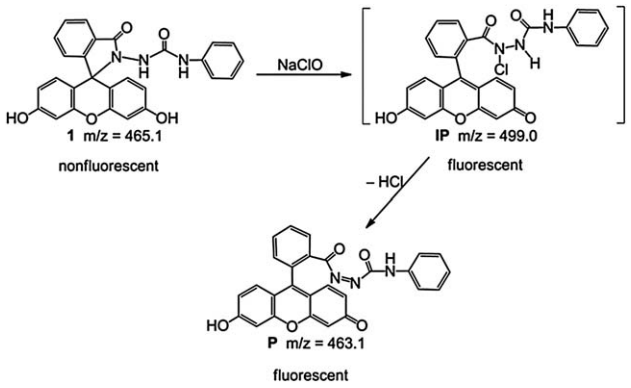


Fig. 4 ESI-MS spectra of the mixture of **1** (10 μM) with 3 equiv. of NaClO in water (pH = 7.4).



Scheme 2 Proposed signaling mechanism of probe **1** to ClO^-.

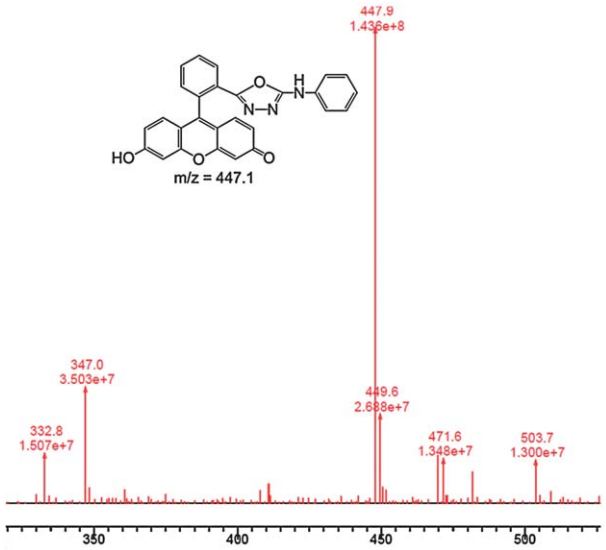
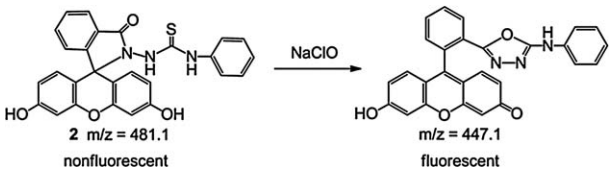


Fig. 5 ESI-MS spectra of the mixture of **2** (10 μM) with 3 equiv. of NaClO in water (pH = 7.4).



Scheme 3 Proposed signaling mechanism of probe **2** to ClO^-.

new fluorescent sensor for selective and rapid detection ClO^- in practical physiological environments.

Conclusion

In conclusion, a new fluorescein semicarbazide-based fluorescent probe **1** was developed for selective and rapid detection of ClO^- in a PBS buffer solution at the physiological pH of 7.4, where the co-solvent EtOH is present at less than 1% and the reaction is complete within 2 minutes. The identification of stable chlorinated intermediate species in the ESI-MS analysis made it possible to propose the signaling mechanism: the nonfluorescent spirocyclic probe **1** is oxidized by NaClO to form a chlorinated intermediate product **IP**, and further elimination of HCl leads to a transformation into the final product **P**, where both **IP** and **P** are open-cycle forms and fluorescent. In contrast, for its thiosemicarbazide counterpart **2**, fluorescence enhancement resulted from the formation of fluorescent fluorescein-1,3,4-oxadiazole by a ClO^--promoted desulfurization and cyclization reaction. The results presented in this work may contribute to the development of new analytical methods and the design of new fluorescent probes that are suitable for ClO^- detection in physiological environments.

Acknowledgements

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