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EDGE ARTICLE

A fluorescent probe for rapid detection of hydrogen sulfide in blood plasma and brain tissues in mice†

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We describe the design, synthesis and application of a sensitive, selective, and fast fluorescent probe for H_2S detection. A linear relationship between emission intensity and sulfide concentration was observed in biological bovine plasma systems. With this probe, we were able to estimate the sulfide concentration in mouse blood plasma and brain tissues.

Introduction

Hydrogen sulfide (H_2S) is a toxic gas best known for its rotten egg smell. Paradoxically, our body produces it in small amounts and H_2S may play key roles in the health of the heart and other organs. H_2S was recently suggested as the third signaling gasotransmitter, along with nitric oxide (NO) and carbon monoxide (CO).[1] Altered levels of H_2S has been linked to many diseases, such as Down syndrome[2] and Alzheimer's diseases. Previous studies have identified a few enzymes, including cystathionine β-synthase (CBS),[3] cystathionine γ-lyase (CSE),[4] and 3-mercaptopyruvate sulfurtransferase (3MST),[5] which produce endogenous H_2S in mammals. It was reported that mitochondrial sulfide quinone oxidoreductase (SQR) and persulfide dioxygenase (ETHE1)[6] are involved in the consumption of H_2S. These findings highlight importance of H_2S homeostasis.

However, the levels of H_2S in biological settings has been reported to span over a broad range of concentrations in the literature.[1b,7] To monitor the fluctuation of H_2S in the body and its cellular site of action, probes with high sensitivity, selectivity, and real-time capability to measure intracellular H_2S are desirable.

Specifically, several challenges are present: (1) the chemistry needs to be bioorthogonal to native cellular processes; (2) due to the transient nature of H_2S and low concentration in most tissues,[8] fast-responding probes are in demand to track this small molecule; (3) one major challenge of developing H_2S-selective probes is thiols (millimolar) in cells, which often interfere with the reaction of H_2S (micromolar or lower). To overcome these obstacles, several groups including us adopted different molecular frameworks to design selective fluorescence probes for H_2S.[9] While the Chang and Wang groups took advantage of the unique reduction reaction between an azide group and H_2S to accomplish selectivity,[9a,9c,9f,9g] our group and the Xian group utilized a bis-electrophilic strategy that took advantage of the double nucleophilic character of H_2S.[9b,9e] Most recently, Nagano *et al.* reported a copper-based coordination complex in which the Cu^{2+} center can be released by binding H_2S to induce fluorescence.[9d,9h]

Our recent strategy to develop H_2S probe[9e] featured: (a) 4,4-difluoro-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY) as the reporter template for its high brightness and photostability;[10] (b) a benzene ring substituted by an acrylate methyl ester moiety and an aldehyde (–CHO) functional group at the *ortho* position as the H_2S targeting site for double nucleophilic reactions.

Though the probe is highly selective, one drawback of this probe is that the reaction time tends to be a little long. Faster response with high sensitivity is highly desirable and practical. Herein, we report a fast response fluorescent probe SFP-3 for the detection of H_2S in blood plasma, which maintains good selectivity and sensitivity. Noting the fact that the nucleophilic SH^- group adds faster to an electron-poor C=C double bond,[11] we hypothesized that, by tuning the electronics, the reaction rate could accelerate while retaining the selectivity. Therefore, we set out to prepare the BODIPY-based probe second-generation reagent SFP-3, replacing the acrylate ester with an α,β-unsaturated phenyl ketone. In this work, we report the synthesis and application of SFP-3 for the measurement of H_2S concentration in mice blood plasma and brain tissues.

Results and discussion

We synthesized the BODIPY-based probe SFP-3 (**8**) as shown in Fig. 1. The synthesis is quite straightforward. The final probe, SFP-3, was characterized by NMR spectroscopy and mass spectrometry (see the ESI†). We then tested the SFP-3 probe

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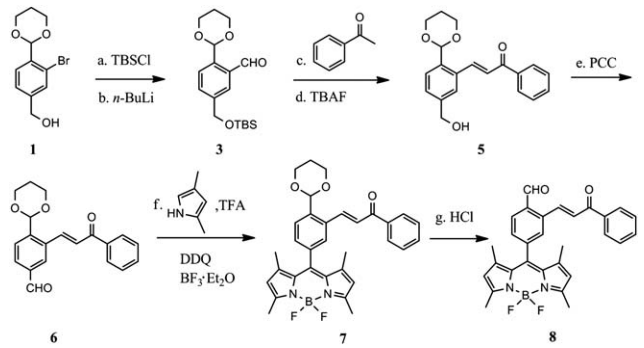

Fig. 1 Probe SFP-3 synthesis: (a) TBSCl (1.2 equiv.), imidazole (2 equiv.), DMF, 25 °C, 6 h, 82%; (b) *n*-BuLi (2 equiv.), THF, −78 °C, 1 h; then DMF (4 equiv.), −78 °C–25 °C , 2 h, 67%; (c) acetophenone (1 equiv.), MeOH, NaOH, 25 °C, 2 h, 74%; (d) TBAF (1 equiv.), THF, 25 °C, 30 min, 82%; (e) PCC (1.5 equiv.), celite, CH_2Cl_2, 25 °C, 1 h, 93%; (f) (1) 2,4-dimethylpyrrole (2 equiv.), TFA (one drop), CH_2Cl_2, 25 °C, 12 h. (2) DDQ (1.1 equiv.), CH_2Cl_2, 25 °C, 1.5 h. (3) Et_3N (5 equiv.), $BF_3 \cdot Et_2O$ (5 equiv.), CH_2Cl_2, 25 °C, 2 h, 22%; (g) HCl, acetone, 25 °C, 2 h, 81%. TBSCl, *tert*-butyl(chloro)dimethylsilane; DMF, *N*,*N*-dimethylformamide; *n*-BuLi, *n*-butyllithium; TBAF, tetrabutylammonium fluoride trihydrate; PCC, pyridinium chlorochromate; Et_3N, triethylamine; TFA, trifluoroacetic acid; DDQ, 2,3-dichloro-5,6-dicyano-*p*-benzoquinone; THF, tetrahydrofuran.

(10 μM) with Na_2S (100 μM) as an aqueous sulfide source at 25 °C in 20 mM PBS buffer (pH 7.4) (Fig. 2a and ESI, Fig. S2†). Gratifyingly, SFP-3 showed a fast, robust increase of the fluorescence intensity (>70-fold) in the emission maximum at 515 nm when excited at 500 nm ($\varepsilon = 1.12 \times 10^5$ M^{-1} cm^{-1}, $\Phi = 0.10$),

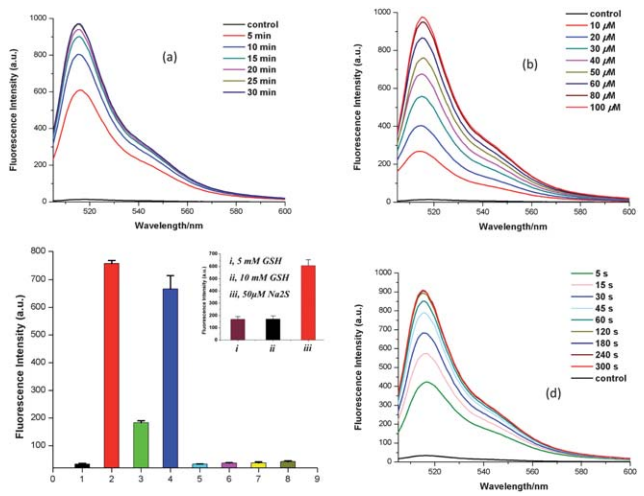

Fig. 2 Fluorescence spectra of the SFP-3 probe (10 μM) in PBS buffer (20 mM, pH 7.0, 40% CH_3CN) at 25 °C. Excitation: 500 nm, emission: 505–600 nm. The data represents the average of three independent experiments. (a) Incubated with 100 μM Na_2S after 5, 10, 15, 20, 25, 30 min. (b) Incubated with different concentrations of Na_2S (10, 20, 30, 40, 50, 60, 80 and 100 μM) for 20 min. (c) Incubated with Na_2S and various thiols or amino acids for 20 min: 1) Na_2S (0 μM); 2) Na_2S (50 μM); 3) GSH (100 μM); 4) GSH (100 μM) + Na_2S (50 μM); 5) Cystine (100 μM); 6) Lys (100 μM); 7) Ala (100 μM); 8) Cys (100 μM). (d) Incubated with 1 μl H_2S buffer (bubbling H_2S 10 min–saturated solution) at 25 °C from 5 s–300 s.

which was completed within 20 min. We also isolated the H_2S addition product **8a** and confirmed its molecular formula by high resolution mass spectrometry (ESI, Fig. S1†).

Next, we examined the sensitivity of SFP-3 for sulfide *via* varying concentrations of Na_2S (10–100 μM). The fluorescent intensity increased about 20–73 fold with the addition of Na_2S (Fig. 2b and Fig. S3†). Moreover, the turn-on fluorescence response was also found to be selective for sulfide over other various biologically relevant thiols and amino acids in the PBS buffer (Fig. 2c and Fig. S4†). SFP-3 probe displayed ~35-fold greater response toward Na_2S than cysteine, and ~8-fold more selective for Na_2S than glutathione. Cystine, lysine, and alanine did not lead to a fluorescent turn-on response. Additionally, exposing SFP-3 to a mixture of GSH and Na_2S still yielded a significant fluorescence signal increase (Fig. 2c). SFP-3 showed good selectivity towards Na_2S in the presence of physiological concentrations (5 mM and 10 mM) of GSH (Fig. 2c insert). The direct response of SFP-3 toward H_2S was also tested. After the addition of 1 μL H_2S buffered solution (10 min H_2S bubbling), a significant fluorescence increase was observed between 5 s to 300 s after mixing, and the reaction was completed in 120 s at 25 °C (Fig. 2d). A smaller amount of H_2S can still induce significant responses, further confirming that SFP-3 is a sensitive probe for H_2S detection (Fig. S5†) under physiological conditions.

To further establish the utility of SFP-3 for the determination of sulfide in a biological sample, we evaluated SFP-3 (100 μM) in commercially available bovine plasma with Na_2S (100 μM) after 30–300 s (Fig. 3a and Fig. S6†). Importantly, the SFP-3 probe was able to retain its significant fluorescence response to sulfide in plasma, and the reaction was completed in 120 s at room temperature. Then, we treated SFP-3 with different Na_2S concentrations (20–200 μM) to obtain a linear relationship of emission intensity *versus* sulfide concentration (Fig. 3b and Fig. S7†). An excellent linear correlation between the added Na_2S concentrations and the fluorescence emission responses was observed in the bovine plasma system. The fluorescence intensity response in plasma was lower than the signal observed in pure PBS buffer system. Interestingly, a small emission wavelength shift was observed, which may due to the fast metabolism of sulfide in plasma. Nevertheless, the fast responses and excellent linear relationship provided a real-time quantitative detection method for sulfide in biological samples.

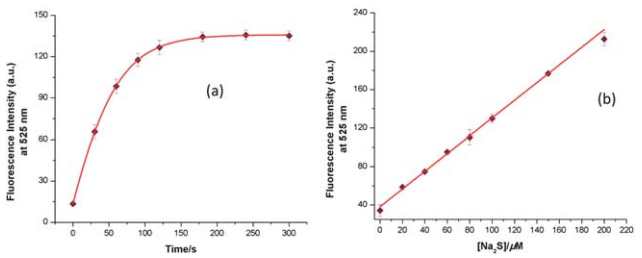

Fig. 3 (a) Fluorescence spectra of the SFP-3 probe (100 μM) incubated with 100 μM Na_2S after 30 s, 60 s, 90 s, 120 s, 180 s, 240 s, 300 s in bovine serum (40% CH_3CN) at 25 °C. (b) SFP-3 probe (100 μM) incubated with 0, 20, 40, 60, 80, 100, 150, 200 μM Na_2S after 3 min in bovine serum (40% CH_3CN) at 25 °C. Excitation: 500 nm, emission: 505–600 nm. The data represents the average of four independent experiments.

Finally, we applied SFP-3 to the measurement of sulfide concentration in mouse blood. Spiked Na_2S was used as internal standard in the blood and we discovered that a significant portion of sulfide was rapidly removed, leading to inconsistent data. These failed attempts confirmed the previous findings, in which sulfide rapidly disappeared in blood by binding to hemoglobin or metabolising.[8]

We next used blood plasma to determine the sulfide concentration. The mouse blood was first centrifuged, Na_2S was then spiked into the blood plasma as an internal standard. The spiked plasma samples were subsequently precipitated by acetonitrile to remove proteins.[12] To the supernatant of the spiked plasma was added SFP-3 (20 μM). The mixture was incubated in PBS buffer at 37°C for 30 min and then monitored. We found that the average sulfide concentration in mice blood plasma is ~36.3 μM (Fig. S8†).

Inspired by the report from the group of Wang,[9c] we conducted our measurements in bovine plasma and were delighted to find that the reaction was significantly faster. The reaction typically completed in 3 min. Considering the short half-life nature of sulfide in plasma,[8] we believe that this fast-response probe could be highly useful in obtaining the accurate value of sulfide concentration in blood. To this end, we tried to directly measure the sulfide concentration in fresh plasma. Fresh mice blood plasma was added to bovine plasma and incubated directly with SFP-3 using Na_2S as the internal standard (see the ESI for details†) at 37 °C. A fluorescence signal increase was observed after 3 min. The average sulfide concentration in four mice blood plasma was 56.0 ± 2.5 μM (Table 1, 2 and Fig. S9†), very close to other reports on sulfide concentrations in blood plasma.[13] Overall, these findings demonstrated that SFP-3 is suitable to detect sulfide in real biological samples in a rapid manner.

Importantly, hydrogen sulfide is recognized as a neuromodulator as well as neuroprotectant in the brain.[14] Several methods have been employed for H_2S measurement in the brain, such as methylene blue colorimetric assay, polarographic H_2S sensor,[15] and gas/ion chromatography.[16] H_2S concentration in the brain were reported as a wide range, from undetectable to more than 100 μM, indicating that methods to obtain accurate measurements are in high demand. We applied SFP-3 in determining the sulfide level in C57BL6/J mice brain tissues. Using a similar method to measuring blood H_2S concentration, the average sulfide concentration in four mice brains was estimated to be 7.1 ± 1.4 μmol g^{-1} protein (Table 1 and Fig. S10†). This is the first probe that could allow parallel measurements of H_2S concentrations in both blood and brain tissues.

Table 1 Determination of H_2S concentration in different mice blood samples with different preparation

Mouse	Spiked blood[a]	Spiked plasma[b]	Plasma[c]
C57BL6/J	Inconsistent	36.3 μM	56.0 ± 2.5 μM

[a] Spiked blood: plasma from whole mouse blood spiked with Na_2S before centrifugation. [b] Spiked plasma: plasma spiked with Na_2S after centrifugation and then precipitated protein to detect sulfide in PBS buffer. [c] Plasma: direct measurement of sulfide in fresh plasma with bovine plasma using Na_2S as internal standard.

Table 2 Measurement of H_2S concentration in mouse blood plasma and brain tissue

Mouse	Blood Plasma (μM)	Brain Tissue (μmol g^{-1} protein)
1	55.4	6.7
2	57.9	8.5
3	52.6	7.7
4	58.0	5.3
Average	56.0 ± 2.5	7.1 ± 1.4

In blood, the levels of thiols can be low. While in brain tissues, thiol levels can be relatively high. We repeated the measurement with probe SFP-2[9e] which is more selective than SFP-3 but with a slower response rate. The sulfide concentration was determined to be 5.0 ± 2.0 μmol g^{-1} protein, confirming the results from the SFP-3 probe.

Conclusions

To conclude, we have developed a new sensitive and selective probe that shows a significant emission increase in the fast response to sulfide over the biologically relevant pH range. The excellent linear relationship between emission intensity and sulfide concentration was obtained in a bovine plasma system. This probe was successfully applied for the parallel measurement of sulfide concentration in mice blood plasma and brain tissues. This fast, sensitive and selective probe has the potential to be a useful tool for the fast and real-time detection of sulfide in more types of biological samples. Current efforts are directed at developing more specific fluorescent probes for optical imaging and mechanistic studies of H_2S metabolism in biological systems.

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