Fluorescent probe for biological gas SO₂ derivatives bisulfite and sulfite†

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Sulfur dioxide (SO₂) is a common air pollutant, and human exposure to SO₂ has become increasingly widespread due to the combustion of fossil fuels. Inhaled SO₂ is easily hydrated to produce sulfurous acid in the respiratory tract and subsequently forms its derivatives sulfite (SO₃²⁻) and bisulfite (HSO₃⁻) (3:1 M/M, in neutral fluid), and the toxicity of SO₂ is mainly affected by the two derivatives. Epidemiological studies implied that SO₂ exposure not only induces many respiratory responses, but is also linked to lung cancer, cardiovascular diseases, and many neurological disorders, such as migraine headaches, stroke, and brain cancer. Toxicological studies further suggested that SO₂ and/or its derivatives could change the characteristics of voltage-gated sodium channels and potassium channels in rat hippocampal neurons, affect thiol levels and hence redox balance in cells, and produce a neuronal insult. However, distinct from its toxicological effects, it was also revealed that the blood pressure of male Wistar rats can be decreased by SO₂ inhalation or by intraperitoneal injection of SO₂ derivatives [Na₂SO₃ and NaHSO₃] in a concentration-dependent manner. Furthermore, SO₂ was shown to be a vasodilator, and might regulate vascular smooth muscle tone in synergy with NO.

Although SO₂ can be produced endogenously from in vivo sulfur-containing amino acids, a large number of its underlying molecular events remain unknown, and the status of SO₂ remains to be confirmed. Since SO₂ exists in aqueous solution at neutral pH as an equilibrium between its two derivatives, i.e., bisulfite and sulfite, the sensitive and selective detection techniques that enable the distribution and function of bisulfite and sulfite in biological systems to be probed are highly valuable.

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After several decades of development, fluorescent probes have been recognized as efficient molecular tools that can help monitor and visualize molecules with high sensitivity and spatial resolution. In recent years, some excellent fluorescent probes for gasotransmitters NO, H₂S and CO have been exploited for applications in biological systems. As for SO₂ derivatives HSO₃⁻ and SO₃²⁻, the previously reported fluorescent probes are mainly focused on the in vitro assay, and function by their specific reactions with an aldehyde or levulinate group (details in the ESI†). However, the aldehyde-based probes can only be operated in acidic conditions, and may suffer from the interference from biothiols (Cys or Hcy); the labile ester linkage in levulinate-type probes may induce a high background signal in biological imaging, as it can potentially be cleaved by proteases and esterases to produce active fluorophores. Therefore, new methods are highly expected to overcome these shortcomings. However, the major challenge is to find an appropriate receptor so that it can specifically bind SO₂ derivatives over other biologically related species, in particular, millimolar concentrations of biothiols found inside most cells, under physiological conditions.

In 1952, it was reported that HSO₃⁻ or SO₃²⁻ could add very rapidly and quantitatively to γ,δ-unsaturated compounds, such as acrylonitrile and methyl acrylate, in aqueous solution. We envisioned that coupling the addition reaction with an appropriate fluorescence sensing mechanism would serve as the foundation for a novel fluorescent probe for HSO₃⁻ or SO₃²⁻. Such consideration reminded us of coumarin–hemicyanine dyes, not only because they possess a similar γ,δ-unsaturated structure to acrylonitrile as well as their favorable photophysical properties, but also because, unlike the common Michael receptors, this type of dye has been indicated to be inert to biothiols, such as Cys and GSH. It was envisioned that the nucleophilic attack of HSO₃⁻ or SO₃²⁻ toward this type of dye will interrupt the π-conjugation and block the ICT process, and, as a result, two well-separated emission peaks before and after adding HSO₃⁻ or SO₃²⁻ could be obtained due to the distinct emission between the hemicyanine dye and the produced coumarin dye (Fig. 1). If so, this will enable the sensing of HSO₃⁻ or SO₃²⁻ using an attractive ratiometric fluorescence strategy. In addition, the high selectivity for HSO₃⁻ or SO₃²⁻
over the main competitive species, i.e., biothiols, was also highly expected.

To test the above-mentioned possibility, we synthesized a diethylaminocoumarin–hemicyanine dye 1 (Fig. 1), and examined its reactivity towards HSO$_3^-$ through time-dependent UV-vis of 1 in PBS buffer (pH 7.4, 10 mM, containing 30% DMF). To our surprise, the reaction is not a simple one-step process, but involves an intermediate before completion. As shown in Fig. 2A (details in Fig. S1, ESI†), upon addition of HSO$_3^-$, the absorption of 1 at 545 nm decreased promptly within 10 s, along with the simultaneous emergence of a new absorption at 463 nm; after that, the absorption at 463 nm gradually decreased from 10 s to 240 s, and, concomitantly, the absorption at 410 nm emerged. Also, similar changes could be observed in time-dependent fluorescence spectra of 1 in the presence of HSO$_3^-$ (Fig. S2, ESI†), where three corresponding emissions at 633 nm, 491 nm and 478 nm could be clearly observed one after another.

The absorption at 410 nm (emission at 478 nm) is due to the 7-diethylaminocoumarin moiety, and could be assigned to the 1–SO$_3^-$ adduct. Moreover, the adduct could be separated and characterized by $^1$H NMR and HRMS (ESI†). However, we speculated that the absorption at 463 nm (emission at 491 nm) probably became constant when the amount of HSO$_3^-$ is enough to complete the reaction. Moreover, a well-defined isosbestic point was noted at 445 nm, suggesting the clean chemical transformation. In addition, the probe is stable in a pH region of 1–8, and displays the best response for HSO$_3^-$ in the physiological pH region (Fig. S4, ESI†). Noteworthy is that when SO$_2^-$ was used, 1 displayed almost the same spectral responses; also, with a synthetic SO$_2$ donor, we could detect real-time SO$_2$ release (Fig. S5 and S6, ESI†) by using 1.

Turning our attention now to the fluorescent properties of 1 towards HSO$_3^-$ under the same conditions (Fig. 3A), the free probe displayed a red emission with the maximum at 633 nm; upon addition of HSO$_3^-$, the emission intensity at 633 nm gradually decreased with the simultaneous appearance of a new blue emission peak at 478 nm, indicating that the chemical reaction interrupted the π-conjugation of 1, after which the fluorescence of the 7-diethylaminocoumarin moiety recovered, in good agreement with the aforementioned ratiometric fluorescent strategy. Essentially, the ratio of the emission intensities ($I_{478}/I_{633}$) became constant when the amount of HSO$_3^-$ added reached 6 equiv. Noteworthy is that the difference between the two emission wavelengths is very large ($\Delta\lambda_{em}: 155$ nm), which not only contributes to the accurate measurement of the intensities of the two emission peaks, but also results in a huge ratiometric value. In the presence of 10 equiv. of HSO$_3^-$, a ca. 1110-fold enhancement in the ratiometric value of $I_{478}/I_{633}$ (from 0.0999 to 110.9548) is achieved with respect to the HSO$_3^-$-free solution. In addition, the detection limit is determined to be 0.38 μM based on $S/N = 3$ (Fig. S7, ESI†), which is sufficient to probe the HSO$_3^-$ concentration in cells (ca. 16 μM).

$Fig. 1$ The proposed mechanism of 1 for SO$_2$ derivatives HSO$_3^-$ and SO$_3^-$.

$Fig. 2$ (A) Time-dependent UV-vis of 1 (10 μM) in the presence of HSO$_3^-$ (10 equiv.) in PBS buffer (pH 7.4, 10 mM, containing 30% DMF). (B) UV-Vis spectra of 1 (10 μM) upon addition of increasing concentrations of NaHSO$_3$ (0–10 equiv.) under the same conditions. Each spectrum was recorded after 5 min.

$Fig. 3$ (A) Fluorescence spectra of 1 (10 μM) upon addition of NaHSO$_3$ (0–10 equiv.) in PBS buffer (pH 7.4, 10 mM, containing 30% DMF). Each spectrum was recorded after 5 min. $\lambda_{ex} = 445$ nm. Slt: 5/10 nm. (B) Fluorescence spectra of 1 upon addition of various species, including AcO$_2^-$, Br$^-$, Cl$^-$, CN$^-$, CO$_3^{2-}$, F$^-$, $\cdot$SO$_2$H$^-$, $\cdot$SO$_2$OH, O$_2^-\cdot$, ClO$^-$, NO$_2^-$ (10 equiv. for each); Cys (1 mM), GSH (5 mM) and HSO$_3^-$ (6 equiv.).
Next, we examined the fluorescence spectral changes of 1 (10 \mu M) incubated with various competitive species under the same conditions. As shown in Fig. 3B (Fig. S8, ESI†), addition of the representative anions, reactive oxygen species (ROS) and biothiols, such as GSH and Cys, did not lead to any significant fluorescence changes of 1.\textsuperscript{25} In contrast, upon treatment of 1 with SO\textsubscript{3}\textsuperscript{−}, a dramatic ratiometric fluorescence response was observed. The high selectivity of 1 towards SO\textsubscript{3}\textsuperscript{−} is also observable by the naked eye. When probe 1 was excited at 365 nm using a UV lamp in the presence of various species, only SO\textsubscript{3}\textsuperscript{−} caused an obvious fluorescence change from red to blue (Fig. S9, ESI†).

In addition, probe 1 is also soluble in pure PBS buffer (pH 7.4, 10 mM), and at least 30 \mu M solution of 1 could be obtained (Fig. S10, ESI†), which is sufficient to stain the cells. Moreover, under the same conditions, probe 1 still displays good reactivity to SO\textsubscript{3}\textsuperscript{−} (Fig. S11, ESI†). Subsequently, we tested the capability of 1 to image SO\textsubscript{2} derivatives in living cells. First, the MTT assay for cells. First, the MTT assay for 1 (10 \mu M) for 30 min from the red channel; (B) fluorescence imaging of (A) from the red channel; (C) overlay of (A) and (B); (D) fluorescence imaging of HeLa cells incubated with 1 for 30 min, and further incubated with NaHSO\textsubscript{3} (200 \mu M) for 30 min from the green channel; (E) fluorescence imaging of (D) from the red channel; (F) overlap of (D) and (E).

In summary, we have reported a coumarin–hemicyanine dye (1) as a ratiometric fluorescence probe for SO\textsubscript{2} derivatives HSO\textsubscript{3}\textsuperscript{−} and SO\textsubscript{2}\textsuperscript{−} based on a novel addition-rearrangement cascade reaction. The probe displays advantages such as being easy-to-make, excellent ratiometric fluorescence response, and high selectivity. Preliminary biological experiments indicate its potential to probe SO\textsubscript{2} chemistry in biological systems.

**Notes and references**

25. We proposed a C–H · · · O hydrogen-binding interaction in intermediate I for explaining the high selectivity of 1 towards SO\textsubscript{3}\textsuperscript{−} over thiols on an anonymous reviewer’s advice.