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Novel turn-on fluorescence sensor for detection and imaging of endogenous H_2S induced by sodium nitroprusside

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Abstract

Currently, fluorescence analysis method has a good application in the detection and imaging of biomarkers and has become an important analytical method. Although there are many fluorescent probes for detecting hydrogen sulfide(H_2S), they are mostly based on fluorophores which already existed, such as 1,8-naphthalimide, coumarin, rhodamine and their derivatives. Here, a new type of fluorescent molecule (BOTD) was synthesized and applied to the detection of H_2S. The probe BOTD could quickly and sensitively detect H_2S and turn on fluorescence. Moreover, the probe BOTD was successfully applied to the detection of exogenous and endogenous H_2S in living cells, and may be expected to become a research tool for studying H_2S-induced drugs.

Keywords: Hydrogen sulfide; Fluorescent probe; H_2S-induced drug; Bioimaging

1. Introduction

Hydrogen sulfide (H_2S), as one of the gas signal molecules, plays an important role in many physiological processes, including: vasodilation, inflammation control, neuroprotection, delaying aging, regulating glucocorticoids and insulin, etc. [1-6] Endogenous H_2S has multiple production pathways in the body. L-cysteine (L-Cys) is used as a substrate in the cytoplasm of the cell. It is produced under the catalytic action of cystathionine β-synthase (CBS), cystathionine-γ-lyase (CSE), and cysteine transferase [7-9]. In mitochondria, β-mercaptopyruvic acid is used as a substrate, and H_2S is produced under the action of mercaptopyruvate trans-sulphurase (MPST) [10-11]. There are usually two forms of H_2S in the body, H_2S and sodium sulfide (NaHS). The changes in the content of H_2S in the body have great potential in the prevention and diagnosis of cardiovascular disease. Unfortunately, it is still difficult to track and accurately quantify H_2S in biological systems, and the regulatory mechanism of H_2S is still unclear. Therefore, it is necessary to develop an analytical method for selective recognition and highly sensitive detection of H_2S in living organisms.

Fluorescence analysis has a wide range of applications in biological detection

[12-14]. The general methods for detecting H_2S are: reduction of azide to amine, nucleophilic addition, precipitation of copper sulfide, reduction of selenium oxide to selenide [15-19]. Under normal physiological conditions, ideal probes should respond to small concentration changes and show sensitive changes, so as to obtain more accurate information while avoiding interference from other substances as much as possible, especially organisms such as glutathione, cysteine, etc. So, the development of fluorescent probes for the perfect detection of H_2S remains a challenging task.

According to previous reports, triphenylamine and its derivatives have good mitochondrial localization selectivity and effective electron donor capabilities [20-23]. In this work, we developed a triphenylamine -based fluorescent probe that reacts with H_2S and detects it through fluorescence activation. The probe (E)-6-(4-(diphenylamino)benzylidene)-5-oxo-5,6,7,8-tetrahydronaphthalen-2-yl 2,4-dinitrobenzenesulfonate (BOTD) could not only detect H_2S sensitively and specifically, but also has low toxicity. This provides an effective means for the detection of exogenous and endogenous H_2S. This fluorescence analysis method can discover that the H_2S induced by sodium nitroprusside in living cells, which also helps to investigate the H_2S metabolism pathway in target cells.

2. Experiments

2.1. Materials and instruments

Both organic compounds and inorganic salts were delivered from Energy Chemical (Shanghai, China), which were analytical grade. The pH was measured by pH meter of model pH400 (Alalis, USA). A560PC UV-VIS spectrophotometer (Xiangyi, China) was used for the UV absorption spectra. The fluorescence spectra were taken on F97xp spectrophotometer (Lengguang Tech, China). All ^1H NMR and ^{13}C NMR of the sample were obtained by Bruker Avance AVIII-400 MHz spectrometer (Bruker Daltonics Corp., USA) at room temperature. High-resolution electrospray ionization mass spectra (HR-ESI-MS) were involved a Q-TOF6510 spectrograph (Agilent). A Zeiss LSM 710 confocal laser scanning microscope (CLSM) was utilized in cell fluorescence imaging experiments.

2.2. Synthesis of the probe BOTD

2.2.1 Synthesis of compound 2

Triphenylamine (3 g, 12.22 mmol) was dissolved in anhydrous N, N-dimethylformamide (20 mL) in a reaction flask and stirred at 0 ℃ for 2 h. Then, to the abovementioned solution, phosphorus oxychloride (5.7 mL, 61 mmol) was added dropwise at 0 ℃. After the addition was completed, the mixture was warmed up to room temperature and the reaction mixture was stirred for 2 h at 45 ℃. The crude product was poured into an ice-water mixture, neutralized with solid sodium bicarbonate. The resulting precipitate was filtered and dried, the crude product was further purified by column chromatography with petroleum ether/ dichloromethane (10:1 v/v) as the eluent to afford 2 as a pale yellow solid (2.70 g, 81%).^{1}H NMR (400 MHz, DMSO-d_6) δ 9.77 (s, 1H), 7.72 (d, J = 8.8 Hz, 2H), 7.42 (t, J = 7.9 Hz, 4H), 7.22 (dd, J = 17.6, 7.4 Hz, 6H), 6.89 (d, J = 8.7 Hz, 2H).

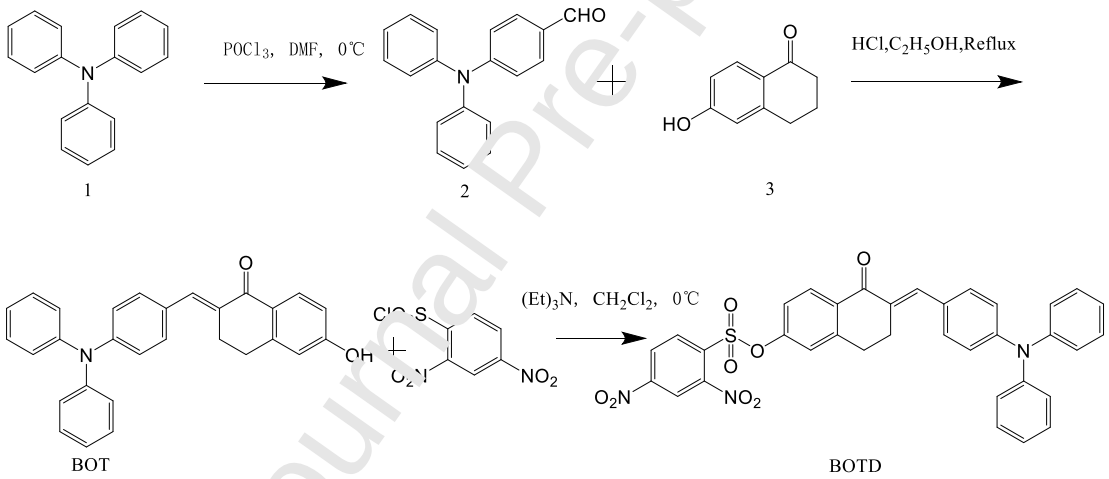
2.2.2 Synthesis of compound BOT

The compound BOT was prepared using an acid-catalyzed method which was named Claisen- Schmidt condensation method [24]. 6-hydroxy-3,4-dihydronaphthalen-1(2H)-one (0.324 g, 2 mmol), the appropriate compound 2 (0.547 g, 2 mmol) and hydrochloric acid (36%; 20 ml) were added in a flask filled with methanol (15 ml), then refluxed for 5 hours. After the reaction was completed, ice cold water (20 ml) was added, and the precipitate was collected by filtration, dried and further purified by silica gel chromatography to obtain BOT (0.6 g, 70.2%).^{1}H NMR (400 MHz, DMSO-d_6) δ 10.38 (s, 1H), 7.83 (d, J = 8.6 Hz, 1H), 7.58 (s, 1H), 7.43 (d, J = 8.7 Hz, 2H), 7.38 – 7.28 (m, 4H), 7.19 – 7.01 (m, 6H), 6.95 (d, J = 8.7 Hz, 2H), 6.77 (d, J = 10.9 Hz, 1H), 6.67 (s, 1H), 3.04 (t, J = 5.9 Hz, 2H), 2.83 (t, J = 6.4 Hz, 2H).

2.2.3 Synthesis of BOTD

As shown in Scheme.1, BOTD is synthesized from BOT and 2,4-dinitrobenzenesulfonyl chloride in 10 ml of dichloromethane solution, and added

with triethylamine and stirred at 0 °C for 1 h, and then stirred at room temperature overnight. The crude product was purified by rotary evaporation and silica gel column chromatography to obtain the pure product. ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.13 (s, 1H), 8.62 (d, *J* = 11.0 Hz, 1H), 8.32 (d, *J* = 8.7 Hz, 1H), 7.97 (d, *J* = 8.6 Hz, 1H), 7.67 (s, 1H), 7.47 (d, *J* = 8.8 Hz, 2H), 7.43 – 7.32 (m, 4H), 7.30 (s, 1H), 7.22 – 7.04 (m, 7H), 6.94 (d, *J* = 8.7 Hz, 2H), 3.09 (t, *J* = 5.9 Hz, 2H), 2.94 (t, *J* = 6.4 Hz, 2H). ^{13}C NMR (101 MHz, DMSO) δ 185.76, 152.04, 151.79, 148.65, 148.55, 146.84, 146.52, 136.97, 134.06, 133.15, 132.86, 132.27, 131.25, 130.59, 130.24, 128.42, 128.08, 125.61, 124.67, 122.07, 121.65, 121.21, 120.89, 60.22, 27.08, 26.88, 21.22, 14.55. MS (ESI) m/z: calcd for $[M+H]^+$, 648.14; Found, 648.14. All ^{1}H NMR, ^{13}C NMR and HRMS are shown in Fig.S1-5.



Scheme.1 Synthesis route of BOTD.

2.3. Analytical procedures

The probe BOTD is prepared as a stock solution of 1 mM / L in DMSO. Solutions for fluorescence and UV measurement is THF-PBS solvent (0.1 μM, pH 7.4, 30% THF, v/v) unless otherwise stated. Both excitation and emission of slit width was 10 nm. And all analytes (F^-, Cl^-, Br^-, I^-, ClO^-, NO_2^-, HSO_3^- , SO_3^{2-}, S^{2-}, HS^-, SO_4^{2-}, HSO_4^-, SCN^-, Cys, Hcy, GSH) were dissolved in double distilled water when used.

2.4. Theoretical calculations

Relevant theoretical calculations were performed using the Gaussian 09 program. The geometry of BOT, DNS and BOTD was optimized using a three-parameter mixed B3LYP density method and a base set of 6-31G. The excited states of all compounds were studied by time-varying density functional theory (DFT).

2.5. Cell culture and cytotoxicity assay

Hela cells were provided by Jiangsu Kaiji Biotechnology Co., Ltd. The cells were cultured in a medium consisting of 90% MEM + 10% FBS, and putted in an incubator at 37 ° C, 5% CO_2, and saturated humidity. The cytotoxic effects of the probe BOTD were tested by the MTT assay by the previous report [25-27]. Differently, the cells were incubated with different concentrations of BOTD (0, 5, 10, 15, 20 μM/L, containing 0.5% DMSO) for 24 h.

2.6. Imaging of H_2S in living Cell

For fluorescence imaging experiments, the cells were divided into three samples; one was incubated with the BOTD probe (10 μM/L) only as a control, the other group was cultured with NaHS (100 μM/L) for 30 min and then the probe was added, and the last group was cultured with 200 μM/L Sodium nitroprusside (SNP) for 60 min and then with the probe, respectively. All cells were washed three times with PBS buffer after incubation. The experiments of cell imaging were conducted by using a laser scanning confocal fluorescence microscope (Zeiss LSM 710). Cell images were obtained using 420 nm excitation and signals were collected from the green channel (500–550 nm).

3.Results and discussions

3.1. The design of BOTD and properties of compound BOT

In order to better understand the role of H_2S at the mitochondrial level, a new type of organelle-based fluorescent probe is urgently needed for detection and imaging. Triphenylamine has good fluorescence characteristics, which is mitochondrial targeted. And triphenylamine as a strong electron donor and 6-hydroxy-3,4-dihydronaphthalen-1 (2H) -one conjugated to form a fluorescent probe

precursor (BOT) and 2,4-dinitrobenzenesulfonyl (DNS) as the H_2S response site. When reacting with H_2S, the electrons of the terminal hydroxyl group are recovered and the fluorescence is released.

We first assessed the ultraviolet absorption and fluorescence spectrum of BOT (10 μM/L) in various solvents including ethanol, acetonitrile, acetone, DMF, DMSO and THF. It could be learned that the maximum ultraviolet absorption of BOT is around 400 nm in all six solvents form Fig.1. with the excitation wavelength at 400 nm, the fluorescence intensity of BOT in DMSO solvent is the highest. This shows that BOT can be utilized as a precursor for fluorescent probe.

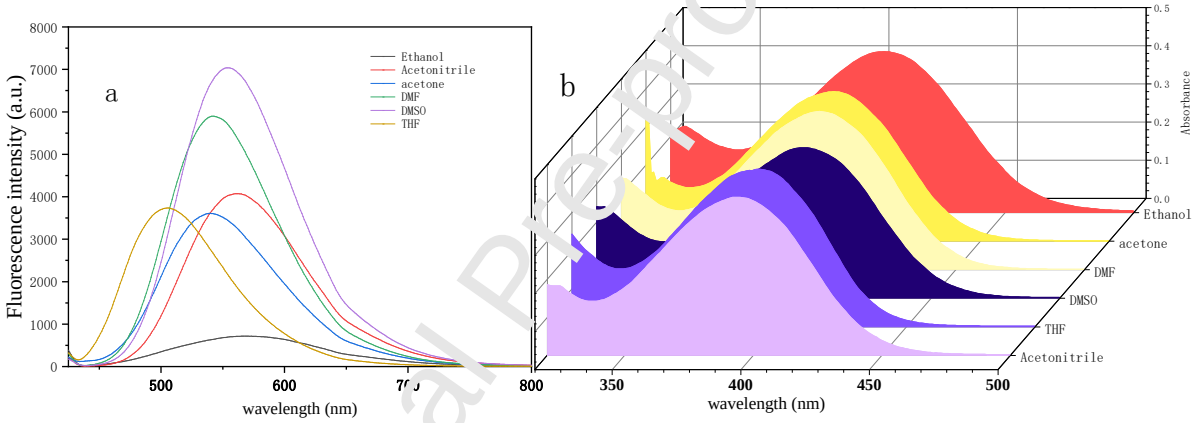


Fig.1 The fluorescence spectrum of BOT (A) and ultraviolet absorption of BOT (B) in different solvents.

3.2. Effects of pH

It is necessary to study the effect of pH on the probe in the environment for determining the applicable environment and stability of the probe BOTD. So, we evaluated the effect of pH on the probe BOTD in the absence and presence of HS^- in various pH buffers. As illustrated in Fig.2, when only the probe is present, the fluorescence of the solution hardly changes, while in the presence of HS^-, the fluorescence intensity of the solution increases sharply at pH 6-8. Consequently, the probe could stably detect HS^- under normal physiological conditions.

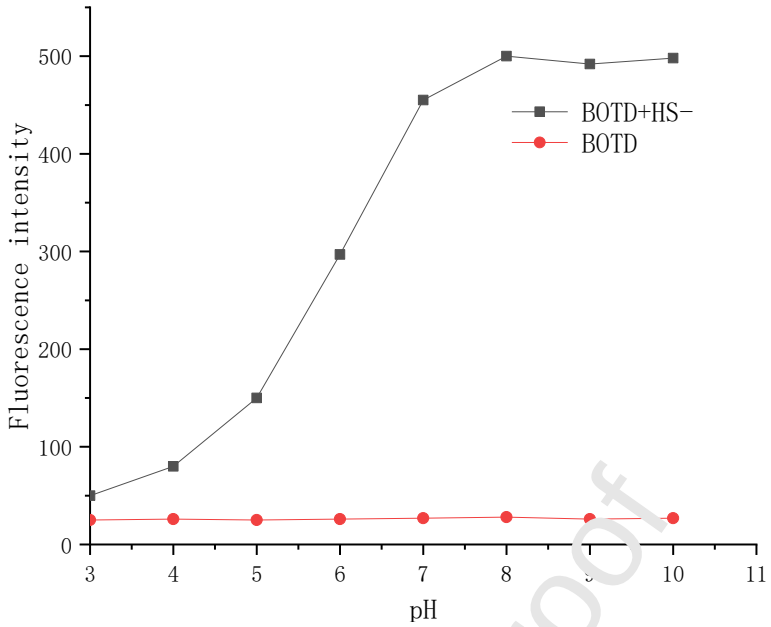


Fig.2 Fluorescence signal changes in the probe BOTD (10 μM/L) treated with HS^- (100 μM/L) at different pH values(λex=420 nm, λem=550 nm).

3.3. Sensing properties of probe BOTD for HS^-

Under normal physiological conditions, the fluorescence response and UV absorption of the probe BOTD to sodium sulfide were recorded in Fig.3. The maximum absorption peak of BOTD was about 420 nm. In Figure 3(a) and Fig.3(b), the fluorescence intensity of BOTD at 550 nm (λex=420 nm) increases from a very weak and significant increase as the concentration of sodium sulfide increases. The fluorescence quantum yield (ΦF) of BOTD increased from 0.02 to 0.23 after reacting with sodium hydrosulfide (Table.S1). And the UV absorption of the probe BOTD shifted to 400 nm in Fig.3(c), indicating that the 2,4-dinitrobenzenesulfonate bond was broken. Moreover, the fluorescence intensity of probe BOTD at 550 nm has a good linear relationship with the concentration of sodium sulfide from 0 to 50 μM/L (R^2 = 0.9947) (Fig. 3(c)). The corresponding detection limit was calculated to be 27.3 nmol/L with the equation of 3σ / k, where k is the slope and σ is the standard deviation of the blank sample, which was lower than other reported probes [28-33] (Table.S2).

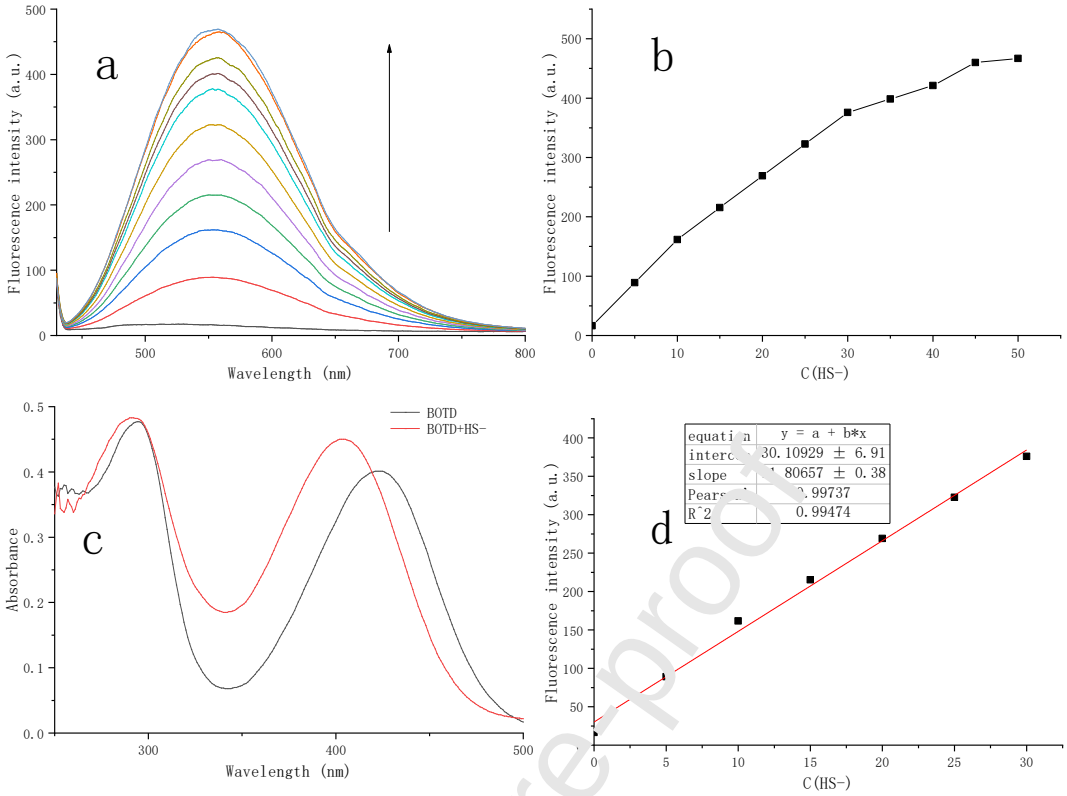


Fig.3 (a) Fluorescence spectra of the probe BOTD (10 μM/L) in aqueous buffer in PBS buffer (pH 7.4, 30% THF) response to NaHS (0–50 μM/L). λex=420 nm, λem=550 nm. (b) The correspondence between fluorescence intensity of probe BOTD and NaHS concentration. (c) The UV absorption of probe BOTD with NaHS (50 μM/L) in aqueous buffer. (d) The linear correlation between the concentration of H_2S (0–30 μM/L) and the fluorescence intensity of BOTD at 550 nm.

3.4. Selective response of BOTD to H_2S

Subsequently, the selectivity of the probe BOTD for H_2S was examined with various analytes in PBS buffer， including F^-, Cl^-, Br^-, I^-, ClO^-, NO_2^-, HSO_3^- , SO_3^{2-}, HS^-, S^{2-}, SO_4^{2-}, HSO_4^-, SCN^-, Cys, Hcy, GSH. As illustrated in Fig.4, the fluorescence of probe BOTD was turn on when detecting HS^- and S^{2-} . While the other analytes showed no significant change in fluorescence and UV absorption (Fig.S6). These results indicated that probe BOTD could detect H_2S with high selectivity.

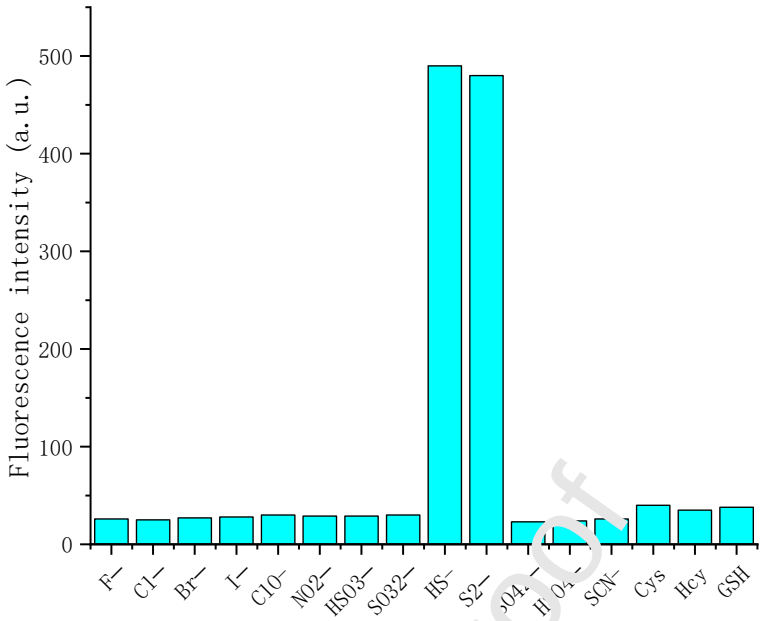


Fig.4 Fluorescence responses of probe BOTD (10 μM/L) towards various analytes (1 mM/L)

3.5. Response time

To obtain the response time of the probe BOTD to H_2S, the data of the fluorescence intensity at 550 nm was measured and plotted in Fig.5. As shown by the results, the fluorescence intensity of free BOTD in PBS buffer is almost unchanged, but only a weak fluorescence is maintained. Conversely, the fluorescence intensity of BOTD gradually increased and reached a peak within 30s after HS^- was added. Because the probe BOTD has extremely short response time to H_2S, it shows excellent potential for real-time detection of H_2S in biological systems.

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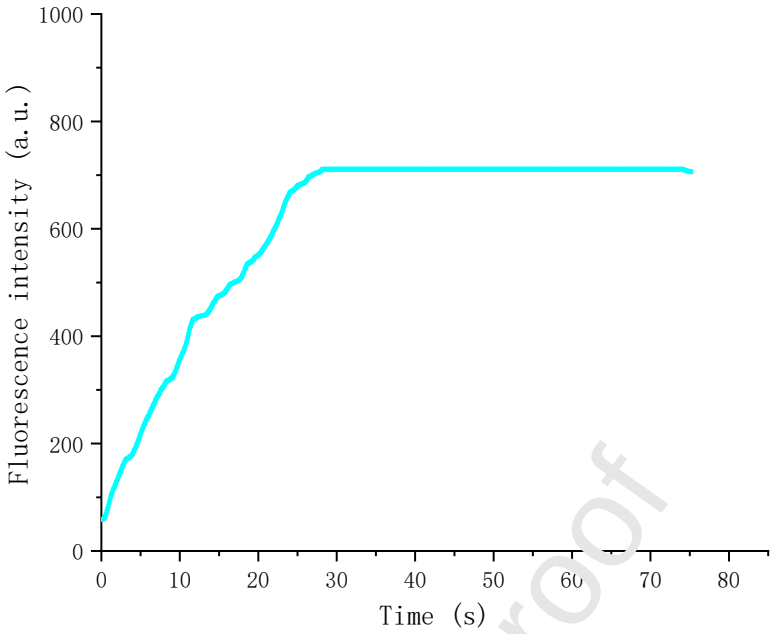

Fig.5 The fluorescence intensity of the probe BOTD (λex = 420 nm, λem = 550 nm) in the presence of H_2S depending on time

3.6. Molecular orbital and mechanism study

To obtained full comprehension of the changes in the photophysical properties of compound BOT and BOTD, the theoretical calculations and geometric optimizations based on DFT were carried out. The spatial distributions and orbital energies of the highest occupied molecular orbital (HOMO) and the lowest unoccupied molecular orbital (LUMO) are shown in Fig. 6. The HOMO energy (-8.34 eV) of DNS is between the HOMO energy (-5.76 eV) and LUMO energy (-8.43 eV) of BOT, and it is lower than the HOMO energy (-8.10 eV) of BOTD, which prevents probe BOTD from emitting fluorescence. Therefore, the molecular orbital energy diagram clearly indicates a PET process.

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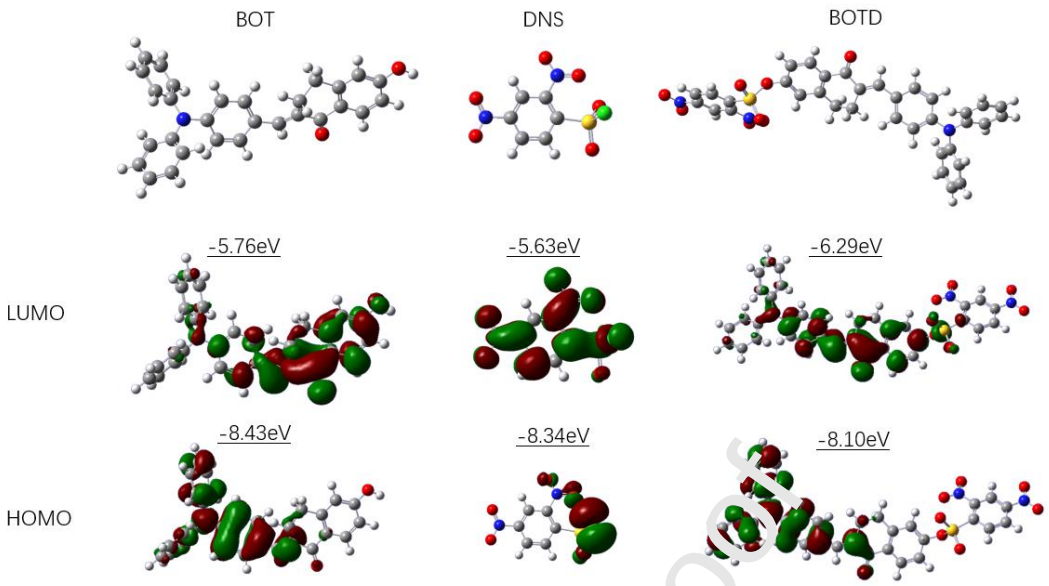
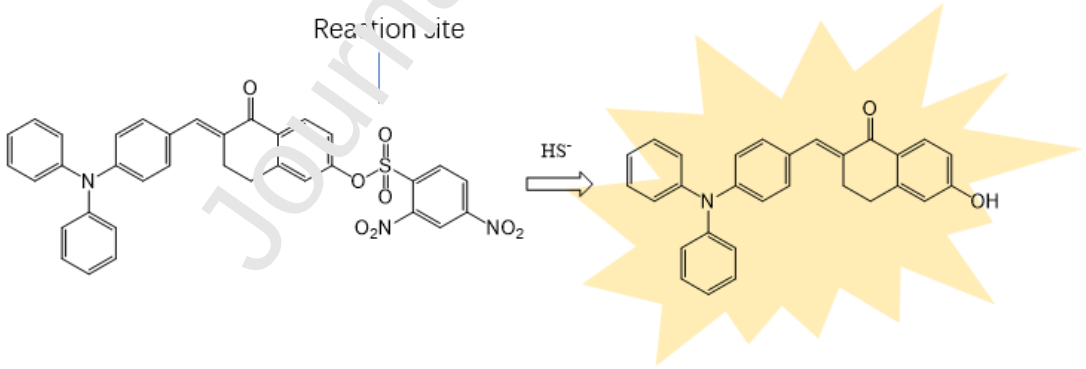


Fig.6 Frontier molecular orbitals of BOT, DNS and BOTD.

The putative detection mechanism was shown in Scheme.2. By analyzing the reaction products of the probe BOTD and HS^-, we found that a peak of m / z 418.18 $[BOT + H]^+$, which was assigned to BOT (calculated as 417.17 for $C_{29}H_{23}NO_2$) (Fig.S7). The ^1H spectrum (Fig.S8) of the BOTD-HS^- product is basically consistent with BOTD, which also proved that the detection mechanism is reasonable.



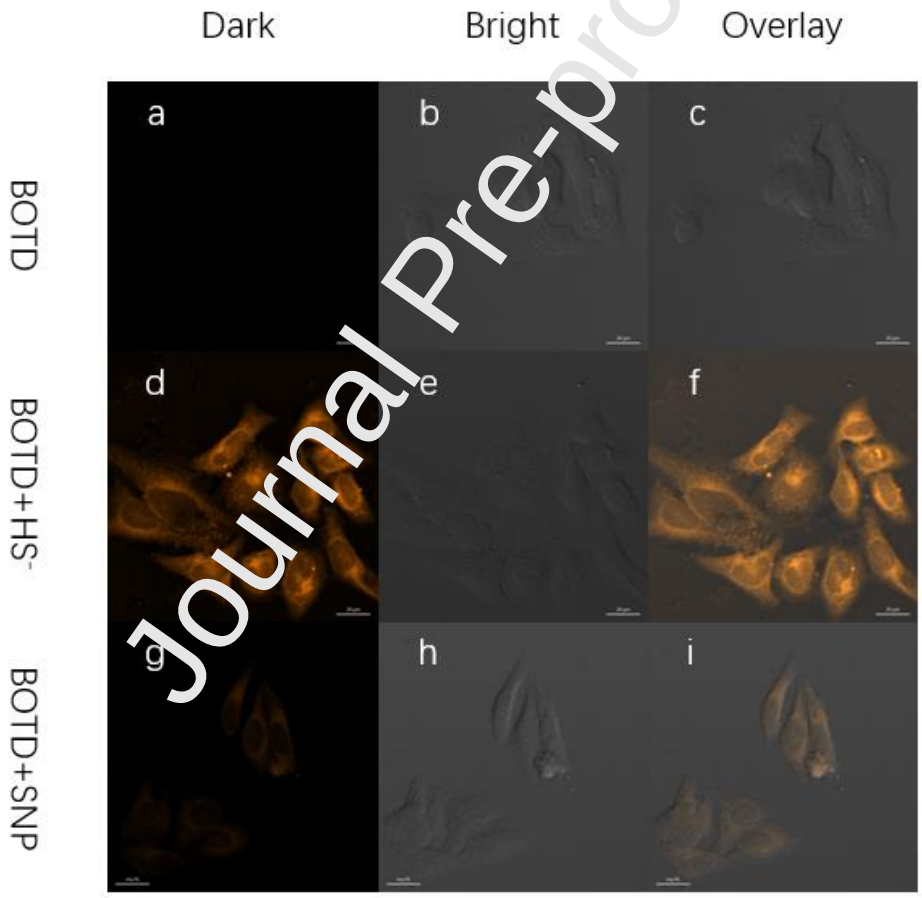
Scheme.2 Schematic illustration of BOTD's sensing towards HS^- cations.

3.7. Toxicity of probe BOTD and cell imaging

Before applying cell imaging to live cells, the MTT assay was performed to verify the low acute toxicity of BOTD to living HeLa cells. It could be seen that even with the probe BOTD concentration of 20 μM/L, the survival rate of HeLa cells is still

85% after 24 h of incubation (Fig.S9). This shows that the probe BOTD has excellent viability and biocompatibility.

Then BOTD is exploited for H_2S detection and imaging in living cell. As described in Fig.7, the probe BOTD has no fluorescent signal without HS^- (Fig.7(a-c)). And the probe BOTD has a yellow-green fluorescence emission after incubation with exogenous H_2S in Fig.7(d-f). More importantly, the HeLa cells incubated with sodium nitroprusside also exhibited fluorescence emission (Fig.7(g-i)), indicating that sodium nitroprusside could induce H_2S production in cells. Sodium nitroprusside is a commonly used drug to regulate blood pressure, but its mechanism of action remains



uncertain. Therefore, the probe BOTD can not only detect H2S in cells, but also provide help for studying the causes of H_2S.

Fig.7 Fluorescence images of HeLa cells (λex = 420nm，λem= 500-550nm). (left) dark field; (middle) bright field and (right) overlay image. (a–c) images of HeLa cells

incubated with BOTD (10 μM/L) for 30 min; (d–f) fluorescence images of cells pre-treated with NaHS(100 μM/L) for 1 h and then incubated with BOTD (10 μM/L) for 30 min; (g–i) images of cells pre-treated with 100 μM/L SNP for 1 h, and then incubated with BOTD (10 μM/L) for 30 min; scale bar was 20 μm.

4. Conclusion

In general, we have successfully constructed a new type of probe BOTD based on PET theory. The probe BOTD is non-fluorescent, but the reaction product with HS^- has fluorescent emission, which is due to blocking the process of PET. Further, the probe could quickly detect H_2S, and has good selectivity and biocompatibility, and detection limits as low as 27.3 nmol/L. Finally, BOTD could detect exogenous and drug-induced endogenous H_2S in living cells. Therefore, the probe BOTD could be a powerful tool for sensitive, real-time detection of H_2S in living cells.

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Author Statement

Li Wang: Conceptualization, Methodology, Writing – review & editing. Wenge Yang: Project administration, Resources. Yiyi Song: Visualization, Investigation. Yonghong Hu: Funding acquisition, Investigation.

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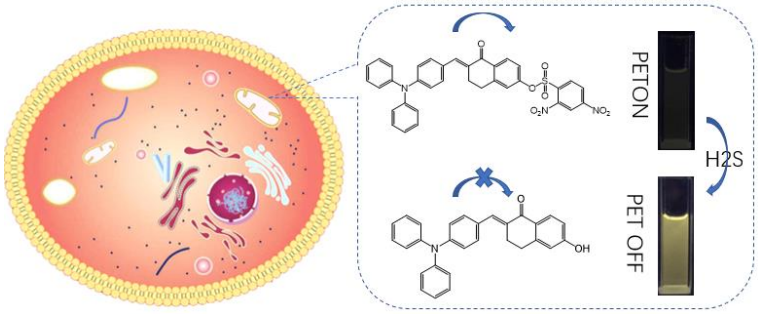
Graphical abstract



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Hightlights

1. A novel fluorescent sensor BOTD was developed for detecting H_2S.
2. BOTD showed high sensitivity and selectivity to H_2S in ultrafast time.
3. BOTD has low detection limits for H_2S.
4. BOTD was successfully applied to the detection of exogenous and endogenous H_2S in living cells

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