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A sensitive and selective fluorescence probe based fluorescein for detection of hypochlorous acid and its application for biological imaging

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A highly sensitive and selective fluorescein-based probe **1** for hypochlorite anion was synthesized. The probe **1** has favorable characteristics for biological imaging, including high water solubility, high fluorescence yield, pH-independent fluorescence, and biocompatibility. Results show that it has a detection limit of 40 nM to hypochlorite anion. In addition, confocal fluorescence microscopy imaging using RAW264.7 cells showed that the new probe **1** could be used as an effective fluorescent probe for detecting HOCl in living cells.

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1. Introduction

Hypochlorite anion (ClO^-) is one of the biologically important reactive oxygen species (ROS), and produces in organisms by the reaction of H_2O_2 with Cl^- ions under the catalysis of a heme enzyme, myeloperoxidase [1,2]. Endogenous ClO^- is essential to life and has important antibacterial properties. However, excessive or misplaced production of ClO^- can lead to tissue damage and diseases, such as atherosclerosis, arthritis, and cancers [3]. Therefore, real-time monitoring and accurate detection of ClO^- anion are attracting increasing attention due to its extremely important role in health and environmental science. Fluorescent probes possess some native advantages over the probes of other types because of their high sensitivity, specificity, simplicity of implementation, and fast response times, offering application methods for not only in vitro assays but also in vivo imaging studies [4–8]. So far, a number of small-molecule fluorescent probes for specific detection of $HOCl/OCl^-$ have been reported [9–21]. However, some of them still face some drawbacks, such as poor water-selectivity, low sensitivity, and pH dependency [22]. Therefore, it is highly desirable to develop new fluorescent probes that can overcome these limitations in detecting $HOCl/OCl^-$. The fluorescein framework seems to be an ideal model to construct “turn-on” fluorescent chemosensors due to its favorable properties such as excitation and emission wavelengths in the visible region with a high fluorescence quantum yield [23–26], easy synthesis and functionalization [27], excellent biocompatibility and cellular membrane-penetrating capacity [28–31]. With these considerations in mind, we synthesized a new fluorescein-based chemosensor bearing a catechol moiety which responded to the amount of ClO^- for detection of hypochlorite anion (Scheme 1). Being a closed spirolactam structure, the probe itself was nearly nonfluorescent, while the strong green fluorescence was restored after addition of HClO. It was ascribed to the opening ring of spirolactam via oxidation of catechol moiety by HClO and then hydrolysis of diacylhydrazine [32]. The probe display highly sensitive and selective detection toward ClO^- over other ROS or reactive nitrogen species (RNS).

2. Experimental

2.1. Materials

Unless otherwise stated all chemicals were commercially obtained and used without further purification. NaOCl was purchased

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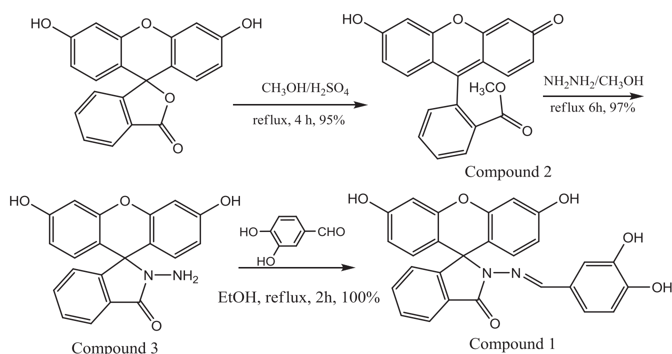

Scheme 1. The routes of synthesis of compound 1.

from Sinopharm Chemical Reagent Co., Ltd (China) and the concentration was determined by titration with $Na_2S_2O_3$.

2.2. Measurements

The UV–vis spectra were recorded on a Perkin Elmer Lambda 35 spectrophotometer. Fluorescence measurements were performed at room temperature on a Perkin Elmer LS-55 spectrophotometer or Hitachi Fluorescence spectrophotometer-F-4600, and Fluorescein was used as a standard for the determination of fluorescence quantum yields. The biological imaging tests were carried out with an Olympus FV-1000 and Leica TCS-SP5 laser scanning confocal fluorescence microscopes. ^{1}H NMR and ^{13}C NMR were measured on a Bruker AV-300 spectrometer with chemical shifts reported in ppm (in DMSO-d$_6$ or $CDCl_3$, TMS as internal standard). Mass spectra were recorded using a Thermo Finnigan LCQ Duo electrospray mass spectrometer in positive ion mode by direct infusion, with spray voltage 4.5 kV and capillary temperature of 200 °C.

2.3. Synthesis of compound **1** (probe **1**)

As shown in Scheme 1, the probe **1** (compound **1**) was readily synthesized by treating fluorescein with methanol using H_2SO_4 as catalyst, which was followed by hydrazine hydrate and 3,4-dihyrdoxybenzaldehyde. The structure of probe **1** was confirmed by ^{1}H NMR, ^{13}C NMR, ESI-MS. Compound 2 and 3 were synthesized according to our previous procedures [33].

2.3.1. Synthesis of compound **2**

To fluorescein (1.0 g, 3.1 mmol) methanol solution (10 mL) in a 25 mL round-bottom flask, was added concentrated sulfuric acid (98%) (1 mL). The solution was refluxed and stirred for 4 h. After cooling, excess methanol was removed under reduced pressure and excess water was added to the residue. The red solid formed was washed with water several times and filtered in vacuum until almost free from fluorescence. After dried in vacuum, 0.95 g red solid fluorescein methyl ester **2** was obtained with a yield of 91%. M.p.: 212–214 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 8.31 (d, 1H, J = 7.8 Hz), 7.79 (m, 2H), 7.34 (d, 1H, J = 7.2 Hz), 7.28 (d, 2H, J = 3.6 Hz), 7.19 (d, 2H, J = 9.0 Hz), 7.08 (dd, 2H, J_1 = 9.3 Hz, J_2 = 2.1 Hz), 3.63 (s, 3H).

2.3.2. Synthesis of compound 3

Compound **2** (0.40 g) and hydrazine hydrate (0.24 g, 4.8 mmol) were added to methanol (5 mL), refluxed and stirred for 6 h. After collecting by filtration, the light brown precipitate was washed by a small amount of methanol and water. 0.41 g straw yellow fluorescein hydrazone **3** was dried in vacuum and then obtained with a yield of 98%. M.p.: 252–253 °C; ^{1}H NMR (DMSO-d_6, 300 MHz) δ (ppm): 7.77 (t, 1H, J = 4.8 Hz), 7.49 (t, 2H, J = 3.6 Hz), 6.98 (m, 1H), 6.59 (d, 2H, J = 2.1 Hz), 6.42 (m, 4H), 4.38 (s, 2H).

2.3.3. Synthesis of compound **1**

Compound **3** (346 mg, 1.0 mmol) was dissolved in absolute methanol (20 mL). 3,4-dihydroxybenzalhyde (138 mg, 1.0 mmol) was added and the mixture was heated at reflux for 4 h. The precipitate produced was filtered and washed with cold ethanol. The crude product was purified by recrystallization from ethanol to afford **1** as brown solid (420 mg, 90%). M.p.: >300 °C (dec); ^{1}H NMR (300 MHz, DMSO-d_6) δ (ppm): 9.88 (s, 2H), 9.39 (s, 1H), 9.18 (s, 1H), 8.76 (s, 1H), 7.86 (dd, J = 6.1, 2.2 Hz, 1H), 7.64–7.47 (m, 2H), 7.06 (dd, J = 5.9, 1.8 Hz, 1H), 6.87 (s, 1H), 6.70–6.58 (m, 4H), 6.45 (m, 4H). ^{13}C NMR (75 MHz, DMSO-d_6), δ 163.32, 158.42, 152.03, 150.85, 150.70, 148.34, 145.56, 133.60, 128.98, 128.90, 127.95, 125.71, 123.53, 122.96, 120.70, 115.46, 112.29, 112.25, 110.21, 102.45, 65.07. ESI-MS (-p) (m/z): 465.75 ($[M-H]^-$), 501.58 ($[M+2H_2O-H]^-$), 525.33 ($[M+CH_3CO_2H-H]^-$), 931.25 ($[2M-H]^-$)

2.4. Determination of quantum yield

The fluorescence quantum yields were determined using fluorescein as a reference with a known Φ value of 0.89 in EtOH [34]. The sample and the reference were excited at the same wavelength (λ_{ex} = 480 nm), maintaining nearly equal absorbance (0.06). The quantum yield was calculated according to the following eqn (1):

$$\Phi_S/\Phi_R = (A_S/A_R) \times (\mathrm{Abs}_R/\mathrm{Abs}_S) \times \left(\eta_S^2/\eta_R^2\right), \quad (1)$$

where Φ_S and Φ_R are the fluorescence quantum yields of the sample and the reference, respectively; A_S and A_R are the emission areas of the sample and the reference, respectively; Abs_S and Abs_R are the corresponding absorbance of the sample and the reference solution at the wavelength of excitation; η_S and η_R are the refractive indices of the sample and the reference, respectively.

2.5. Cell culture

RAW264.7 macrophages were first incubated with LPS (1 μg/mL) and IFN-γ (50 ng/mL) in culture medium for 4.5 h at 37 °C, and then were stimulated with PMA (10 nM) for 45 min at 37 °C. Subsequently, part of the treated cells was incubated with L-methionine (300 μM) for 40 min at 37 °C. After washed with PBS buffer (0.10 M, pH 7.4) for three times, all the cells were incubated with probe **1** (10 μM) in culture medium for 30 min at 37 °C. Before imaging, the cells were washed again with PBS (0.10 M, pH 7.4) for three times.

3. Results and discussion

3.1. Selectivity studies

To evaluate whether probe 1 can selectively respond to OCl^- under simulated physiological conditions (pH = 7.4), the fluorescence responses of probe 1 to other potentially competing ROS/RNS, were also performed. As shown in the selectivity profiles (Fig. 1), only OCl^- incurs a dramatic fluorescence enhancement for probe 1. Other ROS or RNS, including H_2O_2, t-BuOOH, NO_2^-, NO_3^-, and $S_2O_8^{2-}$, exert no obvious spectral changes.

In addition, probe **1** was treated with a wide variety of cations and anions to examine the selectivity. As shown in Fig. 3, the addition of ClO^- induced a significant redshift of the fluorescence emission spectra. However, representative species such as Li^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, Al^{3+}, Cl^-, CH_3COO^-, SO_4^{2-}, CO_3^{2-}, elicited almost no changes in the fluorescence spectra. Furthermore, the

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