This is an Accepted Manuscript, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this Accepted Manuscript with the edited and formatted Advance Article as soon as it is available.

You can find more information about Accepted Manuscripts in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal’s standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this Accepted Manuscript or any consequences arising from the use of any information it contains.
A new fluorescent probe for gasotransmitter H2S: high sensitivity, excellent selectivity, and significant fluorescence off-on response

Jingyu Zhang and Wei Guo*

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

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), 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-mercaptopropionate sulfurransferase, and 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. Also, studies have shown that its deregulation has been correlated with the symptoms of Alzheimer’s disease, Down’s syndrome, diabetes, and liver cirrhosis. H2S has been regarded as the third gasotransmitter besides nitric oxide (NO) and carbon monoxide (CO).

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 characteristic properties of H2S, namely dual nucleophility, good reducing property towards azide, high binding affinity towards copper ion, efficient thiolysis of dinitrophenyl ether as well as specific addition reaction towards unsaturated double bonds. 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 H2S probes more or less fail to meet the requirement. 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 probes display the delayed response time (more than 20 min). Thirdly, because the biologically relevant levels of H2S vary from nanomolar to micromolar levels, it is also important to develop sensitive fluorescent probe that could exhibit obvious signal change to low concentration of H2S to facilitate the in-depth biological research. Indeed, few fluorescence probes could detect H2S at a nanomolar range so far. 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, 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 as well as the resulting ESIPT modulated fluorescence off-on response11 (Fig. 1). The probe exhibits 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.

![Fig. 1](image-url)

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 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 buffer [10 mM, pH 7.4, containing 1 mM CTAB5 (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 the clean chemical transformation. Based on the well-established reduction mechanism, the new absorption at 375 nm could be assigned to product ABT, which was also 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 I (10 μM) in the presence of 100 μM NaHS. (B) Time-dependent fluorescence intensity changes of I (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 I towards H₂S in the same condition. A series of spectra of the solution of I with 0 to 100 μM NaHS were recorded (Fig. 3A). The free probe I 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 I 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), 20 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 I (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 fluorescence intensity and HS⁻ concentration (B and D). Spectra were recorded after incubation with different concentrations of HS⁻ for 10 min at 25 °C. \( \lambda_{ex} = 375 \text{ nm, } \lambda_{em} = 450 \text{ nm} \). Slits: 10/10 nm.

To evaluate the specific nature of I for H₂S, we then examined the fluorescence enhancement of I incubated with various species (Fig. 4), most of which are biologically related. 40 As expected, probe I is considerably inert to the common anions and cations, such as F⁻, Cl⁻, Br⁻, I⁻, SO₄²⁻, CO₃²⁻, NO₃⁻, AcO⁻, H₂PO₄⁻, CN⁻ (0.25 mM for each); K⁺, Na⁺, Ca²⁺, Mg²⁺, Zn²⁺, and Cu²⁺ (1 mM for each). Moreover, probe I did not exhibit any fluorescence enhancement in response to reactive oxygen/sulfur/nitrogen species (ROS/RSS/RNS), such as H₂O₂, Cl₂O, 'OH, O₂⁻, 'O₂, SO₃²⁻, HSO₃⁻, S₂O₃²⁻, S₂O₅²⁻, and NO (0.1 mM for each). Importantly, probe I 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 I, suggesting the high selectivity of I towards H₂S. 13 We also performed the competition experiments in the presence of biothiols as well as Vc-Na. In fact, when H₂S and these species coexisted, we also observed almost the same fluorescence enhancement as that only treated by H₂S (Fig. 5A). Moreover, the H₂S-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 I (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⁺, Ca²⁺, Mg²⁺, Zn²⁺, and Cu²⁺ (1 mM for each); (7–16) F⁻, Cl⁻, Br⁻, I⁻, SO₄²⁻, CO₃²⁻, NO₃⁻, AcO⁻, H₂PO₄⁻, and CN⁻ (0.25 mM for each); (17) Vc-Na (0.1 mM); (18–22) SO₃²⁻, NO, •OH, and 1O₂ (0.1 mM for each); (23–28) H₂O₂, Cl₂O, ‘OH, ‘O₂, NO, ‘OH, and ‘O₂ (0.1 mM for each); (29–32) Cys (0.5 mM), Hcy (0.5 mM), GSH (1 mM), and HS⁻ (0.1 mM). \( \lambda_{ex} = 375 \text{ nm, } \lambda_{em} = 450 \text{ nm} \). Slits: 10/10 nm.

![Fig. 5](A) Fluorescence response of I (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: I + biothiols (or Vc-Na); Blue bar: I + biothiols (or Vc-Na) + HS⁻; \( \lambda_{ex} = 375 \text{ nm, } \lambda_{em} = 450 \text{ nm} \). Slits: 10/10 nm. (B) The corresponding fluorescent images: 1) I only; 2) I + HS⁻; 3) I + Cys; 4) I + Cys + HS⁻; 5) I + Hcy; 6) I + Hcy + HS⁻; 7) I + GSH; 8) I + GSH + HS⁻; 9) I + Vc-Na; 10) I + Vc-Na + HS⁻.

Next, the effect of pH on the fluorescence response of probe I to H₂S was investigated (Fig. S3, ESI†). It was found...
showed a weak fluorescence (Fig. 6A) due to the low H$_2$S 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$_2$S supplemented to the cell cultures. It is well established that H$_2$S could be biosynthesized from Cys by the enzymes CBS and CSE.\textsuperscript{15} Also, it was reported that melanoma cell lines express CSE.\textsuperscript{15} Thus, Cys could be regarded as a precursor to H$_2$S in the cell imaging assays. 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,\textsuperscript{26} when the cells were pre-incubated in a sequence with Cys and phorbol myristate acetate (PMA) that could decrease the H$_2$S level presumably by generating ROS,\textsuperscript{16} and further incubated with 1, almost no fluorescence was observed (Fig. 6D).

![Fig. 6](image)

**Fig. 6** Fluorescence images of H$_2$S 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 replicative 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$_2$S was exploited to couple the azide-based strategy with the excited-state intramolecular proton transfer (ESIPT) sensing mechanism. The probe can highly selectively detect H$_2$S 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$_2$S chemistry in biological systems.

**Notes and references**

School of Chemistry and Chemical Engineering, Shaaxi University, 030606, China. E-mail: gyou@sxu.edu.cn.

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