Micelle-induced multiple performance improvement of fluorescent probes for H2S detection

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HIGHLIGHTS

► Two new H2S probes based on its reducing property were synthesized.
► Rapid response and high sensitivity (the detection limit as low as 20 nM) towards H2S were realized in CTAB micelles.
► The probes could measure H2S levels in fetal bovine serum without any prior sample processing.

GRAPHICAL ABSTRACT

Two colorimetric and turn-on fluorescent probes for the selective recognition of H2S were designed and synthesized. Rapid response and high sensitivity (the detection limit as low as 20 nM) were realized in CTAB micelles. The probes could measure H2S levels in fetal bovine serum without any sample pretreatment.

ARTICLE INFO

Article history:
Received 15 October 2012
Received in revised form 10 January 2013
Accepted 15 January 2013
Available online 23 January 2013

Keywords:
Fluorescent probe
Naphthalimide
H2S
Cetyltrimethyl ammonium bromide (CTAB)
Fetal bovine serum

ABSTRACT

In this paper, two colorimetric and turn-on fluorescent probes N-[2-(2-hydroxy)ethoxy] ethyl-4-azido-1,8-naphthalimide (SS1) and N-butyl-4-azido-1,8-naphthalimide (SS2) for selective recognition of H2S were designed and synthesized. The probes were constructed by incorporating an azido group into the naphthalimide fluorophore as a specific reaction group for sulfide utilizing its reducing property. Once treated with H2S, the azido groups of the probes were converted to amino groups and the solutions' color changed from colorless to yellow companied with a strong yellow-green fluorescence. Rapid and sensitive responses of the probes towards H2S were achieved in the presence of cationic surfactant cetyltrimethyl ammonium bromide (CTAB); the reaction was completed within 10 min in CTAB compared to more than 4 h in buffer solution, and the detection limit decreased from 0.5 μM to 20 nM. High selectivity and good competition of both probes towards H2S over other 11 ions and 2 reducing agents were realized in CTAB micelle. An overall linear concentration range of 0.05 μM to 1 mM was achieved with the assistance of differently charged surfactants CTAB and sodium dodecyl sulfate (SDS). The probes were applied to rapidly and sensitively detect H2S levels in fetal bovine serum without any pretreatment of the sample.

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1. Introduction

Following nitric oxide (NO) and carbon monoxide (CO), hydrogen sulfide (H2S) has been recognized as the third gaseous signaling molecule [1–7], which contributes to many physiological and pathological processes including myocardial contractility and insulin secretion [8,9]. On the other hand, the abnormal concentrations of H2S are related to some diseases such as Alzheimer's...
disease, Down syndrome, liver cirrhosis [10,11]. However, it is challenging to selectively and sensitively track this small molecule in biological settings, because the biologically relevant levels of H₂S are estimated to vary over 10⁵-fold concentration range, i.e., from high nM to low mM levels [12–15] and the metabolism of H₂S in vivo is very fast. Therefore, it is important to develop fast, accurate and real-time detection of H₂S with a linear response to a dynamic concentration range.

Several methods such as colorimetric [16,17], electrochemical [18,19], and chromatographic [20] assays have been developed for H₂S detection, but they usually need tedious sample treatment. Fluorescence technique is superior in H₂S detection for its high sensitivity, selectivity, convenience and real time detection. A few fluorescent probes for H₂S were reported utilizing its chemical properties of metal-induced sulfide precipitation [21], the reducibility [22–26] or the nucleophilicity [27–29]. However, most of the probes need at least half an hour to react with H₂S and their detection limits were at micromolar levels, which were not suitable to monitor H₂S in tissues or cells in real time. Therefore, a rapid reaction system with high sensitivity for the recognition of hydrogen sulfide is greatly desired.

Surfactants have been widely applied in different scientific fields due to their unique physicochemical properties. For instance, surfactant micelles and other aggregates have been employed to catalyze chemical reactions [30,31], adjust the detection sensitivity in fluorescent molecular sensing of ions [32–35], and mimic biological membranes [36,37]. Herein, we explored the use of surfactants in H₂S detection with two azido-based probes. The probes were constructed by incorporating an azido group into the naphthalimide fluorophore as a specifically active reaction group for sulfide utilizing its reducing property [22]; diglycineamide and n-butylamine were used to modulate the hydrophilic or lipophilic properties of the probes (Scheme 1). CTAB and SDS were employed to study their effects on the detection kinetics and sensitivities of the probes for H₂S analysis. Rapidly colorimetric and fluorescent responses of the probes towards H₂S were realized in CTAB micelles, rendering a much lower detection limit of 20 nM and high selectivity over other ions and reducing agents. These merits allow detection of H₂S levels in biological samples without prior sample processing.

2. Experimental

2.1. Reagents and methods

All chemicals of analytical reagent grade were purchased from Aladdin Corporation and were used without further purification. Fetal bovine serum was purchased from the JONLNI Industrial Co., LTD Shanghai. Ultrapure water was prepared through Sartorious Arium 611DI system.

An accurately weighted amount of the dyes were dissolved in MeOH to obtain 2 × 10⁻³ M stock solution. The stock solution was diluted with buffer solution (or surfactant buffer solution) to acquired 1 × 10⁻⁵ M dye aqueous solution. The concentrations of CTAB and SDS buffer solutions were 1 mM and 10 mM, respectively, all higher than their corresponding critical micelle concentrations (0.92 mM for CTAB and 8.9 mM for SDS) [38].

In the kinetic titration measurements, 40 μL of 15 mM freshly prepared Na₂S–PBS was added to 3 mL of 1 × 10⁻⁵ M dye aqueous solution; while in the Na₂S titration experiments, 0–40 μL of Na₂S–PBS with different concentrations were added into 3 mL of 1 × 10⁻⁵ M dye aqueous solution.

Theoretically, the speciation of sulfide-containing species only depends on the solution’s pH. In this work, Na₂S or NaH₂S were employed as the H₂S sources and similar results were obtained.

2.2. Synthesis of the probes

Compounds 1 and 2 were prepared according to the references [39,40]. N-[(2-hydroxy-ethoxy) ethyl-4-azido-1,8-naphthalimide (SS1): compound 1 (500 mg, 1.37 mmol) and sodium azide (200 mg, 3.08 mmol) were dissolved in 20 mL of anhydrous DMF in a 50 mL round bottom flask. The solution was stirred at 80°C under N₂ for 12 h, and then it was poured into 100 mL ultrapure water. The resulted solution was extracted with dichloromethane for 3 times, and the combined organic phase was evaporated under vacuum. The residue was purified by column chromatography (DCM:MeOH = 100:1, v/v) to give SS1 as a pale yellow solid (yield: 62%) and by-product SS1–NH₂ as a yellow solid. ¹H NMR spectrum of SS1 (CDCl₃, 400 MHz): δ: 6.84 (d, 1H), 8.58 (d, 1H), 8.44 (d, 1H), 7.75 (t, 1H), 7.47 (d, 1H), 4.44 (t, 2H), 3.86 (t, 2H), 3.68 (m, 4H), ESI m/z calca for [M+H]⁺: 327.11; found: 327.40. ¹H NMR spectrum of SS1–NH₂ (DMSO-d₆, 400 MHz): δ: 8.62 (d, 1H), 8.43 (d, 1H), 8.19 (d, 1H), 7.65 (t, 1H), 7.46 (s, 2H), 6.85 (d, 1H), 4.58 (m, 1H), 4.20 (t, 2H), 3.61 (t, 2H), 2.51 (s, 4H), ESI m/z calca for [M+Na]⁺: 301.12; found: 301.21 (Fig. S1).

The synthesis procedures of N-butyl-4-azido-1,8-naphthalimide (SS2) were the same as those of SS1 but with compound 2 as the starting material. The residue was purified by column chromatography (PE:DCM = 2:1, v/v) to give SS2 as a pale yellow solid (yield: 30%) and by-product SS2–NH₂ as a yellow solid. ¹H NMR spectrum of SS2 (CDCl₃, 400 MHz): δ: 6.84 (d, 1H), 8.58 (d, 1H), 8.43 (d, 1H), 7.75 (t, 1H), 7.47 (d, 1H), 4.18 (t, 2H), 1.73 (m, 2H), 1.47 (m, 2H), 0.98 (t, 3H), ESI m/z calca for [M+H]⁺: 295.12; found: 295.40. ¹H NMR spectrum of SS2–NH₂ (CDCl₃, 400 MHz): δ: 8.61 (d, 1H), 8.44 (d, 1H), 8.11 (d, 1H), 7.68 (t, 1H), 6.88 (d, 1H), 4.96 (s, 2H), 4.17 (t, 2H), 1.71 (m, 2H), 1.46 (m, 2H), 0.97 (t, 3H), ESI m/z calca for [M+Na]⁺: 269.13; found: 269.1, 291.1 for [M+Na]⁺ (Fig. S2).

2.3. Characterization and measurement

¹H NMR spectra were performed with a Bruker AV-400 spectrometer (400 MHz). Mass spectra were recorded on a MA 1212 Instrument on standard condition (ESI, 70 eV). Absorption spectra were measured with an Evolution 220 UV–vis spectrophotometer (Thermo Scientific). Fluorescence spectra were carried out on a Lumina Fluorescence Spectrometer (Thermo Scientific) with an excitation wavelength of 404 nm; all the fluorescence spectra were uncorrected. The experiments were performed at 25°C using non-degassed samples.

3. Results and discussion

3.1. The photophysical response of the probes towards H₂S in phosphate buffer solution (PBS)

Fig. 1 shows that SS1 has a very weak fluorescence band at 470 nm and displays an absorption band centered at 380 nm in sodium phosphate buffer solution (20 mM PBS, pH 7.4). The presence of H₂S triggered reduction of azide to amine, which concomitantly converted the probe to highly fluorescent 4-amino naphthalimide compound with absorption and emission maxima at 435 nm and 540 nm, respectively. In PBS, upon the addition of 20 eq. of Na₂S (200 μM, as a hydrogen sulfide donor; The pKₐ of H₂S is 6.98, which means that [H₂S] / [HS⁻] ≈ 1.25 under the experimental conditions), the original absorption band of probe SS1 decreased, while a new band at about 435 nm (Fig. 1a) emerged and developed steadily accompanied with the solution color changing from colorless to yellow (Fig. S3). The absorption wavelength shift is caused by
the enhanced internal charge transfer (ICT) process after the reduction. An isobestic point at 404 nm is clearly seen, which indicates a clean 1:1 transformation. The emission band shifted from 470 nm to 540 nm and more than 50-fold enhancement in fluorescence intensity was observed (Fig. 1b). The emission and the absorption spectra (Fig. S4), ESI and the NMR data (Fig. S5) of the reaction product are the same as those of SS1–NH2, which confirm the reaction mechanism (Scheme 1). However, the reaction was not finished within 4 h (the absorbance and fluorescence intensity did not reach the plateau until 4 h after the reaction, Fig. 1), which is a major obstacle for biological application. Though the photophysical properties and the reactive group of SS2 are similar to those of SS1 (Fig. S6), it is difficult to directly measure the spectral changes of SS2 upon the addition of H2S for its low solubility in PBS (less than 0.5 μM). However, SS2 is more soluble in CTAB micelle (higher than 100 μM), and we expect it to behave similarly to SS1 in micellar systems.

### 3.2. The effect of pH on the reduction reaction rate

In general, the reduction product and the reaction rate are related to the pH of the solution. Then we studied the spectral change of probe SS1 upon the addition of H2S in phosphate buffer solution with different pH values. In a defined concentration range, the absorbance in the maximum wavelength of a certain compound is linearly dependent on its concentration. Therefore, we used the A/ΔA0 at 380 nm to estimate the residual amount of the probe, A and ΔA0 are the absorbance of the original absorption peak of SS1 at t and 0 min, respectively. The changes of A/ΔA0 with reaction time under different pH (pH 3.0, 7.4, 11.0, respectively) are shown in Fig. 2. From this figure, it is clearly seen that the A/ΔA0 decreased faster at higher pH, which implies that the reaction rate increases with the increase of pH. That is to say, higher concentration of OH− results in a faster reducing reaction between SS1 and H2S.

In aqueous solution, there exists following equilibrium:

\[
\text{H}_2\text{S} + \text{H}^+ + \text{HS}^- + \text{H}_2\text{O}^+ = 2\text{H}^+ + \text{S}^2 \tag{1}
\]

From Eq. (1), it is known that H2S is the predominant species at pH 3.0; approximately 27.6% of the total sulfide concentration exists as H2S and 72.4% as HS− at physiological pH; and about 90.1% of the total sulfide concentration exists as HS− and 9.9% as S2− at pH 11.0. From the standard electrode potentials of different species [41], we surmised that pH affected the reaction rate mainly by changing the contents of different sulfide-containing species.

### 3.3. The response of probe SS1 towards Na2S in micellar systems

In order to accelerate the spectral response of the probe, the reaction was then conducted in different micellar systems. Fig. 3 demonstrates the time-dependent A/ΔA0 values and fluorescence intensities of SS1 at 540 nm in different micellar systems under

**Scheme 1.** Schematic illustration of synthesis of SS1 and SS2. Both probes are converted to 4-amino products.

**Fig. 1.** Time-dependent absorption (a) and emission spectra (b) of SS1 mixed with Na2S in aqueous buffer. 20 mM PBS, pH 7.4, 25 °C, [SS1] = 10 μM, [Na2S] = 200 μM, λex = 404 nm.

**Fig. 2.** The plots of A/ΔA0 as a function of reaction time at different pH solution. 20 mM PBS, 25 °C, [SS1] = 10 μM, [Na2S] = 200 μM, A and ΔA0 are the absorbance at 380 nm at t min and 0 min, respectively.
the same experimental conditions. It can be seen from Fig. 3a that SDS and CTAB affected the reaction in different ways: the reaction was essentially prohibited in SDS micelle, while it was greatly accelerated and almost completed within 1 min in CTAB micelle, compared to >4 h in PBS (Fig. 1). Compared to PBS, the formation rate of the reduction product was much faster in CTAB, whereas it was slower in SDS micelle (Fig. 3b). Furthermore, the fluorescence intensity of the reaction product was almost 3 times higher in CTAB micelle than in PBS, which reveals that SS1 is more sensitive in CTAB micelle (Fig. 3b).

Surfactants affect the chemical reaction in two ways: changing the local concentrations of the reactants and altering the local pH value [42–44]. As we know, cationic surfactant CTAB can adsorb counter ions OH−, HS− and S2−, so the pH value as well as total sulfide concentration should be much higher on CTAB micelle surface than in the bulk solution. The higher pH leads to the lower ratio of [H2S]/[HS−], and the higher total sulfide concentration results in higher [HS−] around CTAB micelle.

Fig. 57 displays the absorption spectrum of SS1 in different solvents. The absorption maximum of SS1 in CTAB/SDS micelles (373 nm) is shorter than that in PBS (378 nm) but longer than that in apolar solvent ethyl acetate (361 nm), which suggests that the polarity of the position where SS1 existing is weaker than that of PBS but stronger than that of ethyl acetate. Consequently, SS1 may locate in the palisade or around the polar groups of micelles driven by the hydrophobic interaction between its hydrophobic fluorophore and the long alkyl chain in CTAB; it could hardly exist in the apolar oil-like inner core of micelles due to the repulsion from its hydrophilic groups of carbonyl, amino, etc. Therefore, the higher concentrations of HS− and the probe around CTAB micelle (Fig. 4a for speculated Model) resulted in a higher reaction rate. The reduction product could also be incorporated into CTAB micelles, accordingly, the microenvironmental polarity surrounding the product molecules is depressed and the fluorescence intensity is markedly enhanced with about 5 nm blue-shift in the emission wavelength (Fig. 58).

On the contrary, anionic surfactant SDS adsorbs positively charged ions and repels negatively charged ions (Fig. 4b for speculated Model), so the H+ concentration is higher, whereas the target sulfide concentration is lower at the micelle surface than in bulk solution. Accordingly, the apparent reaction rate was hampered in SDS micelle system.

![Fig. 3](image1.png)

**Fig. 3.** The plots of A/A0 (a) and fluorescence intensities (b) of SS1 in the presence of 200 μM Na2S as a function of reaction time in different systems. 20 mM PBS, pH 7.4, [SS1] = 10 μM, [CTAB] = 1 mM, [SDS] = 10 mM, λex/λem = 404 nm/540 nm. A and A0 are the absorbance at 380 nm at t min and 0 min, respectively.

![Fig. 4](image2.png)

**Fig. 4.** Schematic representation of the sponge effect of CTAB (a) and SDS (b) micelles.
3.4. Effect of H$_2$S concentration on the fluorescence intensity of SS1

Based on the above results, a higher sensitivity and a wider linear concentration-signal relationship could be expected in micelle systems. Next, we investigated the fluorescence intensity of SS1 with respect to the H$_2$S concentration in the following 3 systems: CTAB–PBS, PBS and SDS–PBS. The H$_2$S titration experiment revealed that the fluorescence intensity at 540 nm was linearly proportional to the amount of H$_2$S added. Good linear relationship between the fluorescence intensity of SS1 and the total sulfide concentration was obtained in all three systems (Fig. 5). The linear ranges of sulfide concentration were 0.05–10 μM, 1–200 μM and 75–1000 μM in CTAB–PBS, PBS and SDS–PBS systems, respectively, when the reaction time was kept 10 min in CTAB and 1 h in the other two systems (Fig. 5). Therefore, the overall linear concentration range covers almost $10^2$-fold concentration range, that is, from 50 nM to 1 mM with the assistance of the surfactants. The detection limits (3S$_b$/$k$, where S$_b$ is the standard deviation of the blank measurements of 10 times, and $k$ is the slope of the fitted line) are 20 nM, 0.5 μM and 10 μM in CTAB–PBS, PBS and SDS–PBS, respectively. A 20-fold decrease of the detection limit of SS1 in CTAB micelle is caused by the local concentration effect of the micelle. The shorter reaction time and lower detection limit are very important considering the fast metabolism and the dynamic concentration of hydrogen sulfide in biological systems. The reaction profiles of SS2 in micellar systems were similar to those of SS1 (Fig. S9). But the larger linear concentration range of SS2 in CTAB micellar system could be ascribed to the more hydrophobic characteristic of SS2, which made SS2 be located in the more hydrophobic region of micelles. The reaction between SS2 and Na$_2$S in SDS was too slow (Fig. S9) to acquire a detection limit and a linear concentration range.

3.5. The selectivity and competition of the two probes towards H$_2$S over various analytes in CTAB–PBS system

Normally, biological samples are complex and they have relatively high salt concentrations, in which various anions and reducing species could potentially react with the probe and interfere in the detection of H$_2$S. Therefore, the selectivity and the competition are two important issues in biological application of the probe. We further investigated the selectivity of the two probes towards H$_2$S over some other 11 anions and 2 reducing agents (cysteine and glutathione). All the concentrations of the additives were higher than their anticipated concentrations in biological samples. Most of the anions/reducing agents did not induce obvious fluorescence response. The presence of HSO$_3^−$, S$_2$O$_3^{2−}$ and S$_2$O$_4^{2−}$ induced smaller fluorescence enhancements (4 times lower than that caused by sulfide, Fig. 6b and Fig. S10a), and the emission maxima were about 10 nm blue-shift compared to that caused by sulfide, which could be attributed to the different microenvironment surrounding the reduction product. The above results reveal that SS1 and SS2 are highly selective towards H$_2$S and can be used as specific probes for H$_2$S in aqueous solution. The obvious color change enables both probes for naked-eye detection (Fig. S3).

Fig. 7 and Fig. 10b show the effects of some anions/reducing agents on the detection of sulfide. No remarkable interference to H$_2$S determination was found in the presence of a large excess of these chemical species. Thus, SS1 and SS2 display excellent selectivity and competition for hydrogen sulfide in the presence of other ions and reducing agents encountered in biological samples.

3.6. Measurement of H$_2$S in fetal bovine serum

The selectivity, competition and linearity studies imply that SS1 and SS2 might be suitable for the detection of sulfide in
biologically relevant samples. Finally, we investigated the effect of H\textsubscript{2}S on the emission spectra of the probes in fetal bovine serum (FBS). FBS had a weak fluorescence peak at 515 nm, and its emission spectrum changed little upon the addition of the probes; further addition of H\textsubscript{2}S caused evident change in the fluorescence intensity, suggesting that the determination of H\textsubscript{2}S could be carried out directly in FBS without pretreatment. We then added Na\textsubscript{2}S into FBS containing SS\textsubscript{1} or SS\textsubscript{2} and measured the fluorescence after 10 min, and good linear dependence in the range of 1–200 \mu M was observed (Fig. 8). In the presence and absence of CTAB, the respective detection limits were 2 \mu M and 5 \mu M with SS\textsubscript{1} as the probe, and 0.5 \mu M and 2 \mu M with SS\textsubscript{2} as the probe. The above results indicate that SS\textsubscript{2} is more sensitive, and CTAB can also enhance the performance of the probes in the realistic sample. The reduced sensitivity in realistic sample was presumably due to the higher background of FBS. We speculate that design of new probe with longer emission wavelength or with ratiometric signal will lead to enhanced sensitivity in realistic samples. This work is in progress in our lab.

4. Conclusion

In conclusion, we have synthesized two colorimetric and turn-on fluorescent probes for selective and sensitive recognition of hydrogen sulfide. The reaction rate was significantly enhanced (reaction time shortened from 4 h in PBS to 1 min in CTAB–PBS) and the detection limit was about 20-fold lower (from 0.5 \mu M to 0.02 \mu M) in CTAB micelle system. The linear concentration range of hydrogen sulfide could be adjusted by using differently charged surfactants, and the overall linear range can cover five orders of magnitude. The probes were also used to rapidly and sensitively measure H\textsubscript{2}S levels in fetal bovine serum without any pretreatment of the sample. The obtained results reveal that the probes are well suited for in situ analysis of H\textsubscript{2}S concentrations in a variety of biological fluids.

Acknowledgement

This work was financially supported by National 973 Program (No. 2011CB910403), NSFC (No. 21075039) and Open Project Fund of Shanghai Key Laboratory of Scene Material Evidence (2011XCWZK02). We appreciate Prof. A.M. Brouwer (University of Amsterdam) for his kind help.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2013.01.030.

References

[41] The standard electrode potentials for $\varphi_{S^{2-}/S^{2-}}$ and $\varphi_{H_2S/H_2S^{2-}}$ are $-0.508$ V and $0.141$ V, respectively. This implies that the reducing ability of $S^{2-}$ is stronger than that of $H_2S$. pH influences the contents of different sulfide-containing species, and probably thus affects the reaction rate.