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- 1 -

The detection for hypochlorite by UV-Vis and fluorescent spectra based on

oxidized ring opening and successive hydrolysis reaction

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Abstract: In this work, two high selective and sensitive fluorescent probes for ClO⁻,

7-Hydroxycoumarin and 4-Hydroxycoumarin were designed. The reaction mechanism that we speculated was the oxidized ring opening reaction and hydrolysis. The detection could be realized in quasi-aqueous phase and the detection limits of probe (7) and probe (4) for ClO^- were found to be 56.8 nM and 70.5 nM. Furthermore, the probes can be used to cell imagings.

Keywords: UV-Vis and fluorescent spectra, Probes; 7-Hydroxycoumarin; 4-Hydroxycoumarin; Cell imagings.

- 2 -

1. Introduction

The detection and quantification of inorganic anions have been invigorated over the past years from environmental, biological, and medical aspects. And a variety of probes that are able to selectively recognize anions were designed.¹⁻⁶ As one of the reactive oxygen species (ROS), hypochlorite is generated from the reaction of H_2O_2 and chloride ion catalyzed by myeloperoxidase (MPO) enzyme in organisms.⁷⁻⁸ In the physiological pH solution HOCl is partially dissociated into ClO⁻, and it plays a crucial role in vivo due to its antibacterial properties.⁹⁻¹⁰ However, the excessive generation of the potent oxidant also poses a risk to the host, and there is evidence that HOCl contributes to the tissue injury associated with inflammation.¹¹ Neutrophil derived HOCl has been considered to be related to lung injury,¹²⁻¹⁴ rheumatoid arthritis,¹⁵⁻¹⁶ hepatic ischemia-reperfusion injury,¹⁷ and renal disease.¹⁸⁻¹⁹ Current chemo/biosensors for hypochlorous acid or hypochlorite detections are usually limited to the submicromolar level because of their insufficient sensitivity, which is a problem because the concentrations in biological matrices is generally on the nanomolar scale or even lower. The intracellular concentrations of hypochlorite are generally on the submicromolar level. It has been reported that the average production concentration of HOCl in neutrophils is about 0.47 nmol \min^{-1} per 10⁶ cells.²⁰ So it is essential to design some chemosensors with high enough sensitivity for the detection of HOCI/CIO⁻. There have been some successful examples reported very recently such as colorimetric, luminescent, electrochemical and chromatographic methods.²¹⁻³³ Even so, fluorescent probe detection is a promising method for detection of hypochlorous

- 3 -

acid because of the low cytotoxicity of fluorescent probe which can realize detection in living cells.³⁴⁻³⁷

Coumarin and its derivatives are one of the important classes of heterocyclic compounds and are known to possess a wide range of biological activities including anti-HIV, anti-biotic, anti-fungal, anti-bacterial, anti-viral, anti-cancer, anti-clotting activity, and especially as anti-coagulants.³⁸⁻⁴⁵ Meanwhile, 7-Hydroxyl coumarin as a commercial available fluorescent dye can be used for fluorescent indicator and pH indicator; 4-Hydroxyl coumarin as a commercial available dye can be used for a solvent dyes, fluorescent whitening agent and laser dye. In the process of studying their fluorescence properties, we found that they can selective recognition hypochlorite ion.

In this work, 7-hydroxyl coumarin and 4-hydroxy coumarin were developed as two high selective fluorescent probes for hypochlorite ion over other anions. These probes worked well at physiological pH and had low detection limits for ClO⁻.

2. Experimental

2.1 Materials

4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from Sigma-Aldrich (St. Louis, MO). 7-hydroxyl coumarin and 4-hydroxy coumarin were purchased from Aladdin Industrial Corporation (Shanghai, China). Amino acids were purchased from Shanghai Experiment Reagent Co., Ltd (Shanhai, China). All other chemicals used were of analytical grade.

2.1. Physical measurements

- 4 -

A pH meter (Mettler Toledo, Switzerland) was used to determine the pH. Ultraviolet-visible (UV-Vis) spectra were recorded on a Cary 50 Bio UV-Visible spectrophotometer. Fluorescence spectra were measured on Cary Eclipse fluorescence spectrophotometer. A PO-120 quartz cuvette (10 mm) was purchased from Shanhai Huamei Experiment Instrrument Plants, China. ¹H NMR, ¹³C NMR spectra were recorded on a Bruker AVANCE-300 MHz and 75 MHz NMR spectrometer, respectively (Bruker, Billerica, MA). ESI-MS was measured with an LTQ-MS (Thermo) instrument. The ability of probe (7) reacting to OCI⁻ in the living cells was also evaluated by laser confocal fluorescence imaging using an Olympus FV1000 laser scanning microscope.

3. Results and Discussions

3.1. UV–Vis and fluorescence spectra of detecting ClO⁻

The detections of 7-hydroxyl coumarin and 4-hydroxy coumarin for ClO⁻ were investigated by the UV–Vis and fluorescence spectra. Detailed UV–Vis spectral titrations for ClO⁻ were carried out (Fig. 1). Fig. 1a showed the change in the UV-Visible spectra upon addition of hypochlorite solution to a solution of the probe (7) (50 μ M) in HEPES (10 mM, pH 7.4). With increasing concentration of ClO⁻ (0–33.5 μ M), the absorption peak of the probe at 323 nm was gradually decreased and two new absorption peaks appeared at 263 nm and 369 nm. Two isosbestic points appeared at 292 nm and 343 nm, indicating the formation of ClO⁻ to the probe (4) (50 μ M) in HEPES (10 mM, pH 7.4). With increasing concentration of ClO⁻ to the probe (4) μ M), the absorption peak of the probe (4) at 285 nm was gradually decreased and a new absorption peak appeared at 252 nm. An isosbestic point formed at 270 nm, indicating the formation of a new compound. Detailed fluorescence spectral titrations for ClO⁻ were carried out (Fig. 2). Fig. 2a displayed the fluorescence response of the probe (7) to ClO⁻ in HEPES (10 mM, pH 7.4). Initially, the probe (0.1 μ M) had strong fluorescence intensity (λ_{ex} = 323 nm, slit width: 5 nm/5 nm), but the addition of ClO⁻ (0–241.2 μ M) caused a remarkable reduction emission at 463 nm. Fig. 2b showed the change in the fluorescence spectra when the ClO⁻ (0–4.7 μ M) was gradually added to the probe (4) (15 μ M) in HEPES (10 mM, pH 7.4). Upon increasing ClO⁻ concentration, the initial fluorescence intensity of 377 nm gradually decreased (λ_{ex} = 287 nm, slit width: 5 nm/5 nm).



Fig. 1. (a) The UV–Vis absorption spectra of probe (7) (50 μ M) in the presence of various concentrations of ClO⁻ (0–33.5 μ M) in HEPES (10 mM, pH 7.4). (b) The UV–Vis absorption spectra of probe (4) (50 μ M) in the presence of various concentrations of ClO⁻ (0–22.8 μ M) in HEPES (10 mM, pH 7.4).





Fig. 2. (a) Fluorescence spectra of probe (7) (0.1 μ M) in the presence of various concentrations of ClO⁻ (0–241.2 μ M) in HEPES (10 mM, pH 7.4) ($\lambda_{ex} = 323$ nm, slit width: 5 nm/5 nm). Inset: a color change photograph for ClO⁻. (b) Fluorescence spectra of probe (4) (15 μ M) in the presence of various concentrations of ClO⁻ (0–4.7 μ M) in HEPES (10 mM, pH 7.4) ($\lambda_{ex} = 287$ nm, slit width: 5 nm/5 nm). Inset: a color change photograph for ClO⁻.

3.2. The Selective response of probes to ClO⁻

An important feature of probe is that it has special selectivity for a kind of analyte over other substances. In order to study its special recognition ability, we

- 7 -

carried out the experiment by UV-Vis and fluorescence spectrometer. Detailed UV-Vis spectra of probes for ClO⁻ and other analytes were carried out (Fig. 3). Fig. 3(a) showed the absorbance curve changes that probe (7) (50 μ M) undergowent upon the addition of various analytes in HEPES (10 mM, pH 7.4) (2 mL). And they all triggered minor changes except CIO⁻. Upon the addition of CIO⁻, the absorption peak of the probe (7) at 323 nm was decreased and two new absorption peaks appeared at 263 nm and 369 nm. Fig. 3 (b) showed the absorbance curve changes that probe (4) (50 µM) undergowent upon the addition of various analytes in HEPES (10 mM, pH 7.4) (2 mL). And they all triggered minor changes except ClO⁻. Upon the addition of ClO⁻, the absorption peak of the probe (4) at 285 nm was decreased. Detailed fluorescence spectra of probes for ClO⁻ and other analytes were carried out (Fig. 4 and Fig. S1). Fig. 4(a) showed the probe (7) (0.1 μ M) displayed a remarkable fluorescence intensity reduction ($\lambda_{ex} = 323 \text{ nm}$, $\lambda_{em} = 463 \text{ nm}$, slit width: 5 nm/5 nm) in the presence of the ClO^{-} (241.2 μ M). And other analytes only triggered minor changes. Besides, from the Fig. 4a, we could see that the other analytes induced no changes in the solution color, but ClO⁻ induced obvious changes in the solution color (from strong blue fluorescent to colorless) under illumination with a 365 nm UV lamp. Fig. 4b showed the probe (4) (15 μ M) displayed a remarkable fluorescence intensity reduction ($\lambda_{ex} = 287$ nm, $\lambda_{em} = 377$ nm, slit width: 5 nm/5 nm) in the presence of the $ClO^{-}(4.7 \ \mu M)$. Also from Fig. 4b, we could see that the other analytes induced no changes in the fluorescence spectra and solution color, but ClO⁻ induced obvious changes in fluorescence intensity reduction and solution color (from weak blue

- 8 -

0.8 1.0 0.8 0.6 0.6 ∢ other analytes 0.4 analyes ∢ prol 0.4 CIO 0.2 0.2 ció 0.0 0.0 350 400 450 250 300 300 350 Wavelength(nm) 250 400 450 Wavelength(nm) (a) (b)

fluorescent to colorless) under illumination with a 365 nm UV lamp.

Fig. 3. (a) UV-Vis absorption spectra of probe (7) (50 μM) in HEPES (10 mM, pH 7.4) in the presence of F⁻, Cl⁻, Br⁻, NO₃⁻, SCN⁻, CN⁻, SO₄²⁻, HSO₃⁻, S₂O₃²⁻, CO₃²⁻, HCO₃⁻, AcO⁻, H₂PO₄⁻, Cys, H₂O₂, l⁻, ClO₂⁻, C₂O₄²⁻, S₂O₄²⁻, ClO₄⁻, P₂O₇⁴⁻, H₂O₂, HS⁻, GSH, Hcy (600 μM) and ClO⁻ (33.5 μM). (b) UV-Vis absorption spectra of probe (4) (50 μM) in HEPES (10 mM, pH 7.4) in the presence of F⁻, Cl⁻, Br⁻, NO₃⁻, SCN⁻, CN⁻, SO₄²⁻, HSO₃⁻, S₂O₃²⁻, CO₃²⁻, HCO₃⁻, AcO⁻, H₂PO₄⁻, Cys, H₂O₂, l⁻, ClO₂⁻, C₂O₄²⁻, S₂O₄²⁻, ClO₄⁻, P₂O₇⁴⁻, H₂O₂, HS⁻, GSH, Hcy (410.0 μM) and ClO⁻ (22.78 μM).



⁽a)



Fig. 4. (a) Fluorescence spectra of probe (7) (0.1 μ M) with various analytes and ClO⁻ in HEPES (10 mM, pH 7.4) ($\lambda_{ex} = 323$ nm, slit: 5 nm/5 nm). Inset: a visual fluorescence color change photograph for ClO⁻ and other analytes under illumination with a 365 nm UV lamp. (b) Fluorescence spectra of probe (4) (15 μ M) with various analytes and ClO⁻ in HEPES (10 mM, pH 7.4) ($\lambda_{ex} = 287$ nm, slit: 5 nm/5 nm). Inset: a visual fluorescence color change photograph for ClO⁻ and other analytes under illumination with a 365 nm UV lamp.

3.3. pH dependent of the probes

The pH range for the determination of ClO⁻ was also studied. Fig. S2 showed the fluorescence intensity obtained for the free probes and probes-ClO⁻ in different pH values. From Fig. 2S, we could find that both probe (**7**) and probe (**4**) in HEPES (10 mM, pH 7.4) were more effective for spectral detections.

3.4. The kinetic study in the detection process of ClO^{-}

We investigated the reaction time between probes and ClO^- (Fig. 5). From Fig. 5, we could figure out that the minimum fluorescence intensities of probe (**7**)-ClO⁻ and probe (**4**)-ClO⁻ were achieved after 9 s and 17 s, respectively.



Fig.5. (a) Reaction time profile of fluorescence spectra of probe (7) (0.1 μ M) upon addition of ClO⁻ (241.2 μ M) at 463 nm in HEPES (10 mM, pH 7.4) (λ_{ex} = 323 nm, slit width: 5 nm/5 nm). (b) Reaction time profile of fluorescence spectra of probe (4) (15 μ M) upon addition of ClO⁻ (4.7 μ M) at 377 nm in HEPES (10 mM, pH 7.4) (λ_{ex} = 287 nm, slit width: 5 nm/5 nm).

3.5. The detection limit of probes for ClO⁻

To investigate the detection limits of the probes for ClO⁻, probe (7) (0.1 μ M) was treated with various concentrations of ClO⁻ (0–201 μ M) and the fluorescence intensity at 463 nm was plotted as a function of ClO⁻ concentration, probe (4) (15 μ M) was treated with various concentrations of ClO⁻ (0–4.7 μ M) and the fluorescence intensity at 377 nm was plotted as a function of ClO⁻ concentration (Fig. S3). The fluorescence intensities of probe (7) and probe (4) were linearly proportional to the ClO⁻ concentrations, and the detection limits were 56.8 nM and 70.5 nM, respectively, based on the definition by IUPAC (C_{DL}= 3 Sb/m).⁴⁶ The detection limits indicated that commercially available fluorescence probes: 7-hydroxyl coumarin and 4-hydroxy coumarin showed certain sensitivities towards ClO⁻ and their detection limits were compared to the other reported hypochlorite probes (Fig. S4).⁴⁷⁻⁵⁴

3.6. Proposed mechanism

- 11 -

The reaction mechanism that we speculated was the oxidized ring opening reaction and hydrolysis. As we known, hypochlorite is a strong oxidant, it maybe can oxidize ethylenic bond of coumarin to form carboxyl product. Furthermore, the C-O bond of ester group may be disconnected by hydrolysis of ester bond (Scheme 1). To elucidate the detailed signal mechanism, ¹H NMR ¹³C NMR and ESI-MS analyses of probes-ClO⁻ products were carried out (Fig. S5, ESI⁺). The ESI-MS of probe (7)-ClO⁻, m/z: 188.67, [M+Cl]⁻; the ESI-MS of probe (4)-ClO⁻, m/z: 138.83, [M]⁻.



Scheme 1. The reaction mechanism of probes.

3.7. Cellular Imaging

We further investigated the utility of the probe (7) in tissue imaging. The ability of probe (7) to detect ClO⁻ within living cells was also evaluated by laser confocal fluorescence imaging using an Olympus FV1000 laser scanning microscope. The optical window at the channel (400 – 550 nm) was chosen as a signal output. Under selective excitation at 323nm, HepG2 cells incubated with probe (7) (0.3 μ M) for 30 min at 37 °C showed light blue fluorescence (Fig. 6a). In a further experiment it was found that HepG2 cells displayed no fluorescence when the cells were first incubated with 0.3 μ M of probe (7) for 30 min at 37 °C and then incubated with 30 μ M ClO⁻ (Fig. 6b). These cell experiments showed the good cell-membrane permeability of probe (7), thus it could be used to mark ClO⁻ within living cells.

- 12 -



Fig. 6 Confocal fluorescence images in HepG2 cells: (a) Fluorescence image of HepG2 cells with adding probe (**7**) (0.3 μ M) and its bright field image (c); (b) Fluorescence image of HepG2 cells incubated with 0.3 μ M probe (**7**) for 30 mins at 37 °C, then incubated with NaClO (30 μ M) for 30 mins at 37 °C and its bright field image (d).

4. Conclusion

In summary, we have developed two effective fluorescent probes for CIO⁻ in quasi-aqueous phase. The probes are based on commercially available coumarin compounds which have high selectivities and sensitivities for CIO⁻ over other analytes in HEPES (10 mM, pH 7.4). Moreover, the probes are sensitive enough to monitor CIO⁻ comparable to that of other reported CIO⁻ chemosensors (Fig. S4). Interestingly, the practical appilication was realized by cell imagings.

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- 13 -

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- 14 -

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- 18 -



- 19 -

Highlights

- 1. Two new fluorescent probes for hypochlorite based on a novel recognition mechanism: oxidized ring opening reaction and hydrolysis were developed.
- 2. The detection could be realized in quasi-aqueous phase and the detection limits were found to be as low as 56.8 nM and 70.5 nM, respectively.
- 3. The probe could be used to monitor intracellular ClO^{-} in HepG2 cells.