Design and application of a fluorescent probe for rapid detection of H$_2$S

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Abstract. H$_2$S is an endogenous gaseous signal molecule with cell protective function, which plays a critical part in many physiological and pathological processes. On the one hand, abnormal fluctuations of the concentration of hydrogen sulfide in the human body are closely related to diseases such as Down's syndrome, Alzheimer's, diabetes and cirrhosis of the liver. On the other hand, acts as an antioxidant in the body, hydrogen sulfide can protect cells from damage caused by reactive oxygen species. Therefore, it is indispensable to detect hydrogen sulfide in living organisms. A fluorescent probe—NX-H$_2$S, was constructed using azide group as the recognition group and naphthalimide as the framework. The spectroscopic properties of the probe were explored comprehensively. The experiment results show that NX-H$_2$S exhibits not only a fast response (5 min) but also excellent sensitivity and selectivity toward H$_2$S. In addition to the rapid detection of H$_2$S in vitro, NX-H$_2$S can also be successfully applied to the fluorescence imaging analysis of endogenous and exogenous H$_2$S in living cells.

1. Introduction

H$_2$S is a colorless gas that smells like rotten eggs, and it is the third endogenous gas biosignaling molecule with cell protective function that has been discovered. It plays an critical part in a series of pathological and physiological processes involving the regulation of neurotransmission$^{[1, 2]}$, the relaxation of vascular smooth muscle$^{[3, 4]}$, the regulation of inflammation$^{[5, 6]}$ and the inhibition of insulin signaling$^{[7]}$. H$_2$S is mainly produced by three enzymes: cystathionine $\gamma$-lyase, cystathionine $\beta$-synthase (CBS), and mercaptopyruvate sulfurtransferase (3-MST)$^{[8, 9]}$. These three enzymes are ubiquitous in different tissues but differ in expression, which contributes to the important status of H$_2$S in the respiratory, circulatory, nervous, and urinary systems. On the one hand, the content of hydrogen sulfide in human body is closely related to diseases such as Down’s syndrome, Alzheimer's disease, cirrhosis of the liver and diabetes$^{[10-13]}$. On the other hand, acts as an antioxidant in the body, hydrogen sulfide can protect cells from damage caused by reactive oxygen species. Therefore, it is indispensable to develop effective methods to detect hydrogen sulfide in living organisms.

In fact, there are many methods which can detect hydrogen sulfide. Traditional methods include colorimetry$^{[14, 15]}$, mass spectrometry$^{[16, 17]}$, gas chromatography$^{[18, 19]}$, electrochemical analysis$^{[20, 21]}$, metal-induced sulfide precipitation method$^{[22]}$. However, due to the shortcomings of these methods such as poor cell compatibility, low time resolution, high requirements for sample preparation, and complicated procedures, their wide application has been greatly limited. Compared with these traditional methods, fluorescent probe has attracted great attention because it can achieve highly sensitive and highly selective visualization imaging of living species in organisms, what’s more, it is
non-invasive and has high temporal and spatial resolution. At present, fluorescent probe has become a powerful tool to detect the target active species in organisms, and it is also a powerful means to reveal the metabolism of active species in biochemical processes.

Because of the advantages of fluorescence detection technology and the significance of H$_2$S in physiological activities, a variety of H$_2$S fluorescent probes have sprung up in recent years. For one thing, H$_2$S has a good water solubility, with a $P_{ka1}$ value of 6.8 and a $P_{ka2}$ value of more than 13. At pH 7.4, 80% H$_2$S exists in the form of HS$^-$, showing strong nucleophilicity, so many probes based on its nucleophilicity have been designed [23, 24]. For another, H$_2$S has excellent reducing ability, a typical H$_2$S reduction reaction is that H$_2$S can reduce azide groups or nitro groups to amino groups, a lot of fluorescent probes based on H$_2$S reductivity have been designed [25, 26]. However, among various H$_2$S fluorescent probes, the response speed of many H$_2$S fluorescent probes is still not fast enough to meet the needs of rapid and efficient detection of H$_2$S [27, 28]. Therefore, it is necessary to synthesize more fluorescent probes with faster response rate. A H$_2$S fluorescent probe NX-H$_2$S was synthesized by using azide group as the response group and 4-amino-1, 8-naphthalamide as the fluorophore. The ICT process cannot be realized when the strong electron-withdrawing azide group turns up at position 4, so the probe itself does not emit light. However, when the azide group at position 4 is reduced to amino by H$_2$S, the strong ICT effect can restore and the probe will emit green fluorescence (Figure 1), realizing the response to H$_2$S. It can be seen from the experiment results that NX-H$_2$S has a relatively fast detection speed (5 min), high sensitivity, good selectivity and good spectral performance. In addition to the rapid detection of H$_2$S in vitro, NX-H$_2$S can also be successfully applied to the fluorescence imaging analysis of endogenous and exogenous H$_2$S in living cells.

![Fig. 1. Response mechanism of NX-H$_2$S to H$_2$S](image)

2. Experimental

2.1. The synthesis of NX-H$_2$S

5 mL water containing 350 mg (5.4 mmol) sodium azide was added to 30 mL DMF solution containing 2.0 g (5.1 mmol) 4-bromo-N-2-aminoethylpyridine-1,8 naphthalimide. Before being poured into ice water, the reaction mixture got through 8 hours of stirring at 100 °C. Yellow solids were acquired and dried in a vacuum oven, and the crude product was purified by silica gel column chromatography. The eluent (dichloromethane/methanol =100/1, volume/volume) was selected to get the target product (Figure 2).

The molecular weight of C$_{19}$H$_{13}$N$_5$O$_2$ was calculated to be 343.11, and the mass spectrometric
Fig. 2. Synthesis route of NX-H_2S

result was 344.1127 [M+1]^+. 1H NMR (400 MHz, DMSO) δ 8.48 – 8.44 (m, 1H), 8.44 – 8.41 (m, 1H), 8.40 (d, J = 8.0 Hz, 1H), 8.37 (d, J = 8.4 Hz, 1H), 7.86–7.76 (m, 1H), 7.70 (s, 1H), 7.69–7.65 (m, 1H), 7.29 (d, J = 7.8 Hz, 1H), 7.20 (dd, J = 7.0, 5.3 Hz, 1H), 4.42–4.29 (m, 2H), 3.11–3.01 (m, 2H). 13C NMR (101 MHz, CDCl_3) δ 163.75, 163.33, 158.94, 149.31, 143.35, 136.32, 132.16, 129.13, 128.69, 126.79, 124.31, 123.24, 122.56, 121.42, 118.86, 114.62, 77.32, 77.00, 76.68, 40.07, 36.34.

2.2. Cytotoxic assay
HeLa cells were distributed in a 96-well plate and placed in the incubator for 24 h, while the destiny of Hela cells is 1 × 10^4 cells per well. Then DMSO was used to dissolve NX-H_2S firstly, cell medium containing different concentrations of NX-H_2S was prepared and transferred into the wells. After 24 h incubation, the cell medium was removed and replaced by 200 μL MTT stock solution (0.5 mg/mL) in each well. After putting the plate in the incubator for 4 h, the supernatant was removed and 150 μL DMSO was added into each well. The cell viability (%) was calculated using absorbance data at 570 nm.

2.3. Cell experiment
ATCC cell bank is the original source of HeLa cells. RPMI 1640 (Hyclone) medium containing 10% fetal bovine serum and 1% antibiotics was used to culture cells. The cell culture temperature was 37°C, and the gas environment was humidified atmosphere containing 5%CO_2. The probe concentration in the control group was 5 μM, and the incubation time was 30 min. As for experimental group, the cells were pretreated with 50 μM biothiol scavenger NEM (N-ethyl maleimide) for 30 min. Then the cells were treated with 100 μM H_2S for 30 min. Finally, 5 μM NX-H_2S was added into HeLa cells and cells underwent a 30-minute incubation period. Before imaging, the cells were washed three times with phosphate buffer solution (PBS, pH 7.4) to get rid of excess compounds.

3. Results and discussion

3.1. The spectral properties of NX-H_2S

3.1.1. Fluorescence titration spectra of NX-H_2S
In order to investigate the sensitivity of NX-H_2S, fluorescence titration spectra of NX-H_2S were measured in PBS buffer solution (10 mM, pH = 7.4, 50%CH_3CN, V/V). As can be seen from Figure 3, with the incremental concentration of H_2S, the fluorescence intensity of the system at 532 nm gradually increased. There was an excellent linear relationship between H_2S concentration and fluorescence intensity. The detection limit (LOD) of NX-H_2S for H_2S was 0.164 μM, indicating that NX-H_2S has good sensitivity toward H_2S. To verify the reaction mechanism and our analysis of the spectral data, the mixture of NX-H_2S and H_2S was examined by mass spectrometry. A new peak at m/z [M+1]^+ = 318.1246 was attributed to 4-amino-N-2-aminoethylpyridine-1,8-naphthalimide. The
results of mass spectrometry showed that the probe was indeed reduced by H$_2$S, and the target product which could emit green fluorescence was produced successfully.

3.1.2. UV titration spectra of NX-H$_2$S
In the PBS (10 mM, pH = 7.4, 50% CH$_3$CN, V/V) system, the UV absorption curve of 5 μM NX-H$_2$S also changed significantly with the increasing concentration of H$_2$S (Fig. 4). As shown in Figure 4, the probe itself had an obvious absorption peak at 375 nm. After the addition of H$_2$S to the test system, a new absorption peak appeared at 430 nm. With the incremental concentration of H$_2$S, the absorption peak at 375 nm gradually decreased and the absorption peak at 430 nm gradually increased. It was speculated that the absorption peak at 430 nm was actually the characteristic absorption peak of the target product—4-bromo-N-2-aminoethylpyridine-1,8-naphthalimide. Which was confirmed by mass spectrometry result.

3.1.3. The selectivity of NX-H$_2$S toward H$_2$S
Figure. 5 showed the fluorescence intensity of test system at 532 nm when the probe (5 μM) encountered different analytes. As shown in this figure, in the PBS (10 mM, pH=7.4, 50%CH$_3$CN, V/V) system, the fluorescence intensity of NX-H$_2$S at 532 nm was very weak, but it significantly enhanced after the addition of H$_2$S. The sharp change in fluorescence intensity could be attributed to the reaction that H$_2$S reduced the azide group on the probe molecule to an amino group, which restored the ICT process and turned on the green fluorescence. On the contrary, it could be clearly
seen from the histogram that the addition of other biological species had no significant influence on the fluorescence intensity of NX-H$_2$S at 532 nm. Which indicated that NX-H$_2$S had good selectivity to H$_2$S.

3.1.4. Anti-jamming capability of NX-H$_2$S
In order to test the selectivity of NX-H$_2$S in practical applications, we added a variety of other analytes to the fluorescence test system to explore the selective reaction of NX-H$_2$S to H$_2$S (Figure 6). In the interference experiment, 150 μM H$_2$S was first mixed with 150 μM of Mg$^{2+}$, Zn$^{2+}$, K$^+$, Ca$^{2+}$, Ag$^+$, NO$_2^-$, SO$_3^{2-}$, HSO$_3^-$, SO$_4^{2-}$, HSO$_4^-$, CO$_3^{2-}$, HCO$_3^-$, CH$_3$COO$^-$, Br$^-$, I$^-$, NaClO, H$_2$O$_2$, N-acetylcysteine (NAC), ascorbic acid, Lys or 1 mM GSH respectively, and finally 5 μM NX-H$_2$S joined for fluorescence tests. As can be seen from Fig. 6, under the interference of various analytes, the fluorescence responses of NX-H$_2$S to H$_2$S had no significant difference from that in the presence of H$_2$S only. From the results of interference experiments, we found that NX-H$_2$S could be used as a selective fluorescent probe to detect H$_2$S under the interference of various biological species.
3.1.5. Time-dependent response of NX-H2S toward H2S

Fig. 7. Time-dependent fluorescence response of NX-H2S (5 μM) toward H2S (150 μM)

In order to further explore the properties of NX-H2S, we monitor the changes of fluorescence response of NX-H2S toward H2S with the development of time (Figure 7). After the addition of H2S, the fluorescence intensity of the probe at 532nm rapidly increased to a stable state within 5 minutes, and then the fluorescence signal remained basically unchanged until 30th min. It could be seen that NX-H2S had a fast response to H2S and was able to monitor H2S efficiently.

3.1.6. pH stability of NX-H2S

Fig. 8. Fluorescence spectra of NX-H2S (5 μM) in the presence of H2S(150 μM) upon different pH conditions

In order to investigate whether NX-H2S can work under physiological conditions, we investigated the effect of pH value on fluorescence signal. As shown in Figure 8, when NX-H2S encountered H2S upon the pH range of 6.53~10.64, NX-H2S showed significant fluorescence enhancement and the extent of fluorescence enhancement was basically stable. In addition, the fluorescence intensity of the probe itself almost did not change under different pH conditions. These results indicated that the performance of NX-H2S was still reliable under physiological conditions, which could realize the identification and detection of H2S stably.

3.2. Cytotoxicity of NX-H2S and Cell imaging

3.2.1. Cytotoxicity of NX-H2S

The cytotoxicity of NX-H2S needed to be explored before cell imaging analysis. MTT (thiazolium blue) method was used to investigate the cytotoxicity of NX-H2S, and the results were shown in Figure 9. Probe NX-H2S showed very low toxicity to HeLa cells, and the survival rate of cells was 98% when the probe concentration was 5 μM. The results of MTT assay showed that 5 μM NX-H2S
had good biocompatibility and could be used for the imaging detection and analysis of intracellular H₂S.

Fig. 9. MTT assay to evaluate the cell viability of HeLa cells after adding different concentrations of NX-H₂S

3.2.2. Cell imaging
As shown in Figure 10, after the addition of NX-H₂S to HeLa cells, bright fluorescence could be captured in the green channel. What’s more, if the biothiol scavenger NEM (N-ethylmaleimide) was used to pretreat the cells for 30 min, and then NX-H₂S was added to the cells for imaging, there was almost no fluorescence emission in the green channel, which could attribute to the removal of endogenous H₂S in the cells by NEM. It was also proved that the detection of endogenous H₂S in living cells could be achieved by NX-H₂S. However, when exogenous H₂S was added to NEM-pretreated cells for incubation for 30 min, and then NX-H₂S was added to the cells for final fluorescence imaging, bright fluorescent signal restored in the green channel, which suggested that the probe could successfully detect exogenous H₂S. In general, the cell experiment results indicated that NX-H₂S could successfully get through the cell membrane and achieve selective fluorescence imaging of endogenous and exogenous H₂S in living cells.

Fig. 10. Fluorescence images of HeLa cells. A) HeLa cells which were treated with NX-H₂S (5 μM, 30 min); B) NEM-pretreated(50 μM, 30 min) HeLa cells which were treated with NX-H₂S(5 μM, 30 min); C) NEM-pretreated HeLa cells which were incubated with H₂S (150 μM, 30 min) and then treated with NX-H₂S (5 μM, 30 min). λex= 405 nm. Scale = 20 μm.
4. Conclusion
Using the reaction that H$_2$S can reduce azide group to amino group, a H$_2$S fluorescent probe NX-H$_2$S has been constructed. It exhibits fast response (5 min), high sensitivity (0.164 μM) and good selectivity to H$_2$S. In addition to the rapid detection of H$_2$S in vitro, it has also been successfully used for fluorescence imaging analysis of endogenous and exogenous H$_2$S in living cells. We hope this tool will broaden our knowledge about the effects of H$_2$S in living organisms.

Acknowledgements
This work was supported by the National Natural Science Foundation of China (No. 21575015 and 21974009).

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