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A turn-on fluorescent probe for hydrogen sulfide and its application in living cells

Miao Zhao,[a] **Hua Li,**[b] **Hui Li,**[b] **Qinglong Qiao,**[a] **Cheng Cao,**[a] **Zhaochao Xu**[a],*

Hydrogen sulfide (H_2S) is an important endogenous signalling molecule in cellular physiology and pathology. Accordingly, sensitive, selective and reliable methods for H_2S detection are in high demand. Fluorescence-based methods thus have received great attentions in recent years. Herein we describe the design, synthesis and application of a probe for H_2S detection based on the reduction of azide to amine. This probe is highly sensitive and selective toward H_2S over biothiols and other biologically relevant species. Finally, the probe 10 was successfully applied for H_2S fluorescent imaging in living cells.

Introduction

Hydrogen sulfide (H_2S) has been known as a toxic pollutant for many years. However, it now emerges as the third most important gasotransmitter following nitric oxide (NO) and carbon monoxide 15 (CO).[1,2] The endogenous H_2S is biosynthesized from cysteine and homocysteine by three enzymes: cystathionine-γ-synthase (CBS), cystathionine-β-lyase (CSE), and 3-mercaptopyruvate sulfur transferase (3-MST).[3,4] The significance of H_2S has been validated in various physiological processes, such as 20 vasodilation,[5,6] antioxidation, anti-apoptosis and anti-inflammation.[7,8] H_2S has been revealed to relax vascular smooth muscle, induce vasodilation of isolated blood vessels, and reduce blood pressure.[9-11] Additionally, H_2S also performs as an antioxidant or a scavenger of reactive oxygen and nitrogen 25 species in cells.[12] And the abnormal H_2S level is believed to be related with some diseases like Alzheimer's disease,[13] Down's syndrome,[14] diabetes[15] and liver cirrhosis.[16] Thus, visualization of H_2S in living cells seems to be great important and helpful.

Fluorescence bioimaging technology is one of the most 30 attractive molecular imaging methods for *in vivo* detection of biomolecules by its several advantages, such as high sensitivity, excellent selectivity, rapid response, non-invasiveness, and aptness for living cells, tissues, and living model animals.[17-20] As we know, H_2S is a reductant as well as a nucleophilic agent, 35 which can specifically react with some probes. We classify these probes according to the reaction types with H_2S. a) H_2S reductive reactions: reducing azides or nitro to amines. The pioneering reduction of azide to amine in fluorescent probes were reported by Chang[21] and Wang[22] in nearly the same time. Since then, 40 several probes have been developed based on this concept by changing the fluorophores.[23-29] b) H_2S nucleophilic reactions: Michael addition reaction, and tandem nucleophilic reaction. Xian constructed a H_2S probe through a nucleophilic substitution reaction between H_2S and the disulfidemoiety.[30] He reported a 45 probe which sensing H_2S in cells through the tandem nucleophilic

reaction of H_2S with the aldehyde and α,β-unsaturated acrylate methyl ester.[31] Lin constructed new NIR probes based on thiolysis of dinitrophenyl ether.[32,33] c) copper-sulfide precipitation reaction. Nagano reported a fluorescent probe by 50 utilizing azamacrocyclic copper(II) complex for H_2S detection.[34] Shen constructed a red fluorescent probe based phenanthrene fused dipyrromethene copper(II) complex.[35]

In this paper, we develop a new turn-on fluorescent probe for sensing H_2S based on NBD fluorophore. The probe can be easily 55 synthesized from 4-amino-7-nitro-2, 1, 3-benzoxadiazole (NBD-NH_2) (Scheme 1). When introducing the electron withdrawing group carbamate, the electron density of NBD-NH_2 was efficiently decreased, which resulted in a considerably turn-off effect of fluorescence intensity compared to NBD-NH_2. While 60 the azide group reduced to amino by H_2S, the *p*-aminobenzy moiety undergo a self-immolate through an intramolecular 1, 6-elimination to release NBD-NH_2, and the fluorescence would be recovered. The probe exhibits high fluorescence enhancement, relatively fast response and excellent selectivity toward H_2S. 65 Finally, the probe was successfully applied to image H2S in living HT-29 cells.
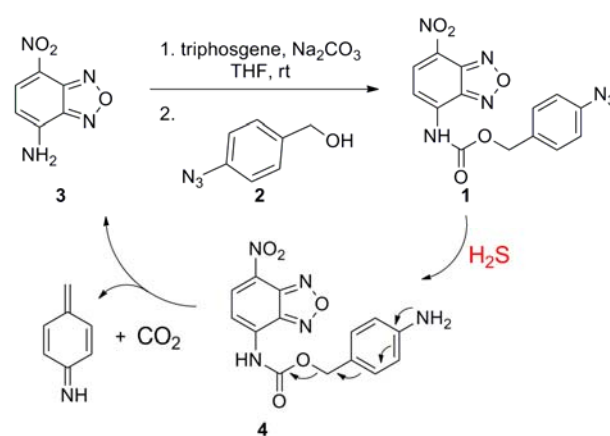
Scheme 1. Proposed detection mechanism of **1**.

Experimental section

Materials and methods

Unless otherwise noted, materials were obtained from Aldrich and were used without further purification. The synthesis of
5 compound 4-amino-7-nitro-2, 1, 3-benzoxadiazole (NBD-NH$_2$) (**3**) was according to the published procedure.[36] Melting points were measured using a Büchi 530 melting point apparatus. ^{1}H NMR and ^{13}C NMR spectra were recorded using Bruker 400 MHz and 100 MHz, UV-Vis absorption spectra were obtained on
10 Agilent Cary 60 UV-Vis Spectrophotometer. Fluorescence emission spectra were obtained using Cary Eclipse Fluorescence Spectrophotometer.

Synthesis and characterization of probe 1

To a mixture of Na$_2$CO$_3$ (636 mg, 6.0 mmol, 3.0 eq) and
15 triphosgene (700 mg 2.4 mmol, 1.2 eq) in THF (10 mL), a solution of **3** (360 mg, 2.0 mmol) in THF (5 mL) was added dropwise in an ice bath for 1 h. Then the solution was stirred for additional 6 h at room temperature. The reaction solution was then filtered. Compound **2** (357 mg, 2,4 mmol, 1.2 eq) was added
20 to the filtrate, and the solution was stirred at room temperature for 6 h. The solvent was evaporated and the crude product was purified by silica gel column chromatography (PE:DCM = 1:1) to afford the final compound **1** as a light yellow solid (220 mg, 31 %); M.p. 164-166 °C. ^{1}H NMR (400 MHz, CDCl$_3$) δ 8.56 (d, J
25 = 8.4 Hz, 1H), 8.15 (d, J = 8.4 Hz, 1H), 8.03 (s, 1H), 7.44 (d, J = 8.4 Hz, 2H), 7.07 (d, J = 8.4 Hz, 2H), 5.29 (s, 2H). ^{13}C NMR (100 MHz, CDCl$_3$) δ 151.8, 144.8, 143.0, 141.0, 134.0, 133.7, 131.2, 130.6, 130.4, 119.4, 111.1, 68.2. HRMS (ESI) calcd for C$_{14}$H$_{10}$N$_7$O$_5$ [MH+] 356.0743, found 356.0747.

30 ### Culture of HT-29 cells and fluorescent imaging

HT-29 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% CO$_2$ and 95% air at 37 °C. The cells were seeded in 24-well flat-bottomed plates and then
35 incubated for 48 h at 37 °C under 5% CO$_2$. **1** (5 μM) was then added to the cells and incubated for 30 min. Then the cells were washed three times with PBS. After incubating with 20 μM H$_2$S, fluorescence imaging was observed under a confocal microscopy (Olympus FV1000) with a 60×objective lens.

40 ## Results and discussion

Characterization of fluorescent probe 1 for H$_2$S recognition

Absorbance and fluorescence studies in aqueous solution (CH$_3$CN:PBS = 9 : 1, pH = 7.4) were performed firstly. The changes of the absorption spectra in the absence and presence of
45 NaHS (a commonly used hydrogen sulfide source) (0-20 eq) were displayed in Fig. 1. The probe **1** showed an absorption band at 520 nm. When we added NaHS to the solution of probe **1**, the absorbance at 520 nm decreased sharply to its limiting value, while an absorption band at 470 nm developed, which was visible
50 to the naked eye with a clear colour change from pink to yellow. Additionally, if the concentration of NaHS reaches 20 equiv., the reaction can be completely finished in minutes.
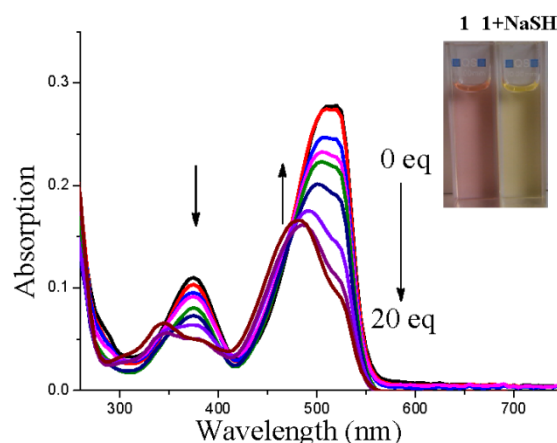

Fig. 1. UV-Vis absorption spectra of 10 μM probe **1** in the presence of 0-20 equiv of H$_2$S in aqueous solution (CH$_3$CN:PBS = 9:1, 20 Mm PBS, pH
55 = 7.4). Inset: cuvette images of probe **1** before and after addition of H$_2$S taken under ambient light.

The emission spectra and fluorescence titration experiments of probe **1** with H$_2$S were then recorded in aqueous solution (CH$_3$CN:PBS = 9:1, pH = 7.4) (Fig. 2). The free probe **1**
60 displayed quite weak fluorescence. When H$_2$S was added progressively from 0 to 20 equiv to the solution of probe **1**, the probe was converted efficiently to fluorescent NBD-NH$_2$. And the fluorescence intensity of the emission band at 550 nm increased significantly. From Fig. 2b, it was found that if the
65 concentration of H$_2$S was over 20 equiv, the reaction can be finished in minutes. Therefore, the time-dependent fluorescence responses were next detected with 20 equiv of H$_2$S from 0 to 55 min, and the results showed that the reaction was completed within 50 min (Fig. 2c-2d).
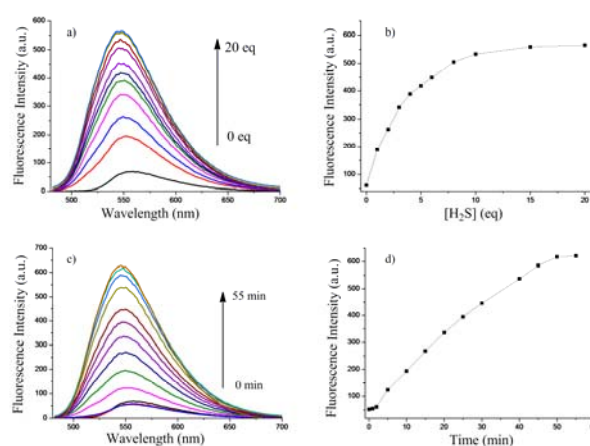
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Fig. 2. a) Fluorescent emission spectra of 10 μM probe **1** in the presence of 0-20 equiv of H$_2$S in aqueous solution (CH$_3$CN:PBS = 9:1, pH = 7.4) (NaHS was dissolved in water in the concentration of 1 mM). Excitation at 470 nm. b) Fluorescence intensity of 10 μM probe **1** in the presence of
75 0-20 equiv of H$_2$S. c) Time dependence of fluorescence profiles of probe **1** (10 μM) with 20 equiv H$_2$S. d) Time dependence of fluorescence intensity of probe **1** (10 μM) at 550 nm with 20 equiv H$_2$S.

In addition, the formation of compound NBD-NH$_2$ was confirmed by ^{1}H-NMR. The cleavage reaction of probe **1** with 20

equiv H₂S revealed the characteristic NMR peaks almost the same as the NBD-NH₂ (Fig. 3). The transformation of **1** to NBD-
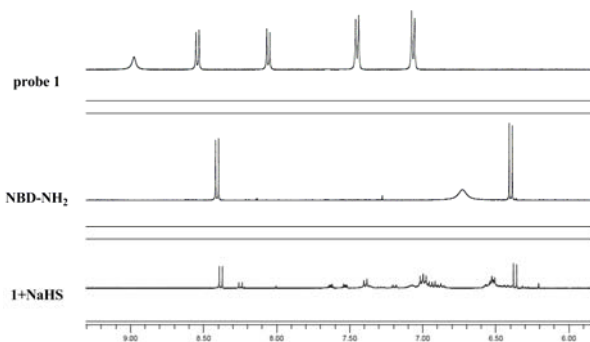
NH₂ was also confirmed by TLC experiments.

Fig 3. Partial ¹H NMR spectra of **1**, NBD-NH₂ and the reaction mixture of
5 **1** with H₂S in CD₃CN.

The selectivity of the fluorescent response of **1** with various anions, sulfur-containing inorganic salts and biothiols including cysteine, homo-cysteine and glutathione were then conducted. The addition of 50 equiv. of F⁻, Cl⁻, Br⁻, I⁻, CO₃²⁻, HCO₃⁻, OAc⁻,
10 NO₃⁻, NO₂⁻, OH⁻, citric acid, hydrogen citrate, dihydrogen citrate, N₃⁻, PO₄³⁻, HPO₄²⁻, H₂PO₄⁻, P₂O₇⁴⁻, SCN⁻, SO₃²⁻, HSO₃⁻, SO₄²⁻, HSO₄⁻S₂O₃²⁻, S₂O₄²⁻, S₂O₅²⁻ produced only a nominal change in the fluorescence spectra of **1**. In addition, both biothiols (50 eq Cys, Hcys and GSH, the main competitive species in
15 biological systems) and reducing conditions ascorbate acid (50 eq.) showed no response to probe **1**. By contrast, only H₂S shows a dramatic fluorescent enhancement, suggesting very high selectivity of **1** towards H₂S (Fig. 4).
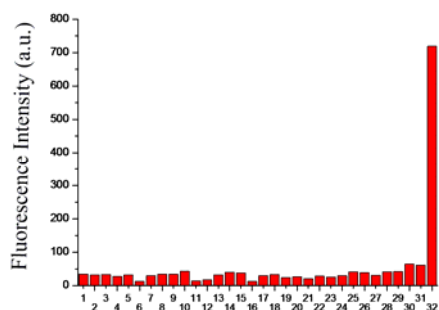

Fig. 4. Fluorescence responses of 10 μM **1** to various analytes in aqueous
20 solution (CH₃CN:PBS = 9:1, pH = 7.4). Excitation at 470 nm. Bars represent the final fluorescence intensity of **1** with 0.5 mM analytes over the original emission of free **1**. 1) free probe **1**; 2) F⁻, 3) Cl⁻, 4) Br⁻, 5) I⁻, 6) CO₃²⁻, 7) HCO₃⁻, 8) OAc⁻, 9) NO₃⁻, 10) NO₂⁻, 11) OH⁻, 12) citric acid, 13) hydrogen citrate, 14) dihydrogen citrate, 15) N₃⁻, 16) PO₄³⁻, 17) HPO₄²⁻,
25 18) H₂PO₄⁻, 19) P₂O₇⁴⁻, 20) SCN⁻, 21) SO₃²⁻, 22) HSO₃⁻, 23) SO₄²⁻, 24) HSO₄⁻, 25) S₂O₃²⁻, 26) S₂O₄²⁻, 27) S₂O₅²⁻, 28) Cys, 29) GSH, 30) Hcy, 31) Ascorbic acid, 32) HS⁻.

We also performed the competition experiments in the presence of biothiols, ascorbate acid and H₂O₂. In fact, when H₂S

30 and these analytes coexisted, almost the same fluorescence enhancement was observed as that only treated with H₂S (Fig. 5).
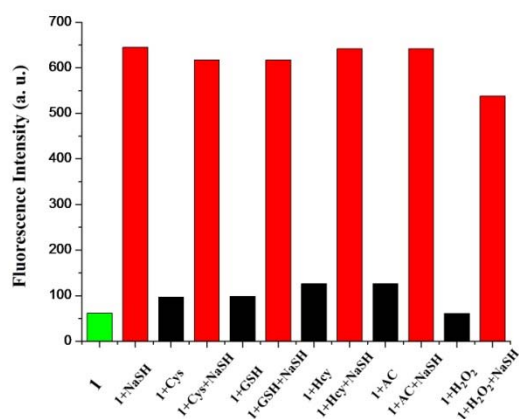

Fig. 5. Fluorescence response of **1** (10 μM) to NaHS (200 μM) and other reactive species, Cys (0.5 mM), GSH (0.5 mM), Hcy (0.5 mM), AC (0.5
35 mM), H₂O₂ (0.5 mM).

Encouraged by the above results, we next sought to apply probe **1** to image H₂S in living cells. Analysis of human colon cancer biopsies and patient-matched normal margin mucosa revealed the selective up-regulation of the H₂S-producing enzyme
40 cystathionine-β-synthase (CBS) in colon cancer, resulting in an increased rate of H₂S production. Then, HT-29 cells was selected to incubate with probe **1**. The cytotoxicity of probe **1** was first examined toward HT-29 cells by a MTT assay (Fig S6). The results showed that >90% HT-29 cells survived after 24 h (5.0
45 μM probe **1** incubation), demonstrating that **1** was of low toxicity toward cultured cell lines. When incubated with 5 μM **1** for 30 min at 37 °C, the cells were washed with PBS (pH = 7.4) to remove excess of **1**. The HT-29 cells exhibited almost no fluorescence as shown in the confocal image Fig. 6a, indicating
50 that biothiols and other biological species showed no interference. While after incubating with 20 μM NaHS for 20 min, the cells displayed enhanced green fluorescence (Fig. 6b). Another 20 min later, a higher turn-on fluorescence response was observed (Fig. 6c). All these experiments indicated that the probe **1** has the
55 potential biological application for imaging H₂S in living cells.
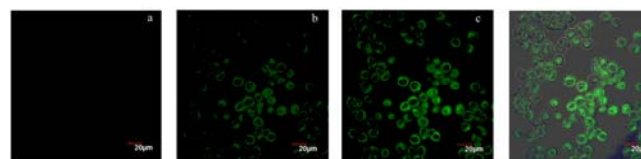

Fig. 6 Images of H₂S detection in HT-29 cells incubated with **1** (5 μM) at 37 °C. a) HT-29 Cells were incubated with **1** for 30 min; After which 20μM H₂S was added, further incubation for 20 min (b) and 40 min (c); d) merged images of c) and bright field.

60 **Conclusion**

In summary, we have reported a reaction-type fluorescent probe **1** for detection of H₂S in aqueous solution based on a specific analyte-induced cleavage of carbamate. This new probe

can be easily prepared and works effectively at physiological pH, which shows high selectivity for H2S over other biological thiols (such as Cys, Hcys and GSH). Concomitantly, the solution colour changes from pink to yellow. This probe can also detect H2S in living cells. The successful application of our probe to detect cellular H2S will help us to study the biological role of H2S and encourage the appearance of new H2S probes suitable for cell imaging.

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Notes and references

[a] *Key Laboratory of Separation Science for Analytical Chemistry of CAS, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, China.*
[b] *Hebei Vocational & Technical College of Building Materials, Hebei, China.*

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‡Footnotes should appear here.

Corresponding Author

E-Mail: zcxu@dicp.ac.cn;Tel: +86-411-84379648. Fax: +86-411-84379648

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