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A new fluorescent probe for gasotransmitter H_2S: high sensitivity, excellent selectivity, and a significant fluorescence *off–on* response†

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A fluorescent *off–on* probe for H_2S was exploited by coupling the azide-based strategy with the excited-state intramolecular proton transfer (ESIPT) sensing mechanism, which exhibits a considerably high fluorescence enhancement (1150-fold), an extremely low detection limit (0.78 nM), and a relatively fast response time (3–10 min) as well as excellent selectivity.

Hydrogen sulfide (H_2S) can be endogenously produced by enzymes such as cystathionine β-synthase, cystathionine γ-lyase, and 3-mercaptopyruvate sulfurtransferase,[1] and it plays important roles in several pathophysiological processes, including vasodilation, angiogenesis, regulation of cell growth, mediation of neurotransmission, inhibition of insulin signaling, and regulation of inflammation.[2] Also, studies have shown that its deregulation is correlated with the symptoms of Alzherimer's disease, Down's syndrome, diabetes, and liver cirrhosis.[3] H_2S has been regarded as the third gasotransmitter besides nitric oxide (NO) and carbon monoxide (CO).[4] Therefore, an efficient method for sensitively and selectively probing H_2S in biological systems is highly required.

Among various methods, fluorescence-based assays have found widespread application especially in biological systems due to the high sensitivity, nondestructive detection, and high spatiotemporal resolution. For fluorescent H_2S probes, the design strategies are commonly based on several significant characteristic properties of H_2S, namely dual nucleophilicity,[5] good reducing property towards azide,[6] high binding affinity towards copper ions,[7] efficient thiolysis of dinitrophenyl ether[8] as well as specific addition reaction towards unsaturated double bonds.[9] Although some advances have been made in the field, there still exist some issues of interest and concern. One is to attain sufficient selectivity over biologically related species, especially biothiols such as glutathione (GSH) that is present at levels of about 1–10 mM in cells. That is to say, these biothiols should neither induce the fluorescent change, nor consume the probe. However, some of the reported H_2S probes more or less fail to meet this requirement. Secondly, considering real-time imaging of H_2S-related biological processes, the designed probe should respond fast with H_2S under mild conditions. However, most of the reported H_2S probes display a delayed response time (more than 20 min). Thirdly, because the biologically relevant levels of H_2S vary from nanomolar to micromolar levels,[10] it is also important to develop a sensitive fluorescent probe that could exhibit an obvious signal change to low concentration of H_2S to facilitate in-depth biological research. Indeed, few fluorescence probes could detect H_2S at a nanomolar range so far.[5f,6k] Furthermore, for the improved spatiotemporal resolution, the low background fluorescence of the probe itself and the high luminescent efficiency upon H_2S treatment are highly desirable. Thus, further efforts to address these concerns are highly required.

Herein, we present a simple and new fluorescent probe **1** for the detection of H_2S based on the strategy of reduction of the azido group by H_2S[6] as well as the resulting ESIPT modulated fluorescence *off–on* response[11] (Fig. 1). The probe exhibits considerably high fluorescence enhancement, extremely low detection limit, and relatively fast response time as well as excellent selectivity toward H_2S, and thus holds great potential for detecting or imaging this important gasotransmitter in biological systems.

Probe **1** could be easily prepared from 2-(2-aminophenyl)benzothiazole (**ABT**) through the Sandmeyer reaction, and its structure was confirmed by ^{1}H NMR, ^{13}C NMR, and HRMS spectra (ESI†). The probe was found to be soluble (up to 12 μM) in PBS buffer (Fig. S1, ESI†). Thus, we first examined the reactivity of **1** (10 μM) towards different concentrations of NaHS (a commonly employed H_2S donor) through time-dependent absorption spectroscopy in

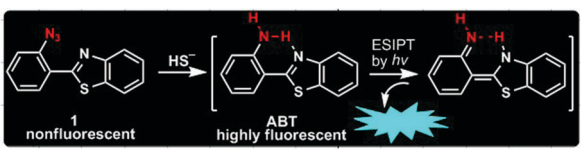

Fig. 1 The proposed sensing mechanism of probe **1** for H_2S.

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† Electronic supplementary information (ESI) available: Experimental procedures, supplemental spectra, and the ^{1}H-, ^{13}C-NMR, and MS spectrum. See DOI: 10.1039/c3cc49605h

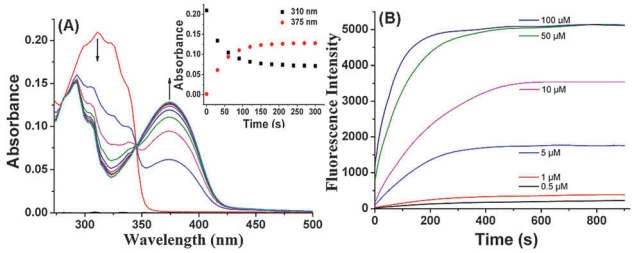

Fig. 2 (A) Time-dependent absorption spectra of **1** (10 μM) in the presence of 100 μM NaHS. (B) Time-dependent fluorescence intensity changes of **1** (10 μM) at 450 nm upon addition of varied concentrations of NaHS. Conditions: PBS buffer (10 mM, pH 7.4, 1 mM CTAB) at 25 °C.

PBS buffer [10 mM, pH 7.4, containing 1 mM CTAB[12] (cetyltrimethylammonium bromide)] at 25 °C (Fig. 2A). The free **1** showed a main absorption at 310 nm. Upon addition of NaHS (100 μM), the absorption of **1** at 310 nm decreased gradually within 3 min, along with the simultaneous emergence of a new absorption at 375 nm. In this process, a well-defined isobestic point was noted at 345 nm, suggesting a clean chemical transformation. Based on the well-established reduction mechanism,[6] the new absorption at 375 nm could be assigned to product **ABT**, which was also supported by HRMS experiment (Fig. S2, ESI†). Furthermore, we performed the time-dependent fluorescent spectra studies (Fig. 2B). As shown in Fig. 2B, although 100 μM NaHS could cause the reaction to be completed within 3 min, the low concentrations of NaHS needed a longer reaction time of 10 min to reach the spectra saturation. Thus, in the subsequent experiments, a time point of 10 min after addition of NaHS was selected.

Subsequently, we performed the fluorescence titration studies of **1** towards H_2S under the same conditions. A series of spectra of the solution of **1** with 0 to 100 μM NaSH were recorded (Fig. 3A). The free probe **1** is almost nonfluorescent in the visible region (fluorescence quantum yield: $\Phi = 0.0064$). Upon treatment with increasing concentrations of NaSH, the fluorescence intensity of **1** at 450 nm gradually increased, and reached saturation when the amount of HS^- was more than 30 μM. In this case, a *ca.* 1150-fold fluorescence enhancement was observed ($\Phi = 0.4138$ with quinine sulfate as a reference), which is in fact bigger than most of those reported in the literature, indicative of a high signal-to-background ratio. Moreover, a linear relationship with the HS^- concentration from 0 to 10 μM could be obtained (Fig. 3B). It is worth noting that the probe is highly sensitive to low concentration of HS^- (Fig. 3C and D), and the detection limit for HS^- was estimated to be 0.78 nM based on $S/N = 3$, which, as far as we know, is the most sensitive fluorescent probe for HS^- to date.

To evaluate the specific nature of **1** for H_2S, we then examined the fluorescence enhancement of **1** incubated with various species (Fig. 4), most of which are biologically related. As expected, probe **1** is considerably inert to the common anions and cations, such as F^-, Cl^-, Br^-, I^-, SO_4^{2-}, CO_3^{2-}, NO_3^-, AcO^-, $H_2PO_4^-$, CN^- (0.25 mM for each); K^+, Na^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, and Cu^{2+} (1 mM for each). Moreover, probe **1** did not exhibit any fluorescence enhancement in response to reactive oxygen/sulfur/nitrogen species (ROS/RSS/RNS), such as H_2O_2, ClO^-, $^{\bullet}OH$, O_2^-, $^1O_2$, SO_3^{2-}, HSO_3^-, $S_2O_3^{2-}$, $S_2O_4^{2-}$, $S_2O_5^{2-}$, and NO (0.1 mM for each). Importantly, probe **1** showed no

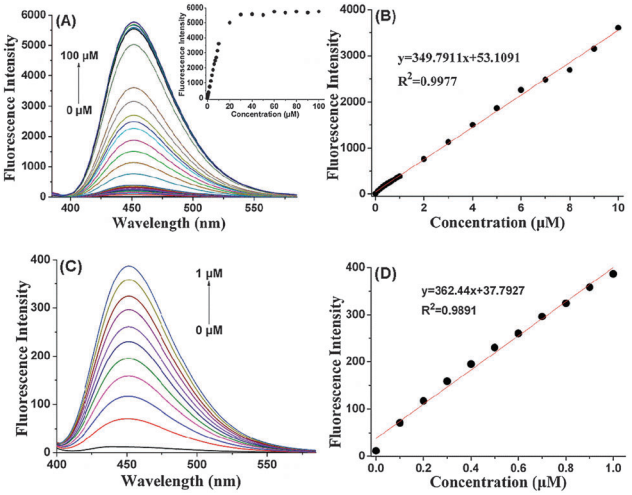

Fig. 3 Fluorescence spectra of **1** (10 μM) upon addition of HS^- (0–100 μM for A and 0–1 μM for C) in PBS buffer (10 mM, pH 7.4, 1 mM CTAB) and the corresponding linear relationship between the fluorescent intensity and HS^- concentration (B and D). Spectra were recorded after incubation with different concentrations of HS^- for 10 min at 25 °C. λ_{ex} = 375 nm, λ_{em} = 450 nm. Slits: 10/10 nm.

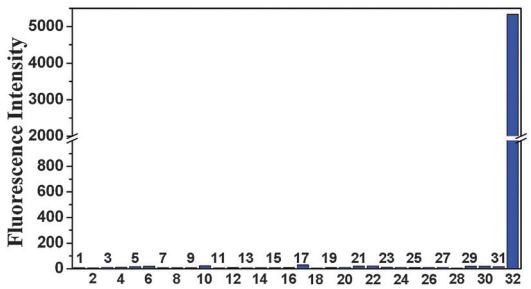

Fig. 4 Fluorescence intensities of **1** (10 μM) upon addition of various species in PBS buffer (10 mM, pH 7.4, 1 mM CTAB) after 10 min at 25 °C. (1–6) K^+, Na^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, and Cu^{2+} (1 mM for each); (7–16) F^-, Cl^-, Br^-, I^-, SO_4^{2-}, CO_3^{2-}, NO_3^-, AcO^-, $H_2PO_4^-$, and CN^- (0.25 mM for each); (17) Vc-Na (0.1 mM); (18–22) SO_3^{2-}, HSO_3^-, $S_2O_3^{2-}$, $S_2O_4^{2-}$, and $S_2O_5^{2-}$ (0.1 mM for each); (23–28) H_2O_2, ClO^-, O_2^-, NO, $^{\bullet}OH$, and $^1O_2$ (0.1 mM for each); (29–32) Cys (0.5 mM), Hcy (0.5 mM), GSH (1 mM), and HS^- (0.1 mM). λ_{ex} = 375 nm, λ_{em} = 450 nm. Slits: 10/10 nm.

response to both biothiols (0.5 mM Cys and Hcy; 1 mM GSH, the main competitive species in biological systems) as well as to reducing conditions (0.1 mM) sodium ascorbate (Vc-Na). By contrast, only HS^- elicited a dramatic increase in the fluorescence intensity of **1**, suggesting the high selectivity of **1** towards H_2S.[13] We also performed the competition experiments in the presence of biothiols as well as Vc-Na. In fact, when H_2S and these species coexisted, we also observed almost the same fluorescence enhancement as that only treated by H_2S (Fig. 5A). Moreover, the H_2S-induced fluorescence enhancement in the presence of biothiols or sodium ascorbate could be clearly observed by the naked eyes (Fig. 5B).

Next, the effect of pH on the fluorescence response of probe **1** to H_2S was investigated (Fig. S3, ESI†). It was found that probe **1** is stable over a wide pH range of 2–12, and displays the best response for H_2S in the region of 7–12. Thus, probe **1** could function properly at physiological pH.

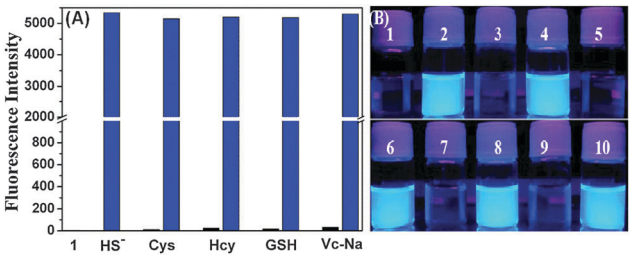

Fig. 5 (A) Fluorescence response of **1** (10 μM) to HS^- (0.1 mM), Cys (0.5 mM), Hcy (0.5 mM), GSH (1 mM), and Vc-Na (0.1 mM) in PBS buffer (10 mM, pH 7.4, 1 mM CTAB) after 10 min at 25 °C. Black bar: **1** + biothiols (or Vc-Na); Blue bar: **1** + biothiols (or Vc-Na) + HS^-. λ_{ex} = 375 nm, λ_{em} = 450 nm. Slits: 10/10 nm. (B) The corresponding fluorescent images: (1) **1** only; (2) **1** + HS^-; (3) **1** + Cys; (4) **1** + Cys + HS^-; (5) **1** + Hcy; (6) **1** + Hcy + HS^-; (7) **1** + GSH; (8) **1** + GSH + HS^-; (9) **1** + Vc-Na; (10) **1** + Vc-Na + HS^-.

Encouraged by these results, we tested the capability of **1** to image H_2S in biological systems. Melanoma B16 cells incubated with **1** in culture medium for 30 min at 37 °C showed a weak fluorescence (Fig. 6A) due to the low H_2S level in the cells. However, strong fluorescence in the cells was observed after the cells were pre-incubated with NaHS and further incubated with **1** (Fig. 6B), suggesting that probe **1** could detect external H_2S supplemented to the cell cultures. It is well established that H_2S could be biosynthesized from Cys by the enzymes CBS and CSE.[14] Also, it was reported that melanoma cell lines express CSE.[15] Thus, Cys could be regarded as a precursor to H_2S in the cell imaging assays.[5c,6c,j] In fact, when B16 cells were incubated with Cys for 30 min and then incubated with **1** for 30 min, we also observed the obvious fluorescence (Fig. 6C). As a control,[6c] when the cells were pre-incubated in a sequence with Cys and phorbol myristate acetate (PMA) that could decrease the H_2S level presumably by generating ROS,[16] and further incubated with **1**, almost no fluorescence was observed (Fig. 6D).

In summary, a reaction-type fluorescent probe **1** for detection of H_2S was exploited by coupling the azide-based strategy with the excited-state intramolecular proton transfer (ESIPT) sensing mechanism. The probe can detect H_2S with high selectivity even in the presence of millimolar concentrations of biothiols with a significant fluorescence *off–on* response and an extremely low detection limit. Preliminary fluorescence imaging experiments in cells indicate its potential to probe H_2S chemistry in biological systems.

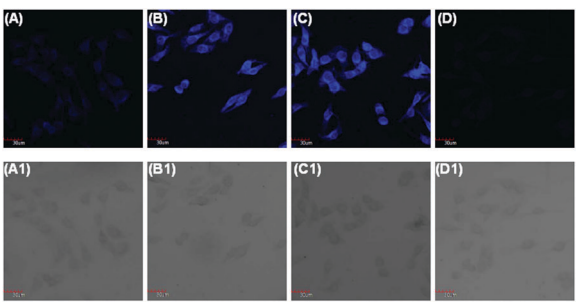

Fig. 6 Fluorescence images of H_2S in B16 cells using **1** (10 μM) at 37 °C. (A) B16 cells incubated with **1** in the presence of CTAB (1 mM) for 30 min. (B) B16 cells pre-incubated with NaHS (100 μM, 30 min) and further incubated with **1** (30 min) in the presence of CTAB (1 mM). (C) B16 cells pre-incubated with Cys (100 μM, 30 min) and further incubated with **1** (30 min) in the presence of CTAB (1 mM). (D) B16 cells pre-incubated in a sequence with Cys (100 μM, 30 min) and PMA (1 μg mL^{-1}), and further incubated with **1** (30 min) in the presence of CTAB (1 mM). (A1–D1) The corresponding bright-field images. Cells shown are representative images from replicate experiments (n = 3). The mean fluorescence intensities in (A–D) are 456.6, 3609.4, 3980.8, and 150.8, respectively. Scale bar: 30 μm.

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