A highly sensitive fluorescent probe for the detection of bisulfite ion and its application in living cells

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Abstract:

A novel direct visualisation, fluorescent probe derived from coumarin for bisulfate ion detection has been designed and synthesized. This probe shows a rapid, high selectivity, and sensitivity in the detection of bisulfate ion. In a solution of DMSO:HEPES (1:9, v:v, 10 mM, pH 7.4) buffer containing the probe, the presence of HSO_3^- made the UV – vis absorption of the probe blue-shift from pink to yellow, and the fluorescence of the probe also changed from red to green. Other investigated analytes and ions did not disturb the determination of HSO_3^- . The reaction mechanism of the determination was proposed based on the nucleophilic attack toward electron-poor C=C bond of the probe. This probe has been successfully applied for determination of HSO_3^- in living cells.

Keywords: Direct visualisation; Fluorescent; Bisulfite; Coumarin

1. Introduction

Bisulfites have been widely used in food and beverage processing as additives because of their ability to prevent oxidation and microbial growth, as well as inhibit browning. Despite these advantages, bisulfites are toxic, and high levels of bisulfites could induce diseases for some individuals, such as allergic reaction, asthma, gastrointestinal, and skin allergy diseases [1]. Furthermore, sulfur dioxide generated from modern industrial processes can cause serious environment problems. SO_2 can dissolve in water solution and form an equilibrium state between sulfite and bisulfite. In view of the deleterious effects of bisulfites, the threshold levels of bisulfite in food and medicines must be limited [2]. The United States Food and Drug Administration required labeling of products that contain more than 10 $\mu\text{g/mL}$ of sulfite [3-4]. Therefore, development of sensitive, selective, precise and low-cost analytical methods is of vital importance for food quality assurance and quality control.

To date, various analysis methods have been reported for bisulfite detection, such as spectrophotometry [5], spectrofluorimetry [6], chemiluminescence measurements [7], chromatography [8], phosphorimetry [9], enzymatic techniques [10], and electrochemical methods [11]. However, most of these methods are time-consuming and require miscellaneous sample pretreatments, multiple reagents, and complicated instruments [12-14]. In contrast, the properties of fluorescent probe method with high selectivity, convenience, fast response, ease of live-cell imaging and inexpensive, making it more advantageous and attractive than other conventional methods [15-16].

In recent years, some fluorescent probes for detecting bisulfite have been

developed mainly based on nucleophilic addition, such as the addition of bisulfite to an aldehyde moiety, polarized C=N bond or C=C bond of the probe [17-27]. However, most of available fluorescent probes for SO_3^{2-} and HSO_3^- detection possess short-wavelength excitation and emission spectra, and can hardly be applied to the direct visualization of SO_3^{2-} and HSO_3^- levels in the living systems because of strong absorption and auto fluorescence of biomolecules in the short wavelength region lead to low signal-to-noise ratios [28-32]. Hence, the development of fluorescent probes with long emission wavelengths is necessary. As we know colorimetric technique possesses a great advantage over other analytical methods because it has the capability to detect target analytes by direct visual observation, without the aid of any advanced instruments [33-37].

In this study, we report a colorimetric and fluorescent probe for the detection of bisulfite based on the coumarin-malononitrile derivative. We chose the electron-poor C=C double bond as the recognition receptor for HSO_3^- . In particular, the probe exhibit several meritorious features as follows: (i) high sensitivity and selectivity for HSO_3^- ; (ii) In the fluorescence emission spectra maximum, blue-shift from 593 to 494 nm, was observed and the color of solution is from purple red to green; (iii) rapid response; and (iv) The addition of HSO_3^- to a physiological aqueous solution of probe can result in an obvious color change from pink to yellow.

2. Experimental

2.1. Reagents and materials

4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased

from Sigma-Aldrich (St. Louis, MO). Sodium hydroxide solution (0.1 mol/L) was added to aqueous HEPES (10 mmol/L) to adjust the pH to 7.4. Anionic salts were purchased from Shanghai Experiment Reagent Co., Ltd (Shanghai, China). All reagents employed for synthesis were obtained commercially and used without further purification. All of the solvents used were of analytical reagent grade. Deionized water was used to prepare all aqueous solutions.

2.2. Instruments

A pH meter (Mettler Toledo, Switzerland) was used to determine the pH. The ^1H -NMR (600 MHz) and ^{13}C -NMR (150 MHz) spectra were recorded in $\text{DMSO}-d_6$ at 20°C on Bruker spectrometer, and tetramethylsilane (TMS) as an internal standard. Melting point (mp) was determined on SGW[®]X-4B (Shanghai INESA Physico optical Instrument Co.,Ltd). IR spectra was performed on a Bruker VERTEX 70 spectrometer. UV - visible spectra were performed on a Thermo Scientific NanoDrop 2000 spectrophotometer. Fluorescence spectra were measured on a Hitachi F-7000 fluorescence spectrophotometer. Electrospray ionization mass spectra (ESI-MS) were measured with Agilent 1260 Series system (Agilent, USA) and AB Sciex 4000Q Trap instrument. The fluorescence images were obtained using Olympus confocal laser scanning microscopy (Olympus Fluoview FV1000). The optical density (OD) of formazan solutions was recorded on a microplate spectrophotometer (Molecular Devices, Versa Max, USA).

2.3. Synthesis of probe 1

The synthesis of probe **1** is summarized in **Scheme 1**.

8-hydroxy-2,3,6,7-tetrahydro-1H,5H-pyrido[3,2,1-ij]quinoline-9-carbaldehyde (2.17 g, 10mmol), diethylmalonate (3.2 g, 20 mmol) and piperidine (10 drops) were combined in absolute ethanol (30 mL). The above solution was refluxed for 24 hours. After completion of the reaction, ethanol was removed under reduced pressure. Then concentrated HCl (20 mL) and glacial acetic acid (20 mL) were added. After the mixture was stirred at 80 °C for another 6h, it was poured into ice water (100 mL). NaOH solution (0.1M) was used to adjust the pH to ~6 to obtain a large amount of yellow precipitate. After stirring for 30 min, the mixture was filtered, washed with water and then purified by column chromatography on silica gel (petroleum ether-ethyl acetate =1:5) to afford yellow crystal (1.75g, 73%)

Under nitrogen, freshly distilled DMF (2.8 mL) was added dropwise to POCl₃ (2.8 mL) at room temperature and stirred for 30 min to obtain a red solution. Then a portion of 2,3,6,7-tetrahydro-1H,5H,11H-pyrano[2,3-f]pyrido[3,2,1-ij]quinolin-11-one (1.21 g, 10 mmol), dissolved in DMF (13 mL) was added dropwise to the above solution to yield a scarlet suspension. After the mixture was stirred at 60 °C for 12 h, it was poured into ice water (100 mL). NaOH solution (20 %) was used to adjust the pH to ~6.0 to obtain a large amount of precipitate. The obtained product was purified by simple washing with hexane afford 11-oxo-2,3,6,7-tetrahydro-1H,5H,11H-pyrano[2,3-f]pyrido[3,2,1-ij]quinoline-10-carbaldehyde (0.98g, 72.9%). To a stirred solution of 11-oxo-2,3,6,7-tetrahydro-1H,5H,11H-pyrano[2,3-f]pyrido[3,2,1-ij]quinoline-10-carbaldehyde (0.54 g, 2 mmol) and malononitrile (0.14 g, 2 mmol) in ethanol (10 mL), piperidine (0.5 mL) was added at room temperature. The reaction mixture was stirred

at room temperature for 3 h. The solvent was evaporated under reduced pressure, and the residue was washed with ethanol to afford 2-((11-oxo-2,3,6,7-tetrahydro-1H,5H,11H-pyrano[2,3-f]pyrido[3,2,1-ij]quinolin-10-yl)-methylene)malononitrile as a purple-dark brown solid (0.47 g, 75.4%). mp 204.6-205.5°C. FT-IR (KBr, cm^{-1}) ν : 2219 (-CN), 1715 (-C=O), 1624 (-C=C), 1520 (-C=C), 1340 (-C-N), 1285 (-C-O), 1215 (-C-O). ^1H NMR (600 Hz, $\text{DMSO}-d_6$) 8.46 (s, 1H), 7.93 (s, 1H) 7.22 (s, 1H), 3.45 (q, $J=5.4\text{Hz}$, 4H), 2.71 (t, $J=6.6\text{Hz}$, 4H), 1.87-1.91 (m, 4H); ^{13}C NMR (150 MHz, CDCl_3) δ 160.6, 153.3, 152.3, 150.7, 143.6, 128.6, 120.8, 114.5, 109.0, 108.8, 106.5, 50.8, 50.4, 27.2, 20.9, 19.9, 19.7. (ESI-MS) m/z : $[\text{M}+\text{H}]^+$ Calcd for $\text{C}_{14}\text{H}_{15}\text{N}_3\text{O}_2$ 318.1237, Found 318.1238.

<Inserted Scheme 1>

2.4. Sample preparation and measurements

Stock solutions of probe **1** (2 mmol/L) were prepared in DMSO. Stock solutions (0.2 mol/L) of the analytes were prepared in water. The stock solutions were used freshly and were diluted to desired concentrations with water when needed. UV-Vis and fluorescence spectra were obtained in DMSO: water (1: 9 v/v, HEPES buffer, pH 7.4) solution. The emission spectrum was scanned from 425 nm to 650 nm at 1200 nm/min. The photo multiplier voltage was set at 700V.

2.5. MTT assay

The effect of probe **1** on cells viability was determined using the MTT assay. In briefly, MCF-7 cells were seeded in 96 well culture plates at density of 2×10^4 cells per well and incubated overnight. Then probe **1** was added to the wells to achieve final

concentrations. Control wells were prepared by addition of culture medium. Wells containing culture medium without cells were used as blanks. At the end of incubation, 10 μL of MTT (5.0 mg/mL) was added into each well and incubation for another 4 h. Then, the supernatant was removed and 100 μL DMSO was added to each well to dissolve the MTT formazan. The cell viability was presented as the fold over the control group and was calculated according to the following formula: cell viability (%) = $(\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}) / (\text{OD}_{\text{control}} - \text{OD}_{\text{blank}}) \times 100$.

2.6. Cell culture and confocal microscopic methods

The MCF-7 cell was maintained in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose and 10% fetal bovine serum in a water-jacketed CO_2 incubator providing a humidified atmosphere containing 5% CO_2 at 37 $^{\circ}\text{C}$, then washed with PBS (pH 7.4) buffer three times. Fluorescence imaging was performed by an Olympus FV1000 confocal laser scanning microscope. Emission was collected at 500-540 nm for the green channel ($\lambda_{\text{ex}}=488$ nm) and at 555-655 nm for the red channel ($\lambda_{\text{ex}} = 568$ nm).

3. Results and discussion

3.1. Selectivity of probe **1** to HSO_3^-

HSO_3^- , F^- , Cl^- , Br^- , NO_3^- , NO_2^- , I^- , SCN^- , $\text{S}_2\text{O}_3^{2-}$, AcO^- , CO_3^{2-} , SO_4^{2-} , ClO^- , HS^- , H_2O_2 , SO_3^{2-} , Cys, Hcy and GSH were added to the DMSO:HEPES (1:9, v/v, pH = 7.4) buffer solutions of probe **1** respectively to investigate the response of **1** to analytes. Most of the analytes showed no significant influence on the UV-vis absorption spectra of the probe solution, while only HSO_3^- was added to the solutions, the absorption

peak at 558 nm gradually decreased and a new peak appeared at 440 nm. A well-defined isosbestic point was noted at 488 nm, which indicate the formation of single new species. We also note that the color of the solution changes from pink to yellow as shown in **Fig. 1a**. On the other hand, as it can be seen that the addition of HSO_3^- caused significant changes in the fluorescence spectra, and the color of solution changed from red to green as shown in **Fig. 1b**. Hence, probe **1** can be used as a directly observable and fluorescent probe for HSO_3^- in DMSO:HEPES buffer (1:9, v/v, pH = 7.4).

<Inserted Figure 1>

3.2. UV - vis and fluorescence spectra of detecting HSO_3^-

The good selectivity inspired us to explore the concentration dependent response of probe **1** toward HSO_3^- . **Fig. 2a** shows the change in the UV - vis spectrum when the HSO_3^- solution is added to DMSO:HEPES buffer (1:9, v/v, pH = 7.4) containing probe **1** (10 $\mu\text{mol/L}$). With increasing HSO_3^- concentration, the absorption peak of probe at 558 nm is gradually decreased with one isosbestic point at 488 nm, indicating the formation of a new compound with the growth of 440 nm, and the color of solution is from pink to yellowish. The probe **1** exhibit strong fluorescence emission at 593 nm ($\lambda_{\text{ex}} = 558 \text{ nm}$), the changes of the fluorescence spectra of probe **1** (1.0 $\mu\text{mol/L}$) in the presence of HSO_3^- are displayed in **Fig. 2b**. The data shown in **Fig. 2b** indicated that the addition of HSO_3^- caused changes in the fluorescence spectra and the color of the solution changed from red to green. We examined the changes of fluorescence intensity at $\lambda_{\text{ex}} = 440 \text{ nm}$ (**Fig. 2b**) and 558 nm (**Fig. S2, ESI**) due to its

red-shift of 99 nm in emission respectively. The fluorescence intensity was linearly proportional to their amount from 0 to 20 equiv. The stokes shift of probe in the red region is 35 nm (fluorescence quantum yield $\Phi = 0.36$, rhodamine B as standard). As depicted in Fig. 2a, the reaction product of probe with HSO_3^- exhibited a 54 nm stokes shift (fluorescence quantum yield $\Phi = 0.54$, fluorescein as standard). The detection limits (DL, S/N = 3) for HSO_3^- was determined to be 0.87-1.56 $\mu\text{mol/L}$ (**Fig. S3, ESI**). This probe is sensitive enough to monitor HSO_3^- comparable to that of other reported HSO_3^- chemosensors [17, 38, 39, 40] (**Table1**).

<Inserted Figure 2>

<Inserted Tbble1>

3.3. Effect of time

Reaction time is an important factor for probe, so the time response of **1** to HSO_3^- was investigated. Time-dependent modulations in the UV – Vis spectra of probe **1** were monitored in the presence of 3 equiv. of HSO_3^- . The kinetic study showed that the reaction was completed within 5 min for HSO_3^- , indicating that probe reacts rapidly with HSO_3^- under the experimental conditions (**Fig. 3**).

<Inserted Figure 3>

3.4. pH dependence of probe

The pH response for probe **1** and **1**- HSO_3^- complexes were investigated using solution with pH values between 3.0 and 13.0. **Fig. 4** showed probe **1** has weak fluorescence emission at 494 nm in the range of pH 3.0~12.0 except for pH 13.0. After addition of HSO_3^- , the fluorescence signal for the probe solution containing bisulfite anion

increased sharply from pH 4.0 to 8.0, and partial fluorescence increased at pH 3 or 9.0~12.0. The physiological pH (7.4) was selected for further research.

<Inserted Figure 4>

3.5. Proposed mechanism

The probe can detect HSO_3^- based on the coumarin derivative as a dye and using the polarized C=C bond as recognition site. The recognition mechanism can be reasonably explained by the nucleophilic addition reaction of HSO_3^- with the polarized C=C bond ((**Fig. 5**). To clarify the sensing mechanism of probe **1**, the sensor solution was investigated by the ^1H NMR-titration experiments (**Fig. 6**). Upon the addition of HSO_3^- to solution (in $\text{DMSO}-d_6$) of probe **1**, the peak at 8.06 ppm (H1) assigned to the alkene proton disappeared and two new peaks at 4.43 and 4.02 ppm (CH alkyl) appeared. Moreover, Mass spectrometry analysis of a product obtained from the reaction of probe **1** with HSO_3^- in CH_3OH , a peak at $m/z = 400.9$, corresponding to $[\text{1} - \text{HSO}_3^- + \text{H}]^+$, is clearly observed (**Figure S4, ESI**). Based on the above results, a possible model of probe **1** with HSO_3^- is proposed.

<Inserted Figure 5>

<Inserted Figure 6>

3.6. Cellular Imaging

Cytotoxicity is an important indicator of the applicability of probe in biological systems. To further evaluate the function of the probe for biological applications, we performed an assay to detect intracellular HSO_3^- in live cells. MCF-7 cells were used in our study. An MTT experiment was conducted and the results showed that the

probe at a concentration of 10 $\mu\text{mol/L}$ has only minimal cytotoxicity. (**Fig. S5, ESI**). Finally, we investigated the potential use of probe **1** in live cell. The ability of **1** to mark HSO_3^- within living cells was also evaluated by laser confocal fluorescence imaging using an Olympus FV1000 laser scanning microscope. The optical window at the green channel (500 – 540 nm) and red channel (555-655 nm) was chosen as a signal output. As shown in **Fig. 7A3**, under selective excitation at 568 nm, MCF-7 cells incubated with 5 $\mu\text{mol/L}$ of probe **1** for 30 min at 37 °C showed green fluorescence. In a further experiment it was found that MCF-7 cells displayed no fluorescence when the cells were first incubated with 20 $\mu\text{mol/L}$ of HSO_3^- for 30 min at 37 °C and then incubated with **1** (**Fig. 7B3**). These cell experiments show **1** can thus be used to mark HSO_3^- within living cells.

<Inserted Figure 7>

4. Conclusions

In summary, we have developed a new probe for HSO_3^- based coumarin-malononitrile derivative. The nucleophilic addition reaction mechanism of **1** with HSO_3^- was also investigated by ^1H NMR and ESI-MS. In UV – vis absorption, blue-shift from 558 to 440 nm with color of solution is from pink to yellow, also in the fluorescence emission spectra, blue-shift from 593 to 494 nm, was observed and the color of solution was changed from red to green. Preliminary biological experiments indicated that this probe has the potential to detect and image HSO_3^- in biological systems.

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Figure captions

Scheme 1 The synthesis of probe 1.

Fig. 1 (a) Above: UV-Vis absorption spectra of probe 1 (10 $\mu\text{mol/L}$) in solution (DMSO : HEPES = 1 : 9, v/v, pH 7.4) in the presence of all kinds of analytes (200 $\mu\text{mol/L}$), Insert: a color change photograph for HSO_3^- and other analytes; (b) fluorescence spectra of probe 1 (1 $\mu\text{mol/L}$) with various analytes (100 $\mu\text{mol/L}$) in solution (DMSO : HEPES = 1 : 9, v/v, pH 7.4) ($\lambda_{\text{ex}} = 440 \text{ nm}$, slit: 5 nm/5 nm), Insert: a visual fluorescence change photograph for HSO_3^- and other analytes under illumination with a 365 nm UV lamp.

Fig. 2 (a) Absorption spectral changes of probe 1 (10 $\mu\text{mol/L}$) in DMSO/HEPES buffer solution DMSO : HEPES = 1 : 9, v/v, pH 7.4) upon addition of HSO_3^- ; HSO_3^- added gradually with $[\text{HSO}_3^-] = 0 - 3 \text{ eq.}$ (b) Fluorescence spectral changes of probe 1 (1 $\mu\text{mol/L}$) DMSO/HEPES buffer solution DMSO : HEPES = 1 : 9, v/v, pH 7.4) upon addition of HSO_3^- ; HSO_3^- added gradually with $[\text{HSO}_3^-] = 0 - 20 \text{ eq.}$ ($\lambda_{\text{ex}} = 440 \text{ nm}$, slit: 5 nm/5 nm).

Table 1 A comparison table about the detection limits for HSO_3^-

Fig. 3 (a) Reaction time profile of probe 1 (10 $\mu\text{mol/L}$) and HSO_3^- (30 $\mu\text{mol/L}$) in absorption spectral at 440 and 558 nm. (b) Reaction time profile of probe 1 (1 $\mu\text{mol/L}$) and HSO_3^- (20 $\mu\text{mol/L}$) in fluorescence spectral at 493 nm ($\lambda_{\text{ex}} = 440 \text{ nm}$, slit: 5 nm/5 nm)

Fig. 4 Effect of pH on the fluorescence intensity of the probe 1. Bisulfite concentration: (a) 0 and (b) 20 $\mu\text{mol/L}$.

Fig. 5 The proposed mechanism for the determination of HSO_3^- .

Fig. 6 The sensing mechanism of probe for HSO_3^- . ^1H NMR spectral change of the probe in the absence (top) and presence (bottom) of exceed equiv. HSO_3^- $\text{DMSO}-d_6$.

Fig. 7 Confocal fluorescence images of MCF-7 cells: A1 and A3: Fluorescence image of MCF-7 cells with probe (0 and 5 $\mu\text{mol/L}$) and their brightfield image (A2 and A4); (c) Fluorescence image of MCF-7 cells incubated with 10 $\mu\text{mol/L}$ HSO_3^- for 30 min at 37°C (B1) and its brightfield image (B2) then incubated with 5 $\mu\text{mol/L}$ probe **1** for 30 min at 37 °C (B3) and its brightfield image (B4).

Scheme 1

Figure 1

(a)

(b)

Figure 2

(a)

(b)

Table 1

Method	Analyte	Signal output	solvent	Detection limit(M)
Ref.[17]	HSO_3^-	Fluorescence	DMSO–acetate	1.0×10^{-7}
Ref.[38]	HSO_3^-	Fluorescence	Citric acid	6.3×10^{-6}
Ref.[39]	HSO_3^-	Absorbance	PBS	3×10^{-6}
Ref.[40]	HSO_3^-	Fluorescence	PBS	3.0×10^{-5}
This work	HSO_3^-	Fluorescence	DMSO–HEPES	8.6×10^{-7}

Figure 3

(a)

(b)

Figure 4

Figure 5

Figure 6

Figure 7

Electronic Supporting Information (ESI)

A highly sensitive fluorescent probe for the detection of bisulfite ion and its application in living cells

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Figure S1: ¹H NMR, ¹³C NMR, ESI-MS, IR spectra of the probe **1**

Figure S2: Fluorescence spectral changes of probe **1** upon addition of HSO₃⁻

Figure S3: Detection limit for sensing of HSO₃⁻

Table 1: A comparison table about the detection limits for HSO₃⁻

Figure S4: ESI-MS spectra of the **1**-HSO₃⁻

Figure S5: MTT assay.

Figure S1: ^1H NMR, ^{13}C NMR, ESI-MS spectra of the probe **1**

EI-MS of the1

IR spectra of the1

Figure S2: Fluorescence spectral changes of probe **1** upon addition of HSO_3^-

Figure S2: Fluorescence spectral changes of probe **1** ($1\mu\text{mol/L}$) DMSO/HEPES buffer solution DMSO : HEPES = 1 : 9, v/v, pH 7.4) upon addition of HSO_3^- ; HSO_3^- added gradually with $[\text{HSO}_3^-] = 0 - 20\text{eq}$ ($\lambda_{\text{ex}} = 558\text{ nm}$, slit: 5 nm/10 nm).

Figure S3: Detection limit for sensing of HSO_3^-

(a)

Figure S3: Calibration curve for the HSO_3^- .

Figure S4: ESI-MS spectra of the **1**- HSO_3^-

Figure S5: MTT assay.

Figure S5: Cell viability of MCF-7 cells treated by probe **1** at different concentrations for different time.