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A fluorescent probe for the biological signaling molecule H_2S based on a specific H_2S trap group†

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A fluorescent turn-on probe for H_2S was exploited based on a H_2S-induced substitution–cyclization cascade reaction towards the bis-electrophilic centers of a new H_2S trap group 2-(iodomethyl)benzoate.

Hydrogen sulfide (H_2S) can be endogenously produced by enzymes such as cystathionine β-synthase, cystathionine γ-lyase, and 3-mercaptopyruvate sulfurtransferase,[1] 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.[2] Also, studies have shown that its deregulation has been correlated with the symptoms of Alzherimer's disease, Down's syndrome, diabetes, and liver cirrhosis.[3] H_2S has been regarded as the third gaseous transmitter besides nitric oxide (NO) and carbon monoxide (CO).[4] Therefore, an efficient method for sensitively and selectively probing H_2S in living 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. Since 2011, fluorescent probes which can potentially be used for H_2S detection in living systems have become available,[5] and the corresponding design strategies are based on several significant characteristic properties of H_2S, namely dual nucleophilicity,[6] good reducing property,[7] high binding affinity towards copper ions,[8] efficient thiolysis of dinitrophenyl ether[9] as well as specific addition reaction towards unsaturated double bonds.[10] Among these, the strategy based on the dual nucleophilicity of H_2S is especially attractive from the selectivity point of view,[6] and fluorescent probes, thus designed, generally contain a potential fluorescent reporter and a H_2S trap group with two electrophilic reaction sites (Fig. 1). The initial nucleophilic attack of H_2S towards site 1 would lead to an intermediate thiol, which spontaneously undergoes a cyclization towards site 2 to either release the fluorophore (Fig. 1A, B and D)[6a,b,e] or inhibit the photo-induced electron transfer (PET) process (Fig. 1C),[6c,d] thereby eliciting the obvious fluorescent signal. Importantly, due to the absence of the dual nucleophilicity, biothiols, such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) (the main competitive species in biological systems), cannot perform the second cyclization reaction, ensuring the high selectivity of this type of probes for H_2S.

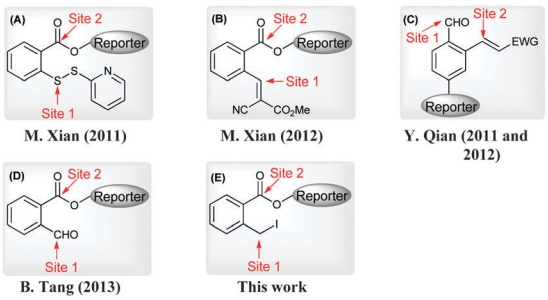

Fig. 1 Design strategies for H_2S fluorescent probes based on the unique dual nucleophilicity of H_2S.

However, one of the main issues of concern in using this strategy is how to avoid the probe consumption by biothiols,[6b] which would otherwise lead to low sensitivity and high probe loading. For example, a 2-pyridinyl disulfide-based H_2S probe (Fig. 1A) could also react with biothiols, leading to a relatively high probe loading;[6a] the aldehyde-based H_2S probes (Fig. 1C and D)[6c–e] may suffer from the same risk, because the aldehyde group could react with Cys/Hcy to form thiazolidines/thiazinanes under mild conditions.[11] The other concern in using this strategy is that the fluorescence changes in this type of probes upon H_2S treatment should result from the second cyclization reaction other than the initial substitution or addition reaction; otherwise, biothiols may more or less elicit the fluorescence changes as well.[6c,d] In view of these, further efforts to refine the dual nucleophilicity of H_2S in this design strategy are highly required.

Herein, we present a fluorescent probe **1** for detection of H_2S in aqueous solution through integration of a new H_2S trap group 2-(iodomethyl)benzoate and a potential fluorescent

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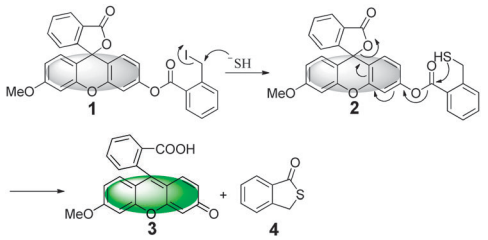

Fig. 2 The proposed sensing mechanism of probe **1** for H_2S.

reporter methylfluorescein. The design rationale is depicted in Fig. 2, and illustrated as follows. Because the hydroxy group is protected by the 2-(iodomethyl)benzoate moiety, the methylfluorescein moiety of **1** exists in a nonfluorescent spirocyclic form. The H_2S-induced substitution–cyclization cascade reaction towards the bis-electrophilic centers (**CH_2**-I and adjacent ester carbonyl) of the 2-(iodomethyl)benzoate moiety would release the ring-opening methylfluorescein **3**, thereby leading to the fluorescence "*off–on*" response of the system. Furthermore, we speculated that biothiols could not perform the initial nucleophilic substitution towards the first electrophilic center **CH_2**-I due to their weak nucleophilicity caused by the relatively high pK_a values compared with H_2S in physiological media (pK_a: for Cys, Hcy and GSH, $\geq$8.5; for H_2S, *ca.* 7.0).[6b,10] If so, this would avoid probe consumption and thus guarantee the high selectivity and sensitivity of probe **1** towards H_2S.

To test the above-mentioned possibility, we prepared probe **1** by a simple coupling procedure with methylfluorescein and 2-(iodomethyl)benzoic acid as starting materials, and DCC (dicyclohexylcarbodiimide) as a coupling reagent (ESI†). Its structure was confirmed by ^{1}H NMR, ^{13}C NMR, and HRMS spectra. In fact, when **1** was treated with excess NaHS (a commonly employed H_2S donor)[12] in CH_3CN/PBS buffer (20 mM, pH = 7.4, 1 : 1), the cyclization product **4** could be separated in 90% yield (ESI†). Moreover, the formation of the ring-opening methylfluorescein **3** in the assay was confirmed by fluorescence emission spectrum analysis of the assay solution, which was identical to that of the authentic sample. Importantly, when **1** was treated with Cys/Hcy/GSH under the same conditions, no new product was found, and only the starting material was recovered. These results strongly supported our speculation, and paved the way for the subsequent sensing studies for H_2S.

Subsequently, we examined the reactivity of **1** (5 μM) towards the different concentrations of NaHS through time-dependent fluorescence spectroscopy in PBS buffer (10 mM, pH 7.4, containing 20 mM CTAB (cetyltrimethylammonium bromide)) at 25 °C. It was found that in the presence of 100 μM NaHS, the reaction could be completed within 5 min, whereas the lower concentrations of NaHS needed a longer reaction time of 30 min to reach the spectral saturation (Fig. S1, ESI†). Thus, with a time point of 30 min after addition of NaHS, we performed the fluorescence titration studies of **1** towards H_2S under the same conditions. As shown in Fig. 3A, the free probe **1** exhibited almost no fluorescence (fluorescence quantum yield: Φ = 0.014) in the visible region due to the closed lactone conformation of the methylfluorescein moiety, meeting the basic requirement to confer the probe with a fluorescence

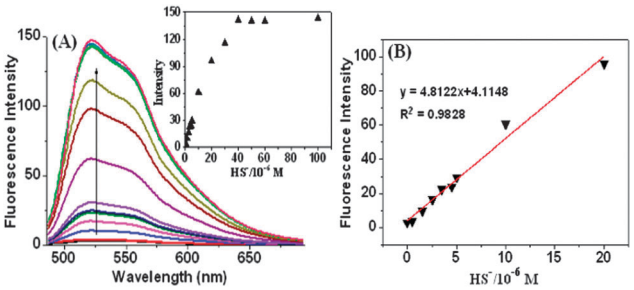

Fig. 3 (A) Fluorescence spectra of **1** (5 μM) upon addition of HS^- (0–100 μM) in PBS buffer (10 mM, pH 7.4, containing 20 mM CTAB). (B) Fluorescence response of **1** at 517 nm to HS^- concentration (0–20 μM). Spectra were recorded after incubation with different concentrations of HS^- for 30 min. λ_{ex} = 455 nm, λ_{em} = 517 nm. Slits: 5/5 nm.

off state. However, treatment with NaHS elicited the obvious fluorescence turn-on at 517 nm, the typical emission of ring-opening methylfluorescein **3**. The changes in the emission intensities became less obvious when the amount of NaHS is more than 40 μM. In this case, a big fluorescence enhancement of approximately 50-fold could be observed (Φ = 0.379, determined in PBS buffer (10 mM, pH 7.4, containing 20 mM CTAB) with fluorescein as a reference), indicative of the high signal-to-noise ratio (*S*/*N*). Moreover, the fluorescence intensities of **1** at 517 nm showed a good linear relationship with the HS^- concentration from 0 to 20 μM. The detection limit for HS^- was estimated to be 0.10 μM based on *S*/*N* = 3 (Fig. 3B), which is lower than the concentration of sulfide required to elicit physiological response (10–600 μM).[7a] In fact, **1** is one of the few fluorescence probes that could detect HS^- at a submicromolar range.[7e,i,j,l,8b]

To evaluate the specific nature of **1** for H_2S, we then examined the fluorescence enhancement of **1** incubated with various species including the representative anion, ROS (reactive oxygen species), cations, and biothiols Cys, Hcy, and GSH. As can be seen in Fig. 4, these competitive species, especially the nucleophilic species, such as AcO^-, CN^-, HSO_3^-, and biothiols, did not lead to significant fluorescence changes in **1**, and only HS^- elicited a dramatic increase in the fluorescence intensity, confirming the high selectivity of probe **1** towards H_2S.

However, the above selectivity experiments could not provide information regarding whether the probe was consumed by biothiols, which is indeed important when considering its use in living systems. Thus, we performed the competition

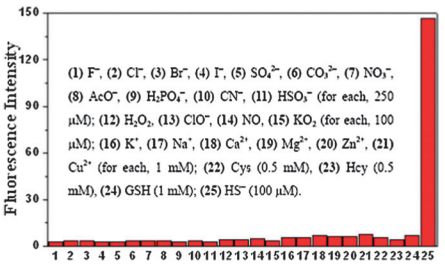

Fig. 4 Fluorescence response of **1** (5 μM) upon addition of various species in PBS buffer (10 mM, pH 7.4, containing 20 mM CTAB). λ_{ex} = 455 nm, λ_{em} = 517 nm. Slits: 5/5 nm.

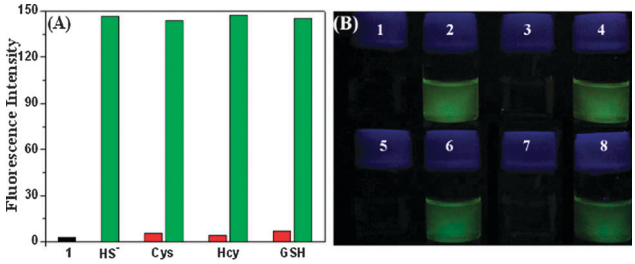

Fig. 5 (A) Fluorescence response of **1** (5 μM) to HS^- (100 μM) and biothiols Cys (0.5 mM), Hcy (0.5 mM), and GSH (1 mM) in PBS buffer (10 mM, pH 7.4, containing 20 mM CTAB). Red bar: **1** + biothiols; green bar: **1** + biothiols + H_2S. λ_{ex} = 455 nm, λ_{em} = 517 nm. Slits: 5/5 nm. (B) The corresponding 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^-.

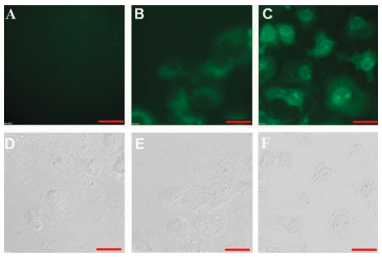

Fig. 6 Images of H_2S in COS-7 cells using probe **1** (5 μM) at 37 °C. (A) Fluorescence image of COS-7 cells incubated with **1** for 30 min. (B) Fluorescence image of COS-7 cells incubated with **1** for 30 min and further incubated with NaHS (100 μM) for 30 min. (C) Fluorescence image of COS-7 cells incubated with **1** for 30 min and further incubated with NaHS (100 μM) for 30 min in the presence of CTAB (1 mM). (D–F) The corresponding bright-field images. Scale bar: 20 μm.

experiments in the presence of biothiols. In fact, when H_2S (100 μM) and biothiols (for Cys and Hcy: 500 μM; for GSH: 1 mM) coexisted, we observed almost the same fluorescence enhancement as that only treated by 100 μM H_2S (Fig. 5A). Moreover, the H_2S-induced obvious fluorescence enhancement in the presence of biothiols was observable by the naked eyes (Fig. 5B). Thus, probe **1** was not consumed by biothiols, and could be used to selective sensing of H_2S in the presence of a high concentration of biothiols.

Encouraged by the above results, we subsequently tested the capability of **1** to image H_2S in biological systems. COS-7 cells, incubated with **1** (5 μM) in culture medium for 30 min at 37 °C, showed almost no fluorescence (Fig. 6A), indicating that biological species, especially biothiols, did not cause interference. However, strong fluorescence in the cells was observed after the cells were pretreated with **1** for 30 min and further incubated with NaHS (100 μM) for 30 min (Fig. 6B). Moreover, we could observe stronger fluorescence when the cells were further treated with 1 mM CTAB (Fig. 6C). These results indicated that probe **1** has the potential to visualize H_2S levels in living cells.

In summary, a reaction-type fluorescent probe **1** for detection of H_2S in aqueous solution was exploited through integration of a new H_2S trap group 2-(iodomethyl)benzoate and a potential fluorescent reporter methylfluorescein. The probe can highly selectively detect H_2S even in the presence of millimolar concentrations of biothiols. Preliminary fluorescence imaging experiments in cells indicate its potential to probe H_2S chemistry in biological systems.

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