Red-Emission Probe for Ratiometric Fluorescent Detection of Bisulfite and Its Application in Live Animals and Food Samples

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ABSTRACT: Key roles of bisulfite (HSO₃⁻) in food quality assurance and human health necessitate a reliable analytical method for rapid, sensitive, and selective detection of HSO₃⁻. Herein, a new red-emitting ratiometric fluorescence probe, BIQ, is reported for sensitive and selective detection of HSO₃⁻ in food samples and live animals. Probe BIQ recognizes HSO₃⁻ via a 1,4-nucleophilic addition reaction. As a result of this specific reaction, emission intensities at 625 and 475 nm are dramatically changed, allowing the detection of HSO₃⁻ in a ratiometric fluorescence model in an aqueous solution. The obvious changes of solution color from pink to transparent and fluorescence color from rose red to cyan allow the detection of HSO₃⁻ by naked eyes. Furthermore, probe BIQ has fast response in color and fluorescence (<2 min), excellent selectivity, and a low detection limit (0.29 μM), which enables its application in HSO₃⁻ detection in food samples and live organisms. The practical applications of probe BIQ are then demonstrated by the visualization of HSO₃⁻ in live animals (zebrafish and nude mouse) as well as the determination of HSO₃⁻ in white wine and sugar.

1. INTRODUCTION

Bisulfite (HSO₃⁻) is an important reactive sulfur species (RSS) that plays a key role either in the physiological system or in the food industry.¹⁻⁷ Under physiological conditions, HSO₃⁻ is mainly generated through reactive oxygen species (ROS)-mediated oxidation of cysteine and glutathione.⁸,⁹ In the biological system, toxicological studies unveil that HSO₃⁻ is of great significance.¹⁰⁻¹³ However, the elevated expression of HSO₃⁻ in vivo is implicated in a series of diseases, such as lung and brain cancers, strokes, and migraine.¹⁴ In the food industry, HSO₃⁻ is as an effective preservative to protect food, beverages, and even pharmaceutical products from spoilage due to its antioxidant and enzyme-inhibition properties.¹⁵⁻¹⁷ Since extensive intake of HSO₃⁻ may potentially trigger allergic reactions or even lead to tissue damage in some individuals, the amount of HSO₃⁻ in food is strictly controlled.¹⁸ For example, the amount of sulfur (calculated by the SO₂ content) in refined white sugar should be lower than 0.1 g/kg in China. While in the USA, products with sulfite level higher than 10 μg/mL must be clearly labeled.¹⁹,²⁰ Hence, developing new methods for HSO₃⁻ detection in both biological system and food samples is of great significance.

Among the reported HSO₃⁻ detection methods,²¹⁻²⁸ such as spectrophotometry, chromatography, and capillary electrophoresis, fluorometry has drawn increasing attention due to its obvious advantages such as excellent sensitivity, selectivity, capability of detection in situ, and easy for naked-eye visualization.²⁹⁻³¹ To date, a number of molecular fluorescence probes for HSO₃⁻ have been developed by exploiting several response reaction mechanisms, such as a nucleophilic reaction with aldehyde,³²⁻³⁵ deprotection of the levulinate group,³⁶ hydrogen bonding,³⁶ and a nucleophilic addition reaction with the C=C bond.³⁷ Of these fluorescence probes, ratiometric fluorescence probes with the improved signal-to-noise ratio are more reliable for the detection of HSO₃⁻.³⁵⁻³⁷ However, some problems of these reported fluorescent probes are (i) relatively long reaction time (30 min to 10 h) and (ii) poor selectivity and sensitivity.³⁶ These limitations seriously retarded the application of the fluorescence probe for the detection of HSO₃⁻ in biological and food samples.

In the present work, a new probe (BIQ) for the ratiometric fluorescence detection of HSO₃⁻ in live animals and in food

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samples is developed (Scheme 1). Probe BIQ was synthesized by a one-step condensation reaction between quinoline derivatives and benzimidazole. The large π-conjugation system, thus formed, with typical intramolecular charge transfer (ICT) character endows probe BIQ with a red emission at 625 nm. In the presence of HSO₃⁻, the electron-deficient C≡C double bond linker is able to react with HSO₃⁻ through an established mechanism of the 1,4-addition reaction. As a result, the π-conjugation system of BIQ is interrupted, resulting in dramatic changes of ultraviolet-visible (UV-vis) absorption and fluorescence spectra. Introducing an electron withdrawing group (−CN) promoted the reaction between C≡C bond and HSO₃⁻ to be completed within 2 min. The ratiometric/colorimetric responses of BIQ toward HSO₃⁻ showed high selectivity over other competitive biologically relevant species. The applications of BIQ for HSO₃⁻ detection in zebrafish, mice, and food samples were then demonstrated.

2. RESULTS AND DISCUSSION

2.1. UV–Vis Spectra Response of the BIQ Toward HSO₃⁻. UV–vis absorption response of probe BIQ to HSO₃⁻ was first evaluated in 20 mM N-(2-hydroxyethyl)piperazine-N’-ethanesulfonic acid (HEPES) buffer of pH 7.4 (containing 30% dimethyl sulfoxide (DMSO) as a cosolvent). As shown in Figure 1A, probe BIQ exhibited a prominent absorption band centered at 460 nm, which can be attributed to the ICT process of probe BIQ. Upon the addition of HSO₃⁻ (0–250 μM), this absorption gradually decreased and the maximum absorption change was obtained when the concentration of HSO₃⁻ reached to 200 μM (Figure 1A, inset). To evaluate the selectivity of probe BIQ toward HSO₃⁻, changes of BIQ’s absorption spectra were recorded in the presence of common interference species, including HSO₃⁻, Br⁻, Cl⁻, F⁻, HSO₄⁻, S²⁻, NO₃⁻, NO₂⁻, O₂⁻, OH⁻, ONOO⁻, PO₄²⁻, PO₃⁻, SO₄²⁻, SO₃²⁻, HCO₃⁻, Pi, Pi, H₂O₂, Cys, Hcy, GSH, HOCl, and AcO⁻. As shown in Figure 1B, negligible UV–vis spectra changes were noticed upon the addition of interference species, indicating high selectivity of probe BIQ for HSO₃⁻ detection. The specificity of the UV–vis response of probe BIQ toward HSO₃⁻ was then validated by a naked-eye colorimetric assay. As shown in Figure 1C, the color of the probe BIQ solution was changed from pale orange to colorless in the presence HSO₃⁻.

2.2. Fluorescence Response of Probe BIQ to HSO₃⁻. Fluorescence response of probe BIQ to HSO₃⁻ was then examined by titration analysis in HEPES buffer. As shown in Figure S4, no obvious changes of emission spectra were obtained with two emission bands at 475 and 625 nm. As shown in Figure 2A, the emission peak at 625 nm (Φ₁ = 0.4314) while nearly nonemissive at 475 nm (Φ₂ = 0.0824) accompanied by a significant increase of fluorescence at 475 nm (Φ₁ = 0.5843) with the increase of HSO₃⁻ concentration. The maximum emission change was obtained when 200 μM HSO₃⁻ was added (Figure 2B). In addition, distinct fluorescence color changes of probe BIQ.
Figure 2. (A) Fluorescence response of probe BIQ (10 μM) toward various amounts of HSO$_3^–$ (0–250 μM) in HEPES aqueous buffer (DMSO/H$_2$O = 3:7, v/v, 20 mM, pH = 7.4). (B) Fluorescence emission intensities of probe BIQ at 475 and 625 nm as a function of different HSO$_3^–$ concentrations. Excitation was performed at 380 and 460 nm, respectively.

Figure 3. (A) Fluorescence responses of probe BIQ (10 μM) at 475 and 625 nm toward various competitive analytes (200 μM). (B) Fluorescence emission ratio $F_{625}/F_{475}$ of probe BIQ (10 μM) toward HSO$_3^–$ in the presence of diverse coexisting competitive analytes (200 μM), including 1. Blank, 2. Br$^–$, 3. Cl$^–$, 4. F$^–$, 5. HSO$_3^–$, 6. SO$_4^{2–}$, 7. NO$_2^–$, 8. NO$_3^–$, 9. O$_2^–$, 10. OH$^–$, 11. ONOO$^–$, 12. P$_2$O$_7^{4–}$, 13. PO$_4^{3–}$, 14. SO$_4^{2–}$, 15. SO$_3^{2–}$, 16. HCO$_3^–$, 17. Pi, 18. PPI, 19. H$_2$O$_2$, 20. Cys, 21. Hcy, 22. GSH, 23. HOCl, 24. AcO$^–$, and 25. HSO$_3^–$. Excitation was performed at 380 and 460 nm, respectively. (C) Fluorescence color photos of probe BIQ (10 μM) in the presence of above analytes.

from rose-red to cyan were obtained upon the addition of HSO$_3^–$ (Figure 2A, inset).

The fluorescence ratio ($F_{625nm}/F_{475nm}$) was linearly proportional to the concentration of HSO$_3^–$ (Figure S6). The detection of limit (LOD) was calculated to be 0.29 μM according to the IUPAC criteria (LOD = 3σ/k). Such a low detection limit allowed HSO$_3^–$ detection in food samples and live animals. The nucleophilic addition reaction between BIQ and HSO$_3^–$ was then studied by Job’s plot analysis. The changes of fluorescence intensities at 625 nm against the mole fraction of HSO$_3^–$ clearly showed the maximum value at around 0.5 (Figure S7), indicating the 1:1 stoichiometry nucleophilic reaction between BIQ and HSO$_3^–$.

Next, the selectivity of probe BIQ to HSO$_3^–$ over other competitive analytes (Br$^–$, Cl$^–$, F$^–$, HSO$_3^–$, SO$_4^{2–}$, NO$_2^–$, NO$_3^–$, O$_2^–$, OH$^–$, ONOO$^–$, P$_2$O$_7^{4–}$, PO$_4^{3–}$, SO$_3^{2–}$, SO$_4^{2–}$, HCO$_3^–$, Pi, PPI, H$_2$O$_2$, Cys, Hcy, GSH, HOCl, AcO$^–$) was evaluated by monitoring the fluorescence spectra and fluorescence intensity at 475 nm ($λ_{ex}$ = 380 nm) and 625 nm ($λ_{ex}$ = 470 nm), respectively. As shown in Figure 3A, no obvious changes in fluorescence spectra of probe BIQ were observed in the presence of other interference species (200 μM). To further investigate the specificity of BIQ, competitive experiments were conducted upon the addition of HSO$_3^–$ to the above interference species-spiked BIQ solution. As shown in Figure 3B, probe BIQ exhibited similar fluorescence response toward HSO$_3^–$ with the coexistence of other interfering species, indicating high selectivity of BIQ to HSO$_3^–$. The specificity of BIQ to HSO$_3^–$ was also examined by fluorescence color changes, where rose-red fluorescence emission faded exclusively in the presence of HSO$_3^–$ (Figure 3C), indicating that probe BIQ is able to detect HSO$_3^–$ in a complex system under a ratiometric fluorescence model.

2.3. Time and pH-Dependent Fluorescence Responses of Probe BIQ toward HSO$_3^–$. The time-dependent fluorescence response ($F_{625nm}/F_{475nm}$) of BIQ in the presence of different amounts of HSO$_3^–$ was then recorded within 200 s in HEPES buffer (Figure 4). Upon the addition of HSO$_3^–$ (0, 67, 124, and 200 μM), changes of fluorescence ratio ($F_{625nm}/F_{475nm}$) reached a plateau within 120 s, indicating that the 1:4-nucleophilic addition reaction between probe BIQ and HSO$_3^–$ is completed within 2 min. The speed of the fluorescence response of BIQ is comparable with some recently reported HSO$_3^–$ fluorescence probes.

In addition, the effect of pH on the fluorescence response of probe BIQ to HSO$_3^–$ was investigated in the absence and the presence of HSO$_3^–$. As shown in Figure 5, in the pH range from 4.0 to 11.5, the fluorescence emission ratio ($F_{625nm}/F_{475nm}$) of BIQ remained consistent and the fluorescence response of BIQ to HSO$_3^–$ was not affected in such a wide pH range. The result indicates that probe BIQ can be employed for the detection of HSO$_3^–$ in weak acid, weak base, and neutral conditions.
Fluorescence images of the BIQ-stained zebrafish showed strong fluorescence in the gill and abdomen. However, this fluorescence signal disappeared when the BIQ-stained zebrafish was further supplied with HSO₃⁻ (Figure 6A).

Furthermore, the mean fluorescence intensity of the gill and abdomen (Figure 6B) also indicates that probe BIQ can be employed as an imaging agent for visualizing HSO₃⁻ in zebrafish.

We then investigated the potential of probe BIQ for the imaging of HSO₃⁻ in mice. Probe BIQ (40 μM, 100 μL) was subcutaneously injected into the left leg of a 6 to 8-week-old nude mouse. After 5 min, the mice were injected with 20 μL of HSO₃⁻ (4 mM) at the same region. The images were then recorded at 5, 10, and 15 min after the HSO₃⁻ injection. As shown in Figure 7, an intense fluorescence signal was detected in the legs of the mice after the probe BIQ injection (Figure 7b), while this fluorescence signal was decreased after the injection of HSO₃⁻ along with time and vanished in 15 min, suggesting that probe BIQ was able to be used as an excellent imaging agent for visualizing HSO₃⁻ in vivo.

2.4. Detection of HSO₃⁻ in Food Samples. The feasibility of probe BIQ for HSO₃⁻ detection in food samples, including sugar and wine, was then evaluated using the calibration curves (Figure S8). As shown in Table 1, for the HSO₃⁻-spiked food samples, the recoveries were determined to be in the range from 96.62 to 101.83%, indicating the high accuracy of probe BIQ for HSO₃⁻ detection in real food samples.

2.5. Fluorescence Imaging of HSO₃⁻ in Vivo. Next, the application of the probe in living animals was evaluated through the imaging of HSO₃⁻ in adult zebrafish (oral feeding) and nude mouse (subcutaneous injection). Prior to the imaging application of BIQ in vivo, the cytotoxicity of probe BIQ to RAW 264.7 cells was evaluated using the MTT assay. As shown in Figure S9, no significant effect on the cell proliferation of BIQ was observed after co-culture with macrophage cells for 24 h, suggesting low cytotoxicity of BIQ. For the visualization of HSO₃⁻ in zebrafish, live adult zebrafish was stained in the probe BIQ solution (40 μM) for 5 min.

Table 1. Results for the Determination of HSO₃⁻ in Food Samples Using BIQ as the Probe

| food samples | C_bisulfito (μM) | bisulfito added (μM) | bisulfito found (μM) | recovery (%) |
|--------------|-----------------|----------------------|---------------------|--------------|
| white wine   | 5.15 ± 0.23     | 26.66                | 31.70 ± 0.33        | 99.66        |
|              | 39.99           | 44.53 ± 0.26         | 98.66               |
|              | 66.65           | 70.19 ± 0.41         | 99.76               |
| sugar        | 6.39 ± 0.54     | 26.66                | 32.29 ± 0.39        | 97.72        |
|              | 39.99           | 47.22 ± 0.32         | 101.83              |
|              | 66.65           | 70.57 ± 0.44         | 96.62               |

Figure 4. Time-profile fluorescence response (F_{425nm}/F_{475nm}) of probe BIQ (10 μM) in HEPES aqueous buffer (DMSO/H₂O = 3:7, v/v, 20 mM, pH = 7.4) upon the addition of various amounts of HSO₃⁻: (a) 0 μM, (b) 67 μM, (c) 124 μM, (d) 200 μM. Excitation was performed at 380 and 460 nm, respectively.

Figure 5. Influence of pH on the fluorescence response (F_{425nm}/F_{475nm}) of probe BIQ (10 μM) in the absence and the presence of HSO₃⁻. Excitation was performed at 380 and 460 nm, respectively.

Figure 6. (A) Fluorescence imaging of HSO₃⁻ intake in live adult zebrafish: (a) control group of zebrafish only; (b) zebrafish incubated with probe BIQ (40 μM) for 5 min; (c) zebrafish in (b) with further incubation of HSO₃⁻ (1 mM) for (c) 10 min and (d) 20 min. (B) Mean fluorescence intensity of zebrafish shown in (a−d). The images of zebrafish were recorded with an excitation filter (465 nm) and an emission filter (630 nm).

Figure 7. Fluorescence imaging of exogenous HSO₃⁻ in nude mice. (a) blank control group; (b) probe BIQ (40 μM, 100 μL) was subcutaneously injected into the left limbs of the mice, followed by the injection of HSO₃⁻ (4 mM, 200 μL) into the same interest area, and images were recorded at different times: (c) 5 min; (d) 10 min; and (e) 15 min, respectively. The mean fluorescence intensities (a−e) were calculated and are shown in (f). The mice were imaged with an excitation filter (465 nm) and an emission filter (625 nm).
3. CONCLUSIONS

In conclusion, we have successfully developed a new red-emitting ratiometric fluorescence probe (BIQ) for HSO₃⁻ detection in food and biological samples. The specific response of probe BIQ toward HSO₃⁻ was attributed to the nucleophilic addition reaction between the electron-deficient C=C bond with HSO₃⁻. A dramatic hypochromic shift of emission spectra from 625 to 475 nm and a color change were obtained within 2.0 min. Probe BIQ exhibits high sensitivity, specificity, strong red emission, and a low detection limit towards HSO₃⁻. The successful applications of BIQ in the imaging of HSO₃⁻ in live animals and the detection of HSO₃⁻ in food samples indicate that probe BIQ could be further used as a tool for investigating the biological roles of HSO₃⁻ in living systems and for evaluating the safety of HSO₃⁻ in food samples.

4. EXPERIMENTAL SECTION

4.1. Materials and Instruments. Selenium dioxide and malononitrile were received from Aladdin reagent Co. (Shanghai, China). N,N-Dimethyl-p-phenylenediamine was obtained from Energy Chemical. Piperidine, 1,2-diaminobenzene, potassium carbonate (K₂CO₃), phosphorus oxychloride, metal ions (nitrate salts), anions (sodium salts), and sodium hypochlorite (NaOCl) were purchased from Sinopharm Chemical Reagent Co., Ltd. (China). All of the experimental reagents were of analytical grade from commercial suppliers and were used without further purification. HSO₃⁻ was freshly prepared by dissolving the corresponding chemicals in deionized water. For the spectroscopic analysis of HSO₃⁻ different concentrations was added into the BIQ solution (total volume 3 mL). The spectroscopic measurements were performed after 2 min of stabilization. Fluorescence slits are 15 nm, 5 nm at 475 nm emission and 10 nm, 12 nm at 625 nm emission, respectively.

4.2. Synthesis of Probe BIQ. 2-(1-H-Benzoyl)imidazol-3-ethyl-2-yl) acetanilide and 6-(dimethylamino) quinoline-2-carbaldehyde were prepared according to published procedures. 2-(1-H-Benzoyl)imidazol-3-ethyl-2-yl) acetanilide (0.186 g, 1 mmol) was dissolved in ethanol, followed by the addition of 6-(dimethylamino) quinoline-2-carbaldehyde (0.200 g, 1 mmol) and a catalytic amount of piperidine. The reaction mixture was then refluxed for 3 h to form a dark precipitate. After filtration, rinsing three times with cold methanol, and vacuum drying, probe BIQ, was obtained with 82% yield. ¹H NMR (600 MHz, CDCl₃): δ (ppm) 8.24 (s, 1H), 8.04 (d, J = 8.8 Hz, 1H), 8.03 (d, J = 7.6 Hz, 1H), 7.81 (d, J = 3.2 Hz, 1H), 7.73 (d, J = 8.4 Hz, 1H), 7.37 (m, 4H), 6.74 (s, 1H), 4.58 (s, 2H), 3.12 (s, 6H), 1.58 (s, 3H). ¹³C NMR (CDCl₃, d₆, 150 Hz, δ (ppm) 150.3, 149.7, 146.2, 142.7, 142.2, 136.0, 133.9, 131.1, 130.5, 123.6, 123.1, 123.0, 120.1, 119.9, 116.8, 110.0, 103.8, 40.5, 40.0, and 15.2. ESI-MS (positive mode, m/z) calc for C₂₃H₂₂N₅⁺: 368.1870, [BIQ]⁺: found, 368.1876. Mp 176.4–177.9 °C.

4.3. General Procedure of Spectra Detection. A stock solution of probe BIQ was prepared by dissolving BIQ in dimethyl sulfoxide (DMSO) at a concentration of 0.5 mM. Before spectroscopic measurements, the test solution was freshly prepared by diluting the stock solution with the corresponding HEPES buffer solution at a concentration of 10 μM (DMSO/H₂O = 3:7, v/v, 20 mM, pH = 7.4). Anions, ROS, and biomolecules (20 mM) were freshly prepared by dissolving the corresponding chemicals in deionized water. For the spectroscopic analysis of HSO₃⁻, HSO₃⁻ at different concentrations was added into the BIQ solution (total volume 3 mL). The spectroscopic measurements were performed after 2 min of stabilization. Fluorescence slits are 15 nm, 5 nm at 475 nm emission and 10 nm, 12 nm at 625 nm emission, respectively.

4.4. Quantum Yield Measurement. The relative fluorescence quantum yields of BIQ before and after reacting with HSO₃⁻ were determined by using fluorescein as the reference (Φ₀ = 0.85 in 0.1 M NaOH aqueous solutions). After the measurements of UV–vis and fluorescence spectra (λₑₓ = 460 nm), the quantum yield of the corresponding compounds were determined using the equation

\[ Φ = \frac{Φ_{\text{std}}}{A_{\text{std}}} \frac{A_{\text{unk}}}{A_{\text{unk}}} \frac{η_{\text{std}}}{η_{\text{unk}}} \]

where Φ₀, Φ₀, F₀, A₀, η₀, A₀, η₀ represent the quantum yield, integrated fluorescence intensity, absorbance, and refraction of the standard solution, respectively. Φ₀, Φ₀, F₀, A₀, η₀, A₀, η₀ represent the quantum yield, integrated fluorescence intensity, absorbance, and refraction of the sample solution, respectively. All of the spectroscopic measurements were performed in triplicate and averaged.

4.5. Determination of HSO₃⁻ in Food Samples. White wine and sugar samples were obtained from a local supermarket, and the samples were prepared to investigate the feasibility of probe BIQ for HSO₃⁻ detection in real food samples. Specifically, the white wine sample was prepared by diluting purchased wine with deionized water into the tenfold diluted test solution, and the sugar sample solution was prepared by dissolving 6.0 g of sugar in deionized water and further diluting to 10 mL. After mixing probe BIQ (10 μM) with food samples spiked with HSO₃⁻ (0, 26.66, 39.99, 66.65, μM, respectively) for 5 min, the emission intensity at 475 and 625 nm of each sample was recorded.

4.6. Fluorescence Imaging of HSO₃⁻ in Adult Zebrafish. Adult zebrafish were incubated with an aqueous solution containing probe BIQ (40 μM) for 5 min, followed by the treatment of HSO₃⁻ through oral feeding for another 5 min. Fluorescence images of the above-treated zebrafish were taken under an Ami Imaging System with a 465 nm excitation filter and a 630 nm emission filter. Untreated zebrafish were used as the control group.

4.7. Fluorescence Imaging of HSO₃⁻ in Nude Mice. Nude mice (6 to 8-week-old) were anesthetized by isoflurane with a flow of oxygen during all of the experiments. For imaging exogenous HSO₃⁻ in live mice, probe BIQ (40 μM,
100 μL was injected into the mice legs, followed by the injection of 20 μL of (4 mM) HSO₃⁻ in the same region. Images on the injection area were recorded every 5 min within 15 min with a 465 nm excitation filter and a 630 nm emission filter.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c00063.

Synthesis of BIQ; HRMS, ¹H NMR, and ¹³C NMR of probe BIQ; photostability of probe BIQ; fluorescence change rate; Job’s plot; standard calibration curves of BIQ in the presence of white wine and sugar samples; viabilities of RAW 264.7 cells after incubation with different concentrations of BIQ; comparison of this work with reported fluorescent probes for HSO₃⁻ detection (PDF)

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Author Contributions
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Notes
The authors declare no competing financial interest.

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