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Fluorescent probe for biological gas SO_2 derivatives bisulfite and sulfite†

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Yuan-Qiang Sun,‡[a] Jing Liu,‡[a] Jingyu Zhang,[a] Ting Yang[b] and Wei Guo*[a]

A coumarin–hemicyanine dye 1 was reported for ratiometric fluorescent detection of SO_2 derivatives HSO_3^- and SO_3^{2-} based on a novel addition-rearrangement cascade reaction.

Sulfur dioxide (SO_2) is a common air pollutant, and human exposure to SO_2 has become increasingly widespread due to the combustion of fossil fuels. Inhaled SO_2 is easily hydrated to produce sulfurous acid in the respiratory tract and subsequently forms its derivatives sulfite (SO_3^{2-}) and bisulfite (HSO_3^-) (3 : 1 M/M, in neutral fluid),[1] and the toxicity of SO_2 is mainly affected by the two derivatives. Epidemiological studies implied that SO_2 exposure not only induces many respiratory responses,[2] but is also linked to lung cancer, cardiovascular diseases, and many neurological disorders, such as migraine headaches, stroke, and brain cancer.[3] Toxicological studies further suggested that SO_2 and/or its derivatives could change the characteristics of voltage-gated sodium channels and potassium channels in rat hippocampal neurons,[4] affect thiol levels and hence redox balance in cells,[5] and produce a neuronal insult.[6] However, distinctive from its toxicological effects, it was also revealed that the blood pressure of male Wistar rats can be decreased by SO_2 inhalation or by intraperitoneal injection of SO_2 derivatives (Na_2SO_3 and $NaHSO_3$) in a concentration-dependent manner.[7] Furthermore, SO_2 was shown to be a vasodilator, and might regulate vascular smooth muscle tone in synergy with NO.[8,9]

Although SO_2 can be produced endogenously from *in vivo* sulfur-containing amino acids,[10] a large number of its underlying molecular events remain unknown, and the status of SO_2 remains to be confirmed. Since SO_2 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.

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,[11] H_2S[12] and CO[13] have been exploited for applications in biological systems. As for SO_2 derivatives HSO_3^- and SO_3^{2-}, the previously reported fluorescent probes are mainly focused on the *in vitro* assay, and function by their specific reactions with an aldehyde[14] or levulinate group[15] (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);[16] 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 SO_2 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 HSO_3^- or SO_3^{2-} could add very rapidly and quantitatively to α,β-unsaturated compounds, such as acrylonitrile and methyl acrylate, in aqueous solution.[17] We envisioned that coupling the addition reaction with an appropriate fluorescence sensing mechanism would serve as the foundation for a novel fluorescent probe for HSO_3^- or SO_3^{2-}. Such consideration reminded us of coumarin–hemicyanine dyes,[18] not only because they possess a similar α,β-unsaturated structure to acrylonitrile as well as their favorable photophysical properties, but also because, unlike the common Michael receptors,[19] this type of dye has been indicated to be inert to biothiols, such as Cys and GSH.[20] It was envisioned that the nucleophilic attack of HSO_3^- or SO_3^{2-} 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 HSO_3^- or SO_3^{2-} 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 HSO_3^- or SO_3^{2-} using an attractive ratiometric fluorescence strategy.[21] In addition, the high selectivity for HSO_3^- or SO_3^{2-}

[a] *School of Chemistry and Chemical Engineering, Shanxi University, Taiyuan 030006, China. E-mail: guow@sxu.edu.cn*

[b] *Institute of Biotechnology, Shanxi University, China*

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‡ These authors contributed equally to this work.

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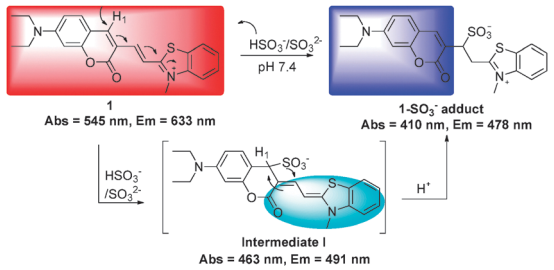

Fig. 1 The proposed mechanism of **1** for SO_2 derivatives HSO_3^- and SO_3^{2-}.

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 resulted from the intermediate **I** (Fig. 1) due to the nucleophilic attack of HSO_3^- to the 4-position of the coumarin moiety of **1**. This can be rationalized by the unique structural motif of **1**, that is, the 4-position of the coumarin moiety in **1** is doubly activated, and, thus, is more reactive.[22] In fact, the intermediate was also observable by ^{1}H NMR spectroscopy (Fig. S3, ESI†). Upon addition of $NaHSO_3$, the signals of **1** disappeared immediately with the concomitant appearance of two sets of new signals. One could be assigned to the **1**–SO_3^- adduct, and the other, which gradually disappeared after 10 min, was due to the intermediate. Moreover, the proton (H_1, at *ca.* δ 8.3 ppm) at the 4-position of the coumarin moiety of **1** was dramatically shifted upfield to 4.9 ppm, corresponding to the saturated –CH– proton adjacent to the SO_3^- group in the intermediate, indicating that the initial nucleophilic attack of HSO_3^- towards **1** occurs at the 4-position of the coumarin moiety. However, the intermediate is unstable because it gradually disappears along with the simultaneous emergence of the **1**–SO_3^- adduct. Thus, we reasoned that the intermediate probably undergoes an intramolecular 1,3-rearrangement reaction, which restores conjugation of the coumarin moiety, and leads to the **1**–SO_3^- adduct (Fig. 1).

Overall, the reaction of **1** with HSO_3^- could be completed within 5 min under the same conditions. With a time point of 5 min upon addition of HSO_3^-, we performed the UV-vis titration studies of **1** towards HSO_3^- (Fig. 2B). The obtained results displayed that 6 equiv. 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_3^{2-} was used, **1** displayed almost the same spectral responses; also, with a synthetic SO_2 donor,[23] 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-diethlyaminocoumarin 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 (ΔE_m: 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_{475}/I_{635} (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).[24]

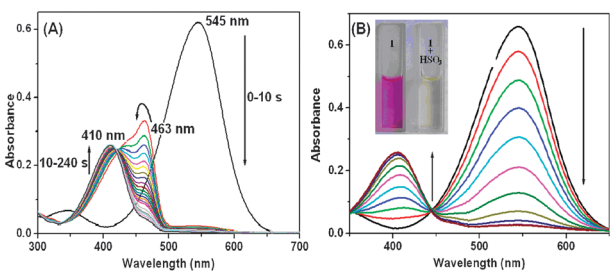

Fig. 2 (A) Time-dependent UV-vis of **1** (10 μM) in the presence of HSO_3^- (10 equiv.) in PBS buffer (pH 7.4, 10 mM, containing 30% DMF). (B) UV-Vis spectra of **1** (10 μM) upon addition of increasing concentrations of $NaHSO_3$ (0–10 equiv.) under the same conditions. Each spectrum was recorded after 5 min.

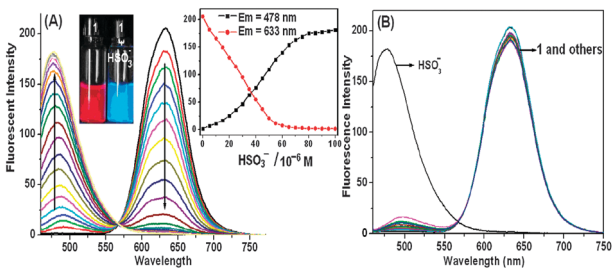

Fig. 3 (A) Fluorescence spectra of **1** (10 μM) upon addition of $NaHSO_3$ (0–10 equiv.) in PBS buffer (pH 7.4, 10 mM, containing 30% DMF). Each spectrum was recorded after 5 min. λ_{ex} = 445 nm. Slits: 5/10 nm. (B) Fluorescence spectra of **1** upon addition of various species, including AcO^-, Br^-, Cl^-, CN^-, CO_3^{2-}, F^-, S^{2-}, I^-, N_3^-, PO_4^{3-}, SCN^-, SO_4^{2-}, NO, O_2^-, H_2O_2, $OH^\bullet$, ClO^-, NO_2^- (10 equiv. for each); Cys (1 mM), GSH (5 mM) and HSO_3^- (6 equiv.).

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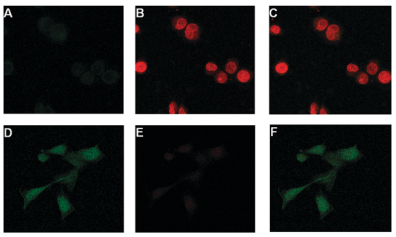

Fig. 4 (A) Fluorescence imaging of HeLa cells incubated with probe **1** (10 μM) from the green channel; (B) fluorescence imaging of (A) from the red channel; (C) overlay of (A) and (B); (D) fluorescence imaging of HeLa cells incubated with **1** for 30 min, and further incubated with $NaHSO_3$ (200 μM) for 30 min from the green channel; (E) fluorescence imaging of (D) from the red channel; (F) overlap of (D) and (E).

Next, we examined the fluorescence spectral changes of **1** (10 μM) 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**.[25] In contrast, upon treatment of **1** with HSO_3^-, a dramatic ratiometric fluorescence response was observed. The high selectivity of **1** towards HSO_3^- 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 HSO_3^- 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 μM 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 HSO_3^- (Fig. S11, ESI†). Subsequently, we tested the capability of **1** to image SO_2 derivatives in living cells. First, the MTT assay for **1** showed that the probe with a concentration range of 2–20 μM has only minimal cytotoxicity after 24 h (Fig. S12, ESI†), and thus the probe at 10 μM was selected for imaging experiments. HeLa cells, incubated with **1** (10 μM) in culture medium for 30 min at 37 °C, showed strong fluorescence in the red channel (Fig. 4B) and weak fluorescence in the green channel (Fig. 4A), confirming that **1** is cell-permeable. Since a certain concentration of SO_2 derivatives has been indicated in cells,[24] the observed weak fluorescence in the green channel is probably a result of the fact that **1** binds the two derivatives in cells. In addition, incubation of excess GSH did not elicit obvious fluorescence changes of **1** in HeLa cells (Fig. S13, ESI†), excluding the possibility that the weak fluorescence in the green channel is due to the cellular biothiols. In a control experiment, the cells were pretreated with **1** for 30 min, and further incubated with $NaHSO_3$ (200 μM) for 30 min, eliciting an obvious fluorescence decrease in the red channel (Fig. 4E) and a fluorescence increase in the green channel (Fig. 4D), in agreement with the HSO_3^--induced ratiometric fluorescence response (Fig. 4C and F).

In summary, we have reported a coumarin–hemicyanine dye (**1**) as a ratiometric fluorescence probe for SO_2 derivatives HSO_3^- and SO_3^{2-} based on a novel addition-rearrangement cascade reaction. The probe displays advantages such as being easy-to-make, excellent ratiometric fluorescent response, and high selectivity. Preliminary biological experiments indicate its potential to probe SO_2 chemistry in biological systems.

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