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A Fluorescent Turn-on H$_2$S-responsive Probe: Design, Synthesis and Application

Yufeng Zhang,$^{1a}$ Haiyan Chen,$^{1b*}$ Dan Chen,$^b$ Di Wu,$^b$ Xiaqiang Chen,$^c$ Sheng Hua Liu$^{*a}$ and Jun Yin$^{*a}$

Hydrogen sulfide (H$_2$S) is considered as the third signaling molecule in vivo and it plays an important role in various physiological processes and pathological processes in vivo, such as vasodilation, apoptosis, neurotransmission, ischemia/reperfusion-induced injury, insulin secretion and inflammation. Developing a highly selective and sensitive method that can detect H$_2$S in biological system is very important. In this work, a colorimetric and “turn-on” fluorescent probe is developed. Furthermore, this probe displays a highly selective response to H$_2$S in aqueous solution and possesses a good capability for bioimaging H$_2$S without interferences in living cells. The results suggest that H$_2$S-selective probe has good water-solubility, biocompatibility and cell-penetrability and can be served as an efficient tool for probing H$_2$S in cell level.

Introduction

Hydrogen sulfide is considered as the third signaling molecule in vivo as well as nitric oxide and carbon monoxide. Endogenous H$_2$S is primarily produced with the catalysis by enzymes such as cystathionine b-synthetase (CBS), cystathionine c-lyase (CSE) and 3-mercaptopyruvate sulphurtransferase (3-MST). On the one hand, H$_2$S plays some important roles in various physiological processes and pathological processes in vivo, such as vasodilation, apoptosis, neurotransmission, ischemia/reperfusion-induced injury, insulin secretion and inflammation. On the other hand, as a poisonous gas with rotten egg smell, high concentrations of H$_2$S can also harm the human body, showed symptoms of central nervous system and suffocation, and even lead to death. Therefore, developing a highly selective and sensitive method that can detect H$_2$S and be applied in biological system is very significant.

Traditional methods such as gas chromatography, electrochemical analysis, colorimetric method, metal-induced sulfide precipitation have been applied in the detection of H$_2$S. However, these methods usually suffer from the limitation in that they can only be used to detect H$_2$S in vitro. As mentioned above, H$_2$S as the signaling molecule in biosome participates in many biological functions. Therefore, developing the efficient approach suitable for the detection in microenvironment such as living cells is very significant. Due to its simplicity, high sensitivity and high selectivity, fluorescent probe technology has become an effective tool in the detection of intracellular H$_2$S in recent years. Numerous H$_2$S-responsive fluorescent probes based on different mechanisms have been widely developed, including redox reaction, nucleophilicity cyclization, and metal sulfide formation.

In the field of environmental sensitive bioimaging, the turn-off fluorescent probes usually involve in some confusion especially for those interferences such as the environment, probe activity, biocompatibility, cellular penetration also result in the fluorescence quenching, except for the detected species. While a turn-on fluorescent probe can overcome these disadvantage. As described above, many H$_2$S-responsive fluorescent probes have been reported, but the same trouble also exists in the design of H$_2$S probes. Herein, we present an efficient strategy for designing the turn-on fluorescent probes, in which a H$_2$S-responsive fluorescent probe is designed and applied for the detection of H$_2$S in living cells.

Results and discussion

Design and synthesis

Over the past years, the mechanism of nucleophilic reaction has been commonly used to design the H$_2$S probes owing to its strong nucleophilic reactivity. Whether the probe is turn-on after treating with H$_2$S depends on the fluorescent emission of cleavage product. If the cleavage product is fluorescent, this probe would be a turn-on fluorescent probe. Accordingly, we propose this design strategy of turn-on fluorescent probe, as presented in Figure 1. In which, a fluorescent compound A is employed as a cleavage product to react with compound B, affording a non-fluorescent or weak fluorescent probe A-B. Upon the treatment of H$_2$S, the nucleophilic substituted reaction takes place, generating the corresponding nucleophilic substitution product B-SH and the cleavage product A. For fluorescent backbone A, it plays dual roles of cleavage product and leaving group while moiety B is required to be an electron-deficient system for meeting the needs of nucleophilic substituted reaction.

In previous work, we found that dansylpiperazine (DP) is a fluorescent moiety with the fluorescence peak at 552 nm under the excitation with 330 nm. In this work, compound DP can be
Scheme 1 Structure of the H$_2$S-responsive turn-on fluorescent probe DP-NBD

Figure 2 (A) Absorption spectra of DP-NBD (10 µM) in the presence of different anions (10 equiv) such as Cys, Hcy, GSH, Ala, Tyr, Lys, Glu, Ser, His, Arg, Met, Gly, HSO$_4^-$, S$_2$O$_3^{2-}$, SO$_2^{2-}$, F, Cl, Br, I, HPO$_4^{2-}$, HSO$_4^-$, NO$_3^-$, ClO$_4^-$, OAc$, CN^-$, NO$_2^-$ and H$_2$S. The UV/Vis absorption response of DP-NBD towards various guests is presented in Figure 2A. Only the addition of H$_2$S to the solution of DP-NBD leads to an obvious red-shift of the second absorption peak. Accordingly, the real optical image shows the color of the solution changes from colorless to pink after H$_2$S is introduced, while other anions and amino acids display few changes, as shown in Figure 2B. The result suggests this probe has good water-solubility, and the UV/Vis absorption spectra strongly indicates that DP-NBD can be used as a colorimetric chemosensor for detecting H$_2$S in aqueous solution.
Next, we investigate the fluorescence behavior of DP-NBD towards the guests described above. The fluorescent responses to various tested guests in a buffer solution of HEPES (pH=7.4, 0.02 M) containing 10% DMSO are exhibited in Figure 3A. Similarly, upon the addition of H₂S, a demonstrable red-shift of the emission peak from 545 nm to 594 nm is observed with an around 5-fold enhancement of the fluorescent intensity under the same condition, whereas no significant response is detected when other guest is added (Figure 3B). As a result, an obvious fluorescent change from “off” to “on” can be observed in Figure 3C. Investigation on the interference from other species suggests DP-NBD has high selectivity for H₂S, as shown in Figure 3D. The results of an experiment probing the time dependent fluorescence response of DP-NBD (10 µM) to H₂S (0-30 equiv.) in a buffer solution of HEPES (pH=7.4, 0.02 M) containing 10% DMSO are exhibited in Figure S3 in ESI. A significant fluorescence enhancement appears as the amount of H₂S increases, and the fluorescence peak intensity changes of DP-NBD correlated well with the concentration of H₂S (Figure S3 in ESI). The sensitivity of DP-NBD for H₂S can be calculated on the basis of the linear relationships between the maximum emission intensity at 595 nm and the concentration of H₂S (Figure S3 in ESI). The probe has a detection limit of 7×10⁻⁸ M, based on 3σ/K, where σ is the standard deviation of the blank measurements, and K is the slope of the intensity versus the sample concentration plot. The results imply that DP-NBD has high sensitivity for H₂S and can be used to detect low levels of H₂S qualitatively.

Bioimaging in living cells

As described above, DP-NBD exhibits good water solubility, high selectivity, low detection limit towards H₂S without interferences from other guests. Furthermore fluorescent spectrum at physiological condition (Figure S4 in ESI) and low cytotoxicity (Figure S5 in ESI) authorizes its further application in living cells. HeLa cells are cultured in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin and are maintained in an incubator at 37 °C with a 5% CO₂/air environment. DP-NBD is incubated with HeLa cells for 30 min at 37 °C, and then the HeLa cells are washed with PBS. The
fluorescent change of the cells is monitored by laser confocal fluorescence microscopy (LCFM). As illustrated by the LCFM images and bright field images (in Figure 4), a red fluorescence image is detected when H$_2$S is added to the HeLa cells stained by DP-NBD and incubated for 1 h. The results suggest that DP-NBD is capable of permeating into cells and visualize H$_2$S. Furthermore, an increasing red fluorescence can be observed when HeLa cells stained by DP-NBD are incubated with H$_2$S for 2 h in Figure 4(H). The results further indicate that probe DP-NBD has excellent cell permeability and bio-compatibility.

![Figure 4](image-url)  
**Figure 4** LCFM images of probe DP-NBD in HeLa cells. (A-D) bright-field images; (E, G) HeLa cells were treated with DP-NBD for 30 min; (F, H) HeLa cells were pre-incubated with DP-NBD for 30 min and then treated with H$_2$S for 1 h or 2h; (I-I) merged images of bright-field and fluorescent image. ($\lambda_{ex} = 365$ nm, $\lambda_{em} = 595$ nm).

**Reaction mechanism**

Subsequent efforts is paid on the sensing mechanism of DP-NBD with H$_2$S by MOLDI-TOF mass spectrometry. According to the mass spectrum, a new peak at 320.14 (m/z) corresponding to DP can be found when 10 equivalents of H$_2$S is added into the solution containing DP-NBD (Figure S6 in ESI). The result confirms that H$_2$S cleaves the C-N bond of DP-NBD to form the cleavage product DP, as described in Scheme 1. Further proof is provided by investigating the fluorescence spectra of fragments NBD-P and DP. For NBD-P, the addition of H$_2$S leads to the decrease of fluorescent intensity at 545 nm. In comparison to NBD-P, DP displays a strong emission at 595 nm and no obvious change is observed upon the addition of H$_2$S. This result further confirms the cleavage product is DP. Consequently, a more detailed analysis of the structure and electron density is performed in an attempt to gain insight into its recognition mechanism. Accordingly, the time-dependent density functional theory (TD-DFT) calculations are used to optimize the structures of DP-NBD, DP and NBD-P at the B3LYP/6-31G* level using a suite of Gaussian 09 programs. The results of the calculations show that the piperazine ring of DP-NBD presents a classic chair conformation while the DP displays a twist-boat conformation, as shown in Figure 5. For their frontier molecular orbital profiles, the electron density of DP-NBD mainly focuses on the dansyl unit in its HOMO orbital while its LUMO orbital lies in the NBD unit. For DP, its HOMO and LUMO orbitals almost focus on the dansyl moiety. The HOMO orbital of intermediate NBD-P is delocalized piperazine unit by extension to NBD unit while its LUMO is more delocalized in NBD unit. Accordingly, we guess that the NBD-P unit possibly plays a role of the PET quencher owing to that the LUMO of DP is higher energy then the LUMO of NBD-P. As a consequence, the lower LUMO orbital of NBD-P can accept an electron from the higher LUMO orbital of DP. Upon the treatment of H$_2$S, the PET process is broken while the DP displays turn-on fluorescence, which is well in agreement with experimental results.

![Figure 5](image-url)  
**Figure 5** Frontier molecular orbital profiles of molecules based on TD-DFT (B3LYP/6-31G*) calculations.

**Conclusion**

In conclusion, we provide an ideal strategy for designing the turn-on fluorescent probes. In which, a fluorescent compound is selected as a cleavage product to construct the H$_2$S-responsive probe based on the nucleophilic substituted reaction. According to this strategy, we design and synthesize a H$_2$S-responsive fluorescent probe which can efficiently detect H$_2$S with high selectivity and low detection limitation. And the probe shows good water solubility, cell permeability and bio-compatibility. The bioimaging in living cells indicates that this probe can monitor H$_2$S at cell level. Considering the efficiency and practicability of this strategy, we believe it will be commonly used to design the turn-on fluorescent probe in near future. Furthermore, the small molecular H$_2$S probe can be used as an efficient tool to investigate the biological functions that are related to H$_2$S.

**Experimental Section**

**Materials and instrumentation.** All manipulations were carried out under an argon atmosphere using standard Schlenk techniques, unless otherwise stated. All commercials were used.
as received without further purification. 1H and 13C spectra were collected on Bruker 400 MHz spectrometer (Bruker, Bremen, Germany) in DMSO-d6 and CDCl3 with Me4Si as an internal reference. UV–Vis spectra were obtained using Hitachi U-3310 visible recording spectrophotometer. Fluorescence spectra were obtained using Perkin Elmer LS-55. Mass spectra were measured in the ESI mode. The theoretical calculations were performed at the B3LYP/6-31G* level by using the Gaussian 09 suite of programs.

Synthesis of DP. To a 25 mL round-bottomed flask was added 134.8 mg (0.5 mmol) of dansyl chloride and 172.2 mg (2 mmol) of piperazine. Then CH3Cl2 and Et3N were added. The reaction was stirred at room temperature for 4 h. The mixture was then evaporated and the residue was purified with column chromatography (silica gel, dichloromethane/methanol = 30:1, v/v). A yellow green solid (154 mg) was obtained, yield: 96%. 1H NMR (400 MHz, DMSO-d6): δ8.54-8.52 (d, J = 8.00 Hz, 1H), 8.35-8.32 (d, J = 12.00 Hz, 1H), 8.12-8.10 (d, J = 8.00 Hz, 1H), 7.69-7.66 (t, J = 8.00 Hz, 1H), 7.63-7.59 (t, J = 8.00 Hz, 1H), 7.28-7.26 (d, J = 8.00 Hz, 1H), 2.95 (s, 4H), 2.84 (s, 6H), 2.65 (s, 4H). EI-MS m/z (M+) calc’d 319.13, found 319.11.

Synthesis of NBD-P. To a 25 mL round-bottomed flask was added 199.5 mg (0.5 mmol) of dansyl chloride and 172.2 mg (2 mmol) of piperazine. Then CDCl3 and Et3N were added. The reaction was stirred at room temperature for 12 h. The mixture was then evaporated and the residue was purified with column chromatography (silica gel, dichloromethane). A orange solid (80.0 mg) was obtained, yield: 55%. 1H NMR (400 MHz, DMSO-d6): δ8.47-8.45 (d, J = 8.00 Hz, 1H), 6.66-6.64 (d, J = 8.00 Hz, 1H), 4.08 (s, 4H), 2.95-2.93 (t, J = 4.00 Hz, 4H). EI-MS m/z (M+) calc’d 249.08, found 249.02.

Synthesis of DP-NBD. To a 25 mL round-bottomed flask was added 65.8 mg (0.33 mmol) of 4-Chloro-7-nitrobenzo-2,1,3-oxadiazole and 172.2 mg (2 mmol) of piperazine. Then DMF was added. The reaction was stirred for 4 h at 85 °C. The mixture was then evaporated and the residue was purified with column chromatography (silica gel, dichloromethane / ethyl acetate = 6:1, v/v). A red solid (97.6 mg) was obtained, yield: 78%. 1H NMR (400 MHz, DMSO-d6): δ8.47-8.45 (d, J = 8.00 Hz, 1H), 6.66-6.64 (d, J = 8.00 Hz, 1H), 4.08 (s, 4H), 2.95-2.93 (t, J = 4.00 Hz, 4H). EI-MS m/z (M+) calc’d 319.13, found 319.11.

DFT calculation. Accordingly, density functional theory (DFT) calculations were used to optimize the structures of DP-NBD, DP and NBD-P at the B3LYP/6-31G* level using a suite of Gaussian 09 programs.

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Notes and references

*Key Laboratory of Pesticide and Chemical Biology of the Ministry of Education, College of Chemistry, Central China Normal University, Wuhan 430079, P. R. China. E-mail: yinji@mail.ccnu.edu.cn
Department of Biomedical Engineering, School of Engineering, China Pharmaceutical University, 24 Tongjiya Lane, Gulou District, Nanjing 210009, China. E-mail: chenhuangyong@cpu.edu.cn

3 State Key Laboratory of Materials-Oriented Chemical Engineering, College of Chemistry and Chemical Engineering, Nanjing Tech University, Nanjing 210009, China.

They contributed equally to this work.

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