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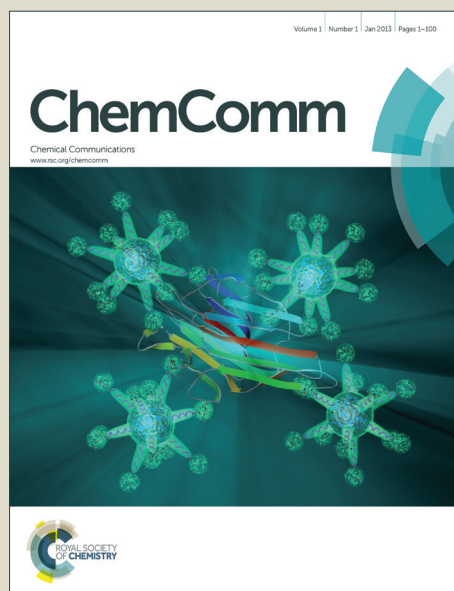
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ARTICLE TYPE

A new fluorescent probe for gasotransmitter H₂S: high sensitivity, excellent selectivity, and significant fluorescence *off-on* response

Jingyu Zhang and Wei Guo*

A fluorescent *off-on* probe for H₂S was exploited by coupling the azide-based strategy with the excited-state intramolecular proton transfer (ESIPT) sensing mechanism, which exhibits considerably high fluorescence enhancement (1150-fold),
10 extremely low detection limit (0.78 nM), and relatively fast response time (3–10 min) as well as excellent selectivity.

Hydrogen sulfide (H₂S) can be endogenously produced by enzymes such as cystathionine β-synthase, cystathionine γ-lyase, and 3-mercaptopyruvate sulfurtransferase,[1] and plays
15 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 has been correlated with the symptoms of
20 Alzherimer's disease, Down's syndrome, diabetes, and liver cirrhosis.[3] H₂S 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 H₂S in biological systems is highly required.

25 Among various methods, fluorescence-based assays have found widespread application especially in biological system due to the high sensitivity, nondestructive detection, and high spatiotemporal resolution. For fluorescent H₂S probes, the design strategies are commonly based on several significant
30 characteristic properties of H₂S, namely dual nucleophilicity,[5] good reducing property towards azide,[6] high binding affinity towards copper ion,[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
35 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,
40 nor consume the probe. However, some of the reported H₂S probes more or less fail to meet the requirment. Secondly, considering real-time imaging of H₂S-related biological processes, the designed probe should response fast with H₂S under mild condition. However, most of the reported H₂S
45 probes display the delayed response time (more than 20 min). Thirdly, because the biologically relevant levels of H₂S vary from nanomolar to micromolar levels,[10] it is also important to develop sensitive fluorescent probe that could exhibit obvious

signal change to low concentration of H₂S to facilitate the in-
50 depth biological research. Indeed, few fluorescence probes could detect H₂S at a nanomolar range so far.[5f,6k] Further, for the improved spatiotemporal resolution, the low background fluorescence of the probe itself and the high luminescent efficiency upon H₂S treatment are highly desirable. Thus,
55 further efforts to address these concerns are highly required.

Herein, we present a simple and new fluorescent probe **1** for the detection of H₂S based on the strategy of reduction of azido group by H₂S[6] as well as the resulting ESIPT modulated fluorescence *off-on* response[11] (Fig. 1). The probe exhibits
60 considerably high fluorescence enhancement, extremely low detection limit, and relatively fast response time as well as the excellent selectivity toward H₂S, and thus holds great potential for detecting or imaging this important gasotransmitter in biological systems.
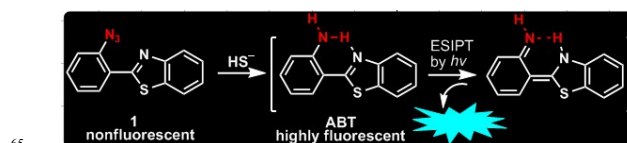

65 **Fig. 1** The proposed sensing mechanism of probe **1** for H₂S.

Probe **1** could be easily prepared from the 2-(2-aminophenyl)benzothiazole (**ABT**) through Sandmeyer reaction, and its structure was confirmed by ¹H NMR, ¹³C
70 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 the different concentrations of NaHS (a commonly employed H₂S donor) through time-dependent absorption spectra in PBS
75 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
80 emergence of a new absorption at 375 nm. In this process, a well-defined isobestic point was noted at 345 nm, suggesting the 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
85 supported by HRMS experiment (Fig. S2, ESI†). Further, we performed the time-dependent fluorescent spectra studies (Fig. 2B). As shown in Fig. 2B, although the 100 μM NaHS could

make 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.
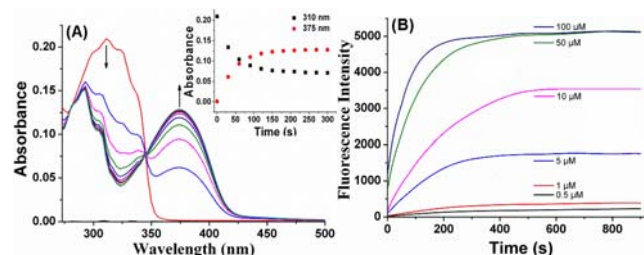

Fig. 2 (A) Time-dependent absorption spectra of **1** (10 μM) in the presence of 100 μM NaHS. (B) Time-dependent fluorescence intensity changes of **1** (10 μM) at 450 nm upon addition of varied concentrations of NaHS. Condition: PBS buffer (10 mM, pH 7.4, 1 mM CTAB) at 25 °C.

Subsequently, we performed the fluorescence titration studies of **1** towards H_2S in the same condition. 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 the increasing concentrations of NaSH, the fluorescence intensity of **1** at 450 nm gradually increased, and reached saturation when the amount of HS^- is 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 literatures, indicative of the high signal-to-background ratio. Moreover, a linear relationship with the HS^- concentration from 0 to 10 μM could be obtained (Fig. 3B). Noteworthy is that the probe is highly sensitive to low concentration of HS^- (Figs. 3C, 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.
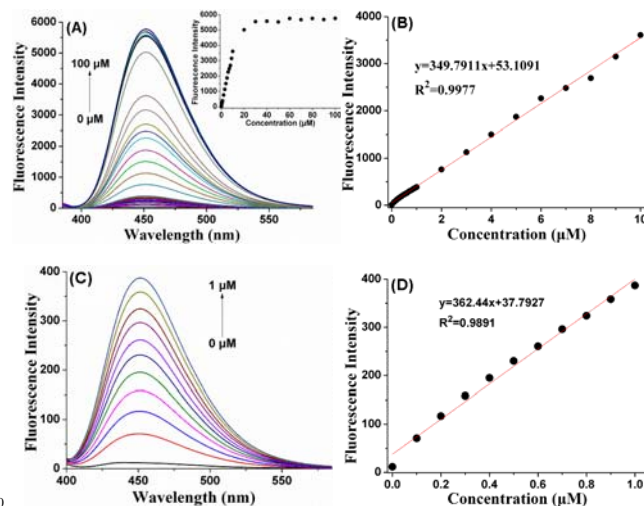

Fig 3 Fluorescence spectra of **1** (10 μM) upon addition of HS^- (0 – 100 μM for A and 0 – 1 μM for C) in PBS buffer (10 mM, pH 7.4, 1 mM CTAB) and the corresponding linear relationship between the fluorescent intensity and HS^- concentration (B and D). Spectra were recorded after incubation with different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

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^-, $^{\cdot}OH$, 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** had no response to both biothiols (0.5 mM Cys and Hcy; 1 mM GSH, the main competitive species in biological system) as well as a reducing condition (0.1 mM sodium ascorbate (Vc-Na). By contrast, only HS^- elicited a dramatic increase of 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).
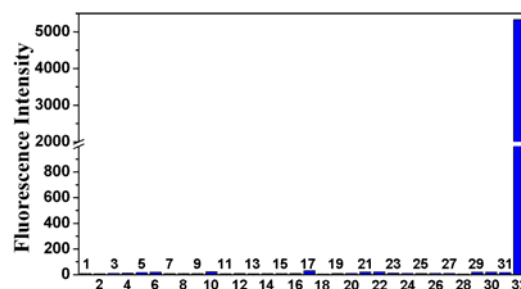

Fig. 4 Fluorescence intensities of **1** (10 μM) upon addition of various species in PBS buffer (10 mM, pH 7.4, 1 mM CTAB) after 10 min at 25 °C. (1–6) K^+, Na^+, Ca^{2+}, Mg^{2+}, Zn^{2+}, and Cu^{2+} (1 mM for each); (7–16) F^-, Cl^-, Br^-, I^-, SO_4^{2-}, CO_3^{2-}, NO_3^-, AcO^-, $H_2PO_4^-$, and 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, $^{\cdot}OH$, 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). $\lambda_{ex} = 375$ nm, $\lambda_{em} = 450$ nm. Slits: 10/10 nm.
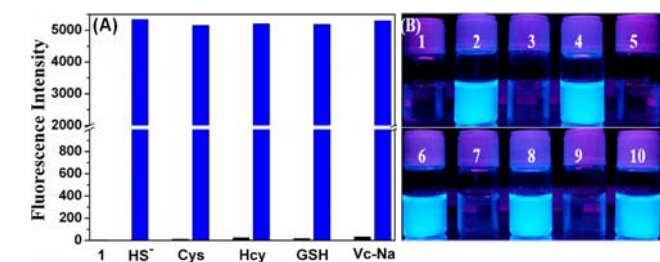

Fig. 5 (A) Fluorescence response of **1** (10 μM) to HS^- (0.1 mM), Cys (0.5 mM), Hcy (0.5 mM), GSH (1 mM), and Vc-Na (0.1 mM) in PBS buffer (10 mM, pH 7.4, 1 mM CTAB) after 10 min at 25 °C.. Black bar: **1** + biothiols (or Vc-Na); Blue bar: **1** + biothiols (or Vc-Na) + HS^-. $\lambda_{ex} = 375$ nm, $\lambda_{em} = 450$ 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^-.

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.

Encouraged by these results, we tested the capability of **1** to image H_2S in biological system. 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).
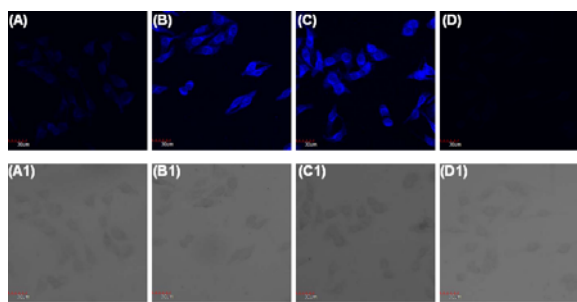

Fig. 6 Fluorescence images of H_2S in B16 cells using **1** (10 μM) at 37 °C. (A) B16 cells incubated with **1** in the presence of CTAB (1 mM) for 30 min. (B) B16 cells pre-incubated with NaHS (100 μM, 30 min) and further incubated with **1** (30 min) in the presence of CTAB (1 mM). (C) B16 cells pre-incubated 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 [2 μL (1 μg/mL)], 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.

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

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† Electronic Supplementary Information (ESI) available: Experimental procedures, supplemental spectra, and the $^1H-$, $^{13}C-$ NMR, and MS spectrum. See DOI: 10.1039/b000000x/

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