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An NBD fluorophore-based colorimetric and fluorescent chemosensor for hydrogen sulfide and its application for bioimaging

Guodong Zhou, Huilin Wang, Yang Ma, Xiaoqiang Chen *

State Key Laboratory of Materials-Oriented Chemical Engineering, College of Chemistry and Chemical Engineering, Nanjing University of Technology, Xinmofan Road 5#, Nanjing 210009, China

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ABSTRACT

Hydrogen sulfide (H_2S) is an important gaseous mediator in cellular physiology and pathology. For understanding the biological action of H_2S to both healthy and disease states, it is important to develop H_2S-selective sensor. Herein we report a colorimetric and fluorescent probe, which employs an NBD moiety as fluorophore, and is equipped with azide group as H_2S-active unit. The probe exhibits selective and sensitive response to H_2S in aqueous solution. By employing 1 μM sensor, the detection limit was evaluated to be 680 nM with a signal-to-noise ratio of 3. Confocal microscopy imaging experiments demonstrate the probe has potential as a powerful tool for the imaging of hydrogen sulfide in living cells.

1. Introduction

Hydrogen sulfide is considered as the third biologically active gas with the other two endogenous gasotransmitter: nitric oxide and carbon monoxide. Most endogenous hydrogen sulfide results from the metabolism of sulfhydryl-containing amino acids (e.g., cysteine) by bacteria present in both the intestinal tract and the mouth.[1] Hydrogen sulfide is also produced via enzymatic reaction in the brain and several smooth muscles.[2] Hydrogen sulfide play important roles in various physiological processes, such as vasodilation,[3] neurotransmission,[2a] apoptosis,[4] inflammation,[5] oxygen sensing,[6] inhibition of insulin signaling.[7] On the other hand, hydrogen sulfide was known as a toxic gas, and inhalation of high concentrations can lead to death in animals. Single, short-term and medium-term inhalation exposures to hydrogen sulfide have also resulted in respiratory, olfactory, cardiovascular, neurological, hepatic, and developmental neurochemical effects and abnormal growth in developing cells.[8] Therefore, it is highly important to develop an imaging probe for hydrogen sulfide with excellent selectivity and sensitivity that is applicable to various biological systems.

To date, several detection methods for hydrogen sulfide have been developed, such as gas chromatography,[9] electrochemical analysis,[10] colorimetric method,[11] metal-induced sulfide precipitation.[12] Although these methods are useful to monitor hydrogen sulfide in environmental samples, such as air, water, sediment, and sludge, they are hard to be applied for intracellular detection due to their limitations to in vivo studies. Fluorescent methods in conjunction with suitable probes are more desirable for the measurement of biological species since they are simple, sensitive, and can afford real information on the localization and quantity of the targets of interest.[13] In the past several years, a few fluorescent probes for hydrogen sulfide based on different mechanisms have been exploited, including hydrogen sulfide-induced reduction of azide to amine,[14] fluorescence recovery after copper dissociation from complex-fluorophore ensemble,[15] removal of 2,4-dinitrobenzenesulfonyl group,[16] cleavage of disulfide bond and H_2S-specific Michael addition–cyclization.[17]

In this work, we introduced azide group to 7-nitrobenz-2-oxa-1,3-diazole (NBD) fluorophore to construct a H_2S-selective sensor. NBD was chosen as the fluorophore because of long emission wavelength and good cell permeability. The azide-functionated NBD derivate was expected to react with H_2S, producing a strongly fluorescent amino-substituted NBD (Scheme 1). The NBD derivate **1** was synthesized as shown in Scheme 1. NBD-Cl was reacted with NaN_3 in EtOH to afford **1** in 18.5% yield. The experimental details and characterization data for **1** are given in Supplementary data.

2. Results and discussion

2.1. Absorbance and fluorescence studies in aqueous solution

To verify the selectivity of **1** to hydrogen sulfide, the absorbance and fluorescence studies in aqueous solution were carried out. The

* Corresponding author. Tel./fax: +86 25 8358 7856; e-mail address: chenxq@njut.edu.cn (X. Chen).

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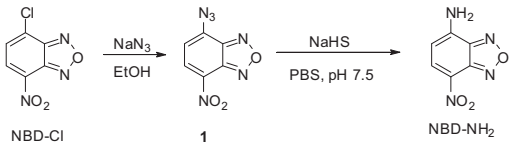

Scheme 1. Synthesis of sensors **1** and the reaction in the presence of H_2S.

fluorescence and absorbance changes that **1** undergoes upon the addition of various analytes were shown in Fig. 1. After adding 100 μM of anions, such as $S_2O_4^{2-}$, HS^-, $S_2O_3^{2-}$, $S_2O_5^{2-}$, HCO_3^-, HPO_4^-, F^-, Cl^-, Br^-, I^-, SO_4^{2-}, HSO_3^-, NO_3^-, $H_2PO_4^-$, OAc^-, and citrate to the PBS buffer (20 mM, pH 7.5, 1% CH_3CN) solution containing 10 μM of **1** for 5 min, only NaHS induced a remarkable fluorescence enhancement at 550 nm. The absorption spectrum also displays that the addition of NaHS lead to a red-shift of absorption peak from 396 nm to 468 nm. Accordingly, the addition of **1** produce a colorimetric change from pale-yellow to deep-yellow, which can be detected by naked-eye (Fig. S1). Interestingly, the mixing of $Na_2S_2O_4$ and **1** creates a blue-shift in maxium absorption wavelength from 396 to 329 nm (Fig. S2), accompanied by the color change from pale-yellow to colorless. It was reasoned that $Na_2S_2O_4$ as a strong reductant, destroyed the conjugated structure of NBD fluorophore, resulting in the fade of color and the blue-shift of absorption wavelength. Besides, other analytes including $Na_2S_2O_3$, $Na_2S_2O_5$, $NaHCO_3$, Na_2HPO_4, NaF, NaCl, NaBr, NaI, Na_2SO_4, $NaHSO_3$, $NaNO_3$, NaH_2PO_4, NaOAc, and sodium citrate show neglectable changes in fluorescence and absorbance spectra under the same conditions.

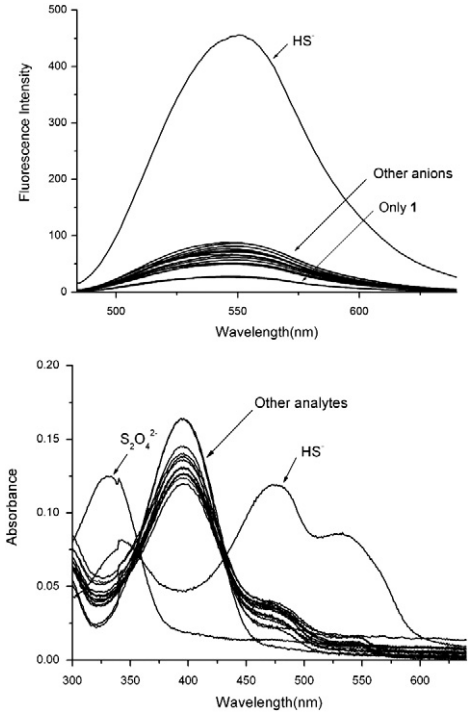

Fig. 1. Top: fluorescence spectra of **1** (10 μM) upon incubation with various analytes (100 μM) for 5 min in PBS buffer (20 mM, pH 7.5, 1% CH_3CN) (λ_{ex}=474 nm, slit: 5/5 nm). Bottom: UV/vis absorption spectra of **1** (10 μM) in PBS buffer (20 mM, pH 7.5, 1% CH_3CN) in the presence of 100 μM analytes including $S_2O_4^{2-}$, HS^-, $S_2O_3^{2-}$, $S_2O_5^{2-}$, HCO_3^-, HPO_4^-, F^-, Cl^-, Br^-, I^-, SO_4^{2-}, HSO_3^-, NO_3^-, $H_2PO_4^-$, OAc^-, citrate.

The changes in the fluorescence spectra of probe **1** (10 μM) in the absence or presence of NaHS (0–60 μM) in PBS buffer are displayed in Fig. 2. The probe **1** is weak-fluorescent in the absence of NaHS; however, the addition of NaHS caused a dramatic change in the fluorescence spectra. A strong new emission peak at 550 nm

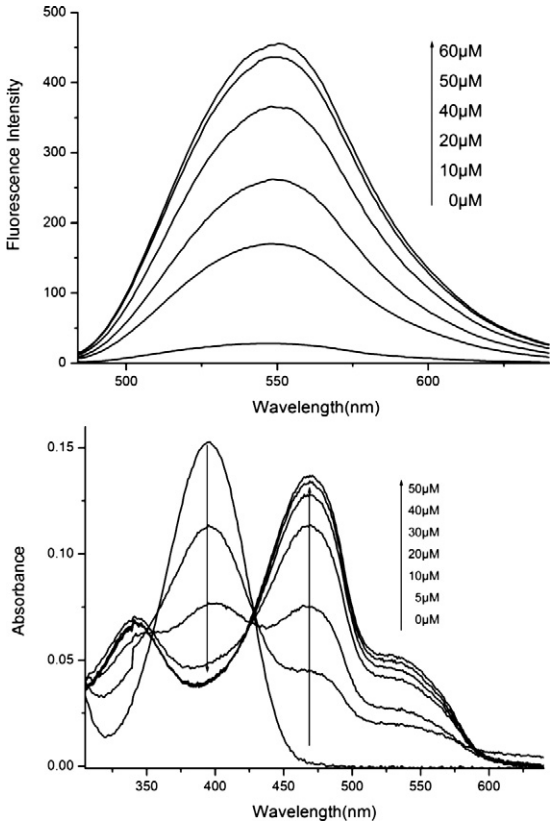

Fig. 2. Fluorescence (top) and absorption spectra (bottom) of **1** (10 μM) in the presence of various concentrations of NaHS (0, 5, 10, 20, 30, 40, 50 and 60 μM) in PBS buffer (20 mM, pH 7.5, 1% CH_3CN) (λ_{ex}=474 nm, slit: 5/5 nm). Each spectrum is recorded at 5 min after addition of NaHS to **1**.

appeared, and an enhancement of the fluorescence intensity by up to 16-fold was observed upon addition of 6 equiv of NaHS. Concomitantly, a gradual decrease of the absorption peak at 396 nm and a progressive increase of a new absorption band at around 468 nm by addition of NaHS were observed (Fig. 2). A well-defined isosbestic point was noted at 428 nm, which may indicate the formation of a new product upon treatment of **1** with NaHS.

The time-dependent fluorescence responses were monitored at 550 nm (Fig. 3, top), and the results show that the reaction is complete within 3 min when 6 equiv of NaHS was mixed with **1**. To investigate the detection limit of **1** for hydrogen sulfide, **1** (1 μM) was treated with various concentrations of NaHS (0–700 nM). The fluorescence intensity at 550 nm was plotted as a function of the NaHS concentration. The fluorescence intensity of **1** is linearly proportional to NaHS concentrations of 0–700 nM, and as low as 680 nM concentration of HS^- was detected by using **1** with a signal-to-noise ratio of 3 (Fig. 3, bottom).

The fluorescence intensity changes of **1** induced by HS^- were measured at various pHs for investigating the effect of pH on the fluorescence response. As shown in Fig. 4 (top), in the absence of NaHS, the fluorescence of **1** was weak in buffers at various pH values from 4.5 to 10. When NaHS was added to the solution containing **1**, strong fluorescence enhancement was detected at pH 6.5 to 9, which indicated that **1** enable to detect NaHS within a wide pH range.

2.2. Sensing mechanism

On the detection mechanism, the fluorescence enhancement and UV–vis spectral change should be attributed to the reduction

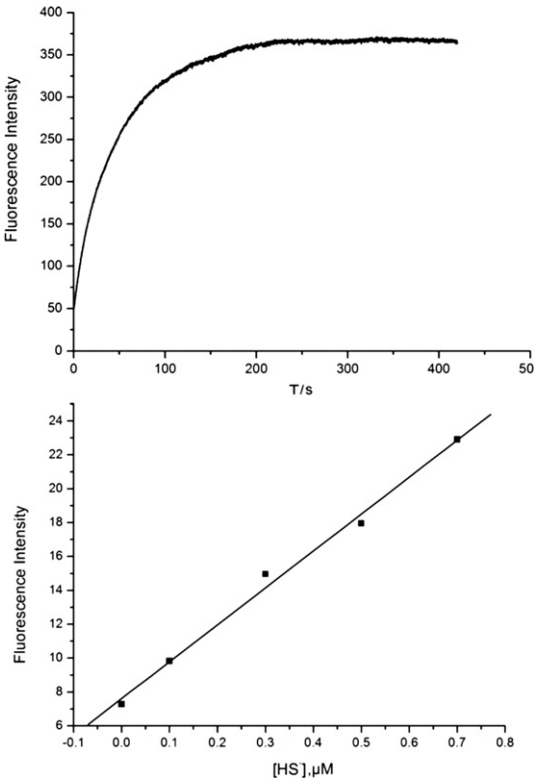

Fig. 3. Top: time-dependent fluorescence intensity of **1** at 10 μM in the presence of 60 μM hydrogen sulfide. Bottom: the change in the fluorescence intensity of **1** at 1 μM against varied concentrations of hydrogen sulphide in CH_3CN–PBS buffer (20 mM, pH 7.5) (1:99, v/v).

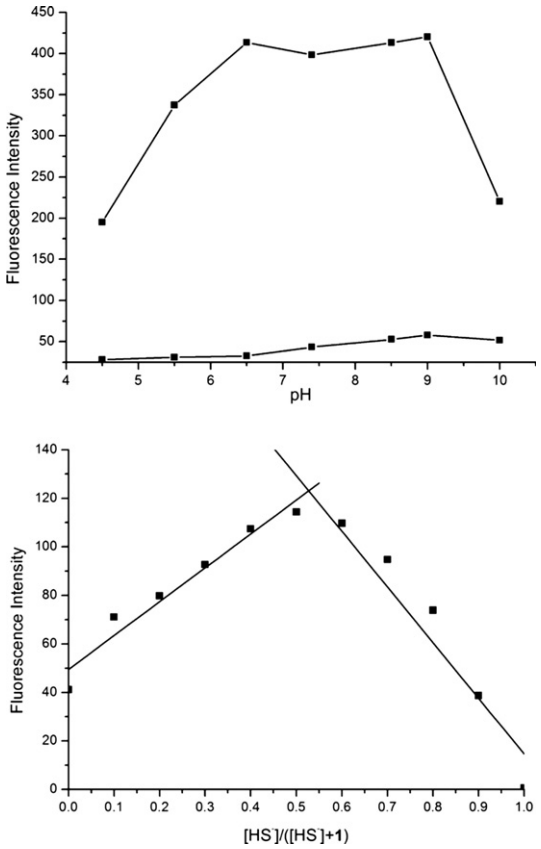

Fig. 4. Top: the fluorescence intensity of **1** (10 μM) at 550 nm in the presence and absence of hydrogen sulfide in CH_3CN–H_2O system with different pHs. (NaH_2PO_4 buffer was used for pH 4.5 and 5.5, MOPS buffer for pH 6.5, HEPES buffer for pH 7.4, Tris–HCl buffer was used for pH 8.5, CHES buffer was used for pH 9.0 and CAPS buffer was used for pH 10.0.) Bottom: Job's plot of the reaction between **1** and hydrogen sulfide in CH_3CN–PBS (20 mM, pH 7.5, 1:99, v/v) solutions at pH 7.5. Total concentration of **1** and hydrogen sulfide was kept constant at 10 μM.

reaction between hydrosulfide anion and azide group in the probe, which afford the amino substituted NBD fluorescein (Scheme 1). To examine this plausible mechanism, the stoichiometry of a reaction event between NaHS and **1** was initially determined. The results obtained from Job's plot show the 1:1 stoichiometry for the reaction between **1** and NaHS (Fig. 4, bottom). NMR analysis of a product NBD–NH_2 obtained from the reaction of **1** with NaHS also supports the formation of amino substituted NBD (Fig. S3 and 4). The peak at 181.0364 corresponding to the resulting product $[NBD–NH_2+H^+]^+$ was clearly observed in the mass spectra (Fig. S5).

2.3. Living cell imaging

In order to further demonstrate that the permeability and the monitoring of hydrogen sulfide in living cells, confocal microscopy experiments were carried out. When MCF-7 cells were incubated with **1** (20 μM), only weak fluorescence was exhibited inside the cells (Fig. 5, top). However, in a control experiment, cells were pretreated with **1** (20 μM), followed by treatment with an excess (150 μM) of NaHS, a resource of hydrogen sulfide. The confocal microscopic images show a significant fluorescence signal (Fig. 5, bottom). This confirms that **1** can be used as the imaging agent for hydrogen sulfide in living cells.

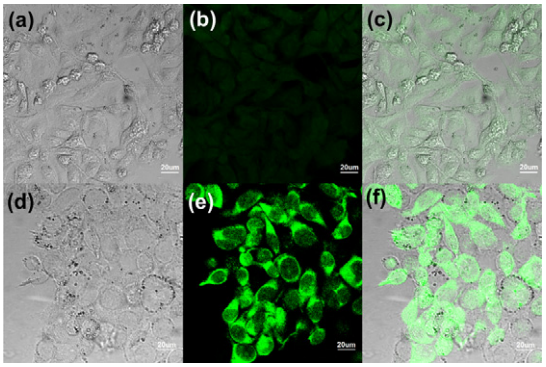

Fig. 5. Phase contrast and fluorescence images of MCF-7 cells. Top: PC-12 cells were treated with 20 μM of **1** for 30 min; Bottom: PC-12 cells were pre-incubated with 20 μM of **1** for 30 min and then treated with 150 μM of NaHS for 30 min (a and d, phase contrast images; b and e, fluorescence images; c and f, merged images).

3. Conclusions

In summary, we have developed a new NBD fluorophore-based colorimetric and fluorescent chemosensor for the detection of hydrogen sulfide with high selectivity and sensitivity. The fluorescence enhancement and UV–vis spectral changes are attributed to reduction of azido group to amine. Confocal microscopy imaging suggested that the present probe has potential as a powerful tool for the imaging of hydrogen sulfide in living cells.

4. Experimental

4.1. Materials and equipments

Unless otherwise noted, materials were obtained from commercial suppliers and were used without further purification.

Chromatography was carried out on silica gel 60 (230–400 mesh ASTM). ^{1}H NMR and ^{13}C NMR spectra were recorded using Bruker 300 or Bruker 500. Mass spectra were obtained using a Waters Micromass Q-Tof micro mass spectrometer. Fluorescence emission spectra were obtained using RF-5301/PC Spectrofluorophotometer. UV absorption spectra were obtained on α-1860A UV–vis Spectrometer. All pH measurements were made with Sartorius PB-10 meter. The IR spectra were obtained using Nicolet 8700 FT-IR Spectrometer.

4.2. Synthesis

Synthesis of sensor 1. A suspension of NBD–Cl (185.6 mg, 0.93 mmol) in 15 mL of EtOH was added dropwise into a stirred solution of sodium azide (130 mg, 2 mmol) in 7 mL of a mixed solvent (H_2O/EtOH, 1:1). Then the reaction mixture was stirred at room temperature for 3 h. The organic solvent was evaporated in vacuum, and the aqueous solution was extracted by CH_2Cl_2. The combined organic layers was washed with brine and then dried over $MgSO_4$ (3 h). Solvent evaporation gave the crude product, which was purified by column chromatography to give **1** (35.5 mg, 18.5%) as a dark yellow solid. Mp 99–100 °C; ^{1}H NMR ($CDCl_3$, 300 MHz) δ (ppm): 8.54 (d, 1H, J=9.0 Hz, ArH), 7.09 (d, 1H, J=9.0 Hz, ArH); ^{13}C NMR ($CDCl_3$, 75 MHz) δ (ppm): 145.87, 143.53, 138.06, 132.09, 114.87; elemental analysis calcd (%) for $C_6H_2N_6O_3$: C 34.96, H 0.98, N 40.77; found: C 34.83, H 1.19, N 40.66; IR ν_{max} (KBr) 3101.1, 2143.8, 2119.4, 1632.6, 1537.2, 1449.8, 1375.4, 1340.9, 1324.1, 1278.5, 1209.0, 1120.1, 1071.8, 1056.0, 1017.2, 993.6, 890.6 cm^{-1}.

The confirmation of the product NBD–NH_2 from the reaction between 1 *and* H_2S. A solution of sodium hydrosulfide (67.2 mg, 1.2 mmol) in 2 mL H_2O was added into a stirred solution of **1** (180 mg, 1 mmol) in 40 mL acetonitrile. The reaction mixture was stirred 3 h at room temperature. Then the organic solvent was evaporated in vacuum, and the aqueous solution was extracted by CH_2Cl_2. The combined organic layers gave the crude product, which was purified by column chromatography to give NBD-NH_2 (30 mg, 16.7%). Mp >200 °C; ^{1}H NMR (DMSO, 300 MHz) δ (ppm): 8.85 (s, 2H, NH_2), 8.49 (d, 1H, J=9.0 Hz, ArH), 6.39 (d, 1H, J=9.0 Hz, ArH); ^{13}C NMR (DMSO, 125 MHz) δ (ppm): 147.24, 144.24, 144.03, 137.86, 120.49, 102.59; ESI-MS m/z=180.0364 $[M+H]^+$, calcd for $C_6H_5N_4O_3$=181.0362; IR ν_{max} (KBr) 3433.7, 3342.5, 3235.7, 2360.3, 2341.9, 1650.0, 1619.7, 1557.5, 1531.3, 1505.3, 1447.3, 1422.5, 1386.5, 1318.0, 1290.7, 1125.2, 1042.1, 994.2 cm^{-1}.

4.3. Absorbance and fluorescence studies in aqueous solution

Stock solutions (10 mM) of analytes including $Na_2S_2O_4$, NaHS, $Na_2S_2O_3$, $Na_2S_2O_5$, $NaHCO_3$, Na_2HPO_4, NaF, NaCl, NaBr, NaI, Na_2SO_4, $NaHSO_3$, $NaNO_3$, NaH_2PO_4, NaOAc, sodium citrate in distilled water were prepared. Stock solution of probe **1** (1 mM) was also prepared in acetonitrile. In a typical experiment, test solution was prepared by placing 30 μL of the probe stock solution into a test tube, diluting the solution to 3 mL with PBS buffer (20 mM, pH 7.5), and adding an appropriate volume of each analyte stock. Normally, excitation was at 474 nm, and both the excitation and emission slit widths were 5 nm. Fluorescence spectra were measured after the addition of analyte for 5 min. For low concentration titration of hydrogen sulfide, fluorescence spectra were measured after addition of hydrogen sulfide for 5 min, and both the excitation and emission slit widths were 5 nm.

4.4. Cell culture and fluorescence imaging

MCF-7 cells were purchased from American Type Culture Collection (ATCC, USA), and were seeded in Laser scanning confocal microscope (LSCM) culture dishes with a density of 5×10^5 cells/well. The cell lines were cultured in RPMI-1640 medium supplemented with 10% (v/v) calf serum, penicillin (100 U mL^{-1}) and streptomycin (100 mg mL^{-1}). Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. When the whole cells took up 70–80% space of culture dishes, the cells were first incubated with 20 μM of **1** in culture media for 30 min at 37 °C. Then the cells were further treated without or with 150 μM hydrogen sulfide in culture media for 30 min at 37 °C. After washing with phosphate buffered saline (PBS) to remove the remaining hydrogen sulfide, the cells were imaged by confocal laser scanning microscopy (Olympus FV-1000).

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Supplementary data

These data include characterization of **1** and **2**, mass spectra of **2**, absorption spectra of **1** (10 μM) in the presence of $Na_2S_2O_4$. Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.tet.2012.10.106.

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