Colorimetric and Ratiometric Fluorescence Detection of \( \text{HSO}_3^- \) With a NIR Fluorescent Dye

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Abstract

Bisulfite (\( \text{HSO}_3^- \)) has been widely used in food and industry, which has brought convenience to human life, but also seriously endangered human health. In this work, the probe PBI was designed and synthesized to detect bisulfite (\( \text{HSO}_3^- \)) through nucleophilic addition reaction. The probe PBI showed a selective reaction to \( \text{HSO}_3^- \) and can quantitatively detect \( \text{HSO}_3^- \). At the same time, the color of the probe PBI changed significantly, which provided a simple method for the naked eye to identify \( \text{HSO}_3^- \). Finally, it was successfully applied to the fluorescence imaging of \( \text{HSO}_3^- \) in living cells.

Keywords Colorimetric · Ratiometric fluorescence · \( \text{HSO}_3^- \) · NIR fluorescent dye

Introduction

In food, \( \text{HSO}_3^- \) can be used as a bleaching agent, preservative, and antioxidant [1–3]. \( \text{HSO}_3^- \) is a bleaching agent to improve food color and antibacterial effects, and widely used in food processing [4]. Then it can be used as a preservative to inhibit microbial activity and prevent food spoilage [5]. In terms of antioxidants, \( \text{HSO}_3^- \) can prevent or delay food oxidation, improve food stability and extend storage life [6]. As a large amount of \( \text{HSO}_3^- \) may cause tissue damage in individuals, it is necessary to strictly control the amount of \( \text{HSO}_3^- \) in food [7–9]. \( \text{HSO}_3^- \) is a reducing agent that plays the key role in industries such as dyes, papermaking, leather making, and chemical synthesis [10–13]. \( \text{HSO}_3^- \) can bleach cotton fabrics and organic substrate [14], treat chromium-containing waste water [15], and be used as an electroplating additive [16]. In the physiological system, \( \text{HSO}_3^- \) is mainly produced by the oxidation of cysteine and glutathione, and this process is mediated through reactive oxygen species (ROS) [17, 18]. Toxicological studies had shown that low concentrations (<450 μM) of \( \text{HSO}_3^- \) have a significant promoting effect on the vasodilation of the cardiovascular system [19]. However, when the expression of \( \text{HSO}_3^- \) rises in the vivo, it can cause a series of diseases [20–23]. Therefore, it is of great significance to study new methods of \( \text{HSO}_3^- \) detection.

Some conventional analysis techniques for the detection of bisulfite have been developed, such as spectrophotometry [24, 25], chemiluminescence measurements [26, 27], chromatography [28], electrochemistry [29, 30], and phosphorimetry [31]. However, the detection process of these methods is more complicated, and some of them are not sensitive enough. So far, many fluorescent probes have been developed for the detection of \( \text{HSO}_3^- \), because they have obvious advantages, including admirable sensitivity, in-situ detection ability and easy visualization with the naked eye [32–34]. These probes mainly react with \( \text{HSO}_3^- \) by using several kinds of reaction mechanisms. For example, the nucleophilic reaction with aldehydes [35], hydrogen bonding [36], and the nucleophilic addition reaction with the carbon–carbon double bond [37, 38].

In this work, a new colorimetric and ratiometric fluorescent probe PBI for detecting \( \text{HSO}_3^- \) was designed based on the nucleophilic addition reaction (Scheme 1). Through experiments, the detection properties of the probe PBI and
its application in biological fluorescence imaging have been studied.

**Experimental**

**Materials**

4-Bromonaphthalene-1-carbonitrile, hydrazine hydrate, 2-methoxyethanol, 3-methyl-2-butanone, 3-aminophenol, 1-bromo-3-chloropropane, N,N-dimethylformamide, acetone, petroleum ether, dichloromethane, anhydrous ethanol, dimethyl sulfoxide, NaHCO₃, POCl₃, K₂CO₃, Na₂SO₄, NaF, NaCl, NaBr, NaI, NaHSO₃, NaOH, KCl, CaCl₂, AlCl₃, ZnCl₂, SnCl₂, Pb(NO₃)₂, CuCl, CuCl₂, FeCl₃, MgCl₂, AgCl, Ni(NO₃)₂, MnCl₂, Na₃PO₄, Na₃HPO₄, NaH₂PO₄, Co(NO₃)₂, Cd(NO₃)₂, Na₂CO₃, NaBF₄, NaI, NaNO₂, NaNO₃, NaHSO₄, Ala, Arg, Asp, Cys, Hcy, Gln, Glu, Gly, His, Ile, Leu, Met, Phe, Pro, Ser, Urea, Val.

**Laboratory Apparatus**

Dual-beam UV–vis Spectrophotometer (TU-1901), Fluorospectrophotometer (F-4600), 400 M NMR spectrometer (AVIII HD 400), High-resolution mass spectrometer (IonSpec4.7), pH meter (PHS-2F), Rotary evaporator (RE-2000B), Electronic analytical balance (FA2004), Constant temperature magnetic stirrer (85–2), Vacuum drying oven (DZF-6020), Circulating water vacuum pump (SHB-3), Vacuum oil pump (2XZ-4), Digital camera (D3300), Portable UV analyzer (ZF-5).

**Synthesis and Methods**

The synthetic route was shown in Scheme 1. Compound 3 was prepared according to previously reported method [39].

**Synthesis of Compound 1**

4-Bromonaphthalene-1-carbonitrile (692.9 mg, 3 mmol) was dissolved in a mixed solution of hydrazine hydrate 80% (1.5 mL, 30 mmol) and 2-Methoxyethanol (25 mL), the resulting mixture was then heated to 125 °C, and reacted under reflux for 8 h. After that, the solution was cooled, filtered with suction, and washed with ethanol to obtain the compound 1 (440 mg, 72.8%). The specific characterization of compound 1 (¹H NMR, ¹³C NMR, mass spectrum) in Figs. S1, S2, and S3. ¹H NMR (600 MHz, DMSO-d₆): δH 8.57 (d, J = 8.2 Hz, 1H), 8.16 (d, J = 8.0 Hz, 1H), 7.84–7.74 (m, 2H), 7.43 (s, 3H), 1.38 (s, 6H). ¹³C NMR (151 MHz, DMSO-d₆): δC 195.00, 152.79, 142.04, 133.14, 129.18, 128.26, 127.97,
125.92, 125.07, 124.41, 118.76, 105.43, 55.75, 22.10, 16.19.

**Synthesis of Compound 2**

Compound 1 (402.75 mg, 2.2 mmol) was added to 3-methyl-2-butanone (20 mL), then concentrated sulfuric acid (0.5 mL) was mixed to get a white turbid liquid. The mixture was heated up to 125 °C and reacted for 8 h. After the reaction, the mixture was cooled to room temperature, a solid precipitated out, and suction filtration to get the compound 2 (310 mg, 60.2%). The specific characterization of compound 2 (1H NMR, 13C NMR, mass spectrum) in Figs. S4, S5, and S6. 1H NMR (400 MHz, DMSO-d$_6$): δH 8.61 (s, 1H), 8.26 (d, J = 8.5 Hz, 1H), 7.90 (dd, J = 19.5, 8.2 Hz, 2H), 7.71–7.63 (m, 1H), 7.55–7.46 (m, 1H), 7.07 (d, J = 8.3 Hz, 1H), 4.48 (s, 2H). 13C NMR (151 MHz, DMSO-d$_6$): δC 151.91, 135.49, 133.43, 128.93, 125.62, 124.84, 122.86, 120.78, 120.27, 102.89, 93.4.

**Synthesis of PBI**

Compound 2 (217.11 mg, 1 mmol) and compound 3 (234.11 mg, 1 mmol) were dissolved in ethanol (30 mL), and the reaction mixture was heated to reflux for 12 h. Then the resulting mixture was cooled to room temperature and the product was collected by suction, washed with ethanol, and dried in vacuo (207.9 mg, 48.0%). The specific characterization of PBI (1H NMR, 13C NMR, mass spectrum) in Figs. S7, S8, and S9. 1H NMR (400 MHz, DMSO-d$_6$): δH 8.64 (d, J = 7.7 Hz, 1H), 8.55 (s, 1H), 8.39 (s, 1H), 8.14 (d, J = 7.9 Hz, 1H), 7.94–7.73 (m, 2H), 7.52 (s, 1H), 7.00–6.85 (m, 1H), 3.37 (s, 5H), 2.77–2.55 (m, 4H), 1.89 (s, 4H), 1.57 (s, 6H). 13C NMR (151 MHz, DMSO-d$_6$): δC 133.37, 129.58, 127.98, 125.24, 124.38, 118.99, 103.25, 50.03, 27.15, 25.10, 21.59, 21.24, 20.59, 15.61, 0.57.

**Results and Discussion**

**Detection Properties of Probe PBI**

For better experimental results, we must first select the most suitable experimental system. The DMSO content was changed from 10%, 20%, to 60% in the test system, and HSO$_3$\(^-\) was added to test under the same conditions. From Fig. 1a, when the DMSO content increased from 10 to 60%, the I$_{397}$/I$_{646}$ decreased. The I$_{397}$/I$_{646}$ reached the maximum as the DMSO content was 10%, but in comparison, when the DMSO content was 20%, the ΔI$_{397}$/I$_{646}$ was the largest before and after the reaction. So we chose the test system as $V_{\text{water}}:V_{\text{DMSO}}$ = 8:2.
In order to understand the properties of fluorescent probe, it is very important to keep the properties of probe stable. As shown in Fig. 1b, the \( \text{I}_{397}/\text{I}_{646} \) showed a stable trend within 60 min, and the fluctuation range could be ignored. It indicated that the probe will not be disturbed by the illumination time, and showed good light stability.

For a better understanding of the practical application of the probe, the detection time of \( \text{HSO}_3^- \) with PBI was studied. Different concentrations of \( \text{HSO}_3^- \) (0 mmol/L, 0.2 mmol/L, 0.3 mmol/L, 0.4 mmol/L) were added to the fluorescent probe PBI solution, and the fluorescence spectra were tested with reaction time changing. It can be seen from Fig. 1c that when 0.2 mmol/L and 0.3 mmol/L \( \text{HSO}_3^- \) were added respectively, the \( \text{I}_{397}/\text{I}_{646} \) increased quickly in the first 12 min, and then reached the maximum when the time was 60 min. when 0.4 mmol/L \( \text{HSO}_3^- \) was added, the \( \text{I}_{397}/\text{I}_{646} \) increased rapidly and the reaction almost finished within 60 min. such results indicates that concentration of \( \text{HSO}_3^- \) can affect the detection rate at the...
first period and have no influence on the overall reaction time of probe with $\text{HSO}_3^-$. The probe should have a wide range of pH for better detection of $\text{HSO}_3^-$. Test system separately was prepared with DMSO and deionized water of different pH, the pH ranges from 1 to 14, and fluorescence intensity of probe PBI was recorded in the absence and presence of $\text{HSO}_3^-$. As shown in Fig. 1d, in the pH range of 4 to 8, great difference of the $I_{397}/I_{646}$ of probe PBI with and without $\text{HSO}_3^-$ was obtained, so the optimal pH test range for the probe PBI to detect $\text{HSO}_3^-$ was 4–8. Such result illustrated that probe PBI can detect $\text{HSO}_3^-$ in a wide pH range and have potential applications in real sample detection.

The UV–vis spectral response of probe PBI to $\text{HSO}_3^-$ was tested firstly. As shown in Fig. 2a, probe PBI showed the absorption maximum at 520 nm originally. Upon addition of $\text{HSO}_3^-$ to the solution, the absorption peak at 520 nm gradually decreased, and the absorption peak progressively increased at 350 nm. When the $\text{HSO}_3^-$ concentration in the test system was $0.07 \text{ mmol/L}$ to $0.22 \text{ mmol/L}$, the $A_{350}/A_{520}$ showed a good linear relationship with the $\text{HSO}_3^-$ concentrations (Fig. 2b), this means that in this interval, we can achieved quantitative detection of $\text{HSO}_3^-$. After adding $\text{HSO}_3^-$, the color of PBI solution changed from pink to colorless under daylight (Fig. 2c), the change was so obvious that it provides an easy way to detect $\text{HSO}_3^-$ with the naked eye.

Fluorescence spectra of probe PBI over various concentrations of $\text{HSO}_3^-$ were recorded. As for PBI, We used double excitation mode that was to choose 350 nm and 520 nm as the excitation wavelength. As shown in Fig. 3a, after the titration of $\text{HSO}_3^-$, the fluorescence intensity of PBI at 392 nm increased gradually, while fluorescence intensity of PBI progressively decreased at 646 nm. In order to more directly express the relationship between fluorescence intensity and concentration of $\text{HSO}_3^-$, the $I_{397}/I_{646}$ was plotted as a function of the concentration of $\text{HSO}_3^-$ (Fig. 3b). In the range of $0.04 \text{ mmol/L}$ to $0.19 \text{ mmol/L}$, the linear increase of $I_{397}/I_{646}$ could be used in the quantitative detection of $\text{HSO}_3^-$. Upon addition of $\text{HSO}_3^-$, the color also changed significantly under portable UV lamps, gradually changing from pink to blue purple (Fig. 3c), which can be more convenient for $\text{HSO}_3^-$ detection in practical applications.

**Selectivity and Anti-interference Ability Studies of Probe PBI**

The special selectivity of fluorescent probes for analytes was higher than other substances is an important feature of it. To understand the selectivity of probe PBI toward $\text{HSO}_3^-$, we conducted a series of controlled experiments with anions. As can be seen from Fig. 4, The $A_{350}/A_{520}$ and $I_{397}/I_{646}$ with only the above anions added had no big difference compared with that of probe PBI only except $\text{HSO}_3^-$, which showed all these anions could not respond to probe PBI. The above anions and $\text{HSO}_3^-$ were added to probe PBI solution at the same time. The $A_{350}/A_{520}$ and $I_{397}/I_{646}$ were significantly increased compared with that of probe PBI only. Obviously, probe PBI responds to $\text{HSO}_3^-$ only, and when other anions and $\text{HSO}_3^-$ coexist, there was no interference on the detection of $\text{HSO}_3^-$ with probe PBI.
Meanwhile, similar to the above method, the influence of metal ions (Fig. S10) and amino acids (Fig. S11) on the detection of \(\text{HSO}_3^-\) with probe PBI was also explored. From the Figs. S10 and S11, it is clear that probe PBI can specifically select \(\text{HSO}_3^-\) in the presence of cations and amino acids, and thus probe PBI had good selectivity and strong anti-interference ability.

**The Reaction Mechanism of Probe to Detect \(\text{HSO}_3^-\)**

The reaction mixture of PBI and NaHSO\(_3\) was analyzed by ESI–MS spectroscopy to explore the recognition mechanism of probe PBI to \(\text{HSO}_3^-\). As shown in Fig. S12, \(m/z = 516.1957\) (theoretical molecular weight = 516.1952) \([\text{M} + \text{HSO}_3^- + \text{H}]^+\) was the peak after the carbon–carbon double bond nucleophilic addition reaction between probe PBI and \(\text{HSO}_3^-\). So we proposed the detection mechanism of probe PBI for \(\text{HSO}_3^-\): in the condition of \(\text{HSO}_3^-\), six-membered ring of probe PBI was broken, and \(\text{HSO}_3^-\) was added to the position of the original carbon–carbon double bond (Scheme 1).

**The Detection of \(\text{HSO}_3^-\) in Living Cells**

In order to explore the potential application of probe PBI in the detection of \(\text{HSO}_3^-\) in living cells, fluorescence confocal imaging experiments were performed in HeLa cells with probe PBI, and the images were captured under a confocal fluorescence microscope. We studied the feasibility of the probe PBI to detect exogenous \(\text{HSO}_3^-\) in HeLa cells. The bright field pictures showed the position in the living cells (Fig. 5a, d). The sole probe PBI emitted clear red fluorescence (Fig. 5b), when the cells were treated with \(\text{HSO}_3^-\), the red fluorescence disappeared (Fig. 5e), and they overlaid well (Fig. 5c, f). The results indicated that probe PBI can effectively detect exogenous \(\text{HSO}_3^-\), which can be used to detect \(\text{HSO}_3^-\) in the living cells.

**Comparison of Probe PBI with Some Reported Probes Toward \(\text{HSO}_3^-\)**

As shown in Table 1, in terms of excitation wavelength/emission wavelength, detection medium, response type, pH, detection limit and cell application, probe PBI had good analytical performance compared with other \(\text{HSO}_3^-\) fluorescent probes reported recently.\(^{40–45}\) The result showed that probe PBI was a ratiometric fluorescent probe with a low detection limit (208 nmol/L). Moreover, biological experiments have demonstrated the application of probe PBI in monitoring intracellular \(\text{HSO}_3^-\).

![Fluorescence confocal images of probe PBI (1 µmol/L) to detect exogenous \(\text{HSO}_3^-\) (0.4 mmol/L) in HeLa cells.](image-url)

**Table 1** Comparison of various fluorescent probes for \(\text{HSO}_3^-\) detection

| Probe | \(\lambda_ex/\lambda_em\) (nm) | Detection medium | Response type | pH range | LOD (µM) | Cellular applications | Ref |
|-------|-----------------------------|-----------------|--------------|----------|----------|-----------------------|-----|
| PPA   | 395/455/604                 | DMSO/PBS (1:9, v/v) | ratiometric | 7–9      | 11.03    | exogenous/endogenous  | [40]|
| probe | 440/493/593                 | DMSO/HEPES (1:9, v/v) | off–on      | 4–8      | 0.87     | exogenous             | [41]|
| EIM   | 440/476/579                 | PBS             | ratiometric | 5–11     | 0.20     | exogenous             | [42]|
| BIQ   | 380/460/475/625            | DMSO/H\(_2\)O (3:7, v/v) | ratiometric | 4–11     | 0.29     | exogenous             | [33]|
| probe | 525/520/600                 | DMSO/PBS (5:5, v/v) | on–off      | 5–11     | 2.01     | exogenous             | [43]|
| probe | 366/420/530                 | Water/Ethanol (9:1, v/v) | ratiometric | Not good | 0.77     | exogenous/endogenous | [44]|
| PBI   | 350/397/520                 | DMSO/PBS (2:8, v/v) | ratiometric | 4–8      | 208      | exogenous             | This work |
Conclusion

In summary, we have developed a new colorimetric and ratiometric fluorescence probe PBI to detect HSO$_3^−$. The reaction mechanism of probe PBI to detect HSO$_3^−$ was attributed to the nucleophilic addition reaction. When the probe was added with HSO$_3^−$, the absorption and fluorescence emission changed significantly, and the color change was so obvious that it provided an easy way to detect HSO$_3^−$ with the naked eye. In addition, probe PBI shows good selectivity and strong anti-interference ability for naked eye. In addition, probe PBI shows good selectivity and strong anti-interference ability for HSO$_3^−$. The probe PBI can in HeLa cells, which would be potential candidates to track HSO$_3^−$ in live cells.

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Authors’ Contributions  All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Wenjie Liu, Chenchen Yang and Hongyan Zhang. Findings were acquired by Zhanxian Li and Mingming Yu. The first draft of the manuscript was written by Wenjie Liu, all authors commented on previous versions of the manuscript and the manuscript was revised by Zhanxian Li and Mingming Yu. All authors read and approved the final manuscript.

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Data Availability  All data generated or analysed during this study are included in this published article and its supplementary information files.

Code Availability  All data were obtained using word, origin and chemBioDraw.

Declarations

Conflicts of Interest  The authors declare that they have no conflict of interest.

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