A two-photon fluorescent probe for imaging hydrogen sulfide in living cells

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Abstract: Fluorescent probes for hydrogen sulfide have received considerable attention because of the biological significance of H2S recognized recently. Two-photo microscopy offers advantages of increased penetration depth, localized excitation, and prolonged observation time. However, two-photon fluorescent probes for H2S are still rare. In this work, we introduced a dinitrophenyl ether group into the 4-position of 1,8-naphthalimide, which acts as the H2S reactive site, to efficiently yield compound \textbf{NI-NHS} as a two-photo fluorescent probe for H2S. The probe \textbf{NI-NHS} has a high selectivity for H2S over competitive anions and sulfide-containing analytes. This probe exhibits turn-on fluorescence detection of H2S in bovine serum and two-photon fluorescent imaging of H2S in living cells.

Keywords: Two-photon fluorescence microscopy; fluorescent probes; Hydrogen sulfide; Thiolysis; High selectivity; Cells imaging

1 Introduction

Hydrogen sulfide (H2S) is well known for its unpleasant odor of rotten eggs. Just recently, H2S is recognized as the third most important gasotransmitter for regulating cardiovascular, neuronal, immune, endocrine and gastrointestinal systems, along with nitric oxide and carbon
monoxide[1-3]. Altered levels of H₂S have been linked to many diseases, such as Alzheimer’s disease, Down’s syndrome, diabetes and liver cirrhosis[4-6]. The endogenous levels of H₂S are achieved by enzymes such as cystathionine-β-synthase (CBS) in the brain and cystathionine-γ-lyase (CSE) in the liver and vascular and nonvascular smooth muscle [7-8]. Some reports showed that mitochondrial sulfide quinone oxidoreductase (SQR) and persulfide dioxygenase (ETHE1) are involved in the consumption of H₂S[9]. These findings in living systems would be very important and helpful to elucidate the biological roles of H₂S.

For the detection of H₂S, a variety of fluorescent probes have been developed, [10-14] featured with high sensitivity, high spatial and temporal resolution.[15-18] These fluorescent probes for H₂S are based on specific chemical reactions by taking advantage of the reducing or nucleophilic properties of H₂S. In accordance with the fluorophores, the reported probes are mainly derived from rhodamine,[19] fluorescein,[20] dansyl,[21] BODIPY,[22] naphthalimide,[23] resorufamine,[24] NBD,[25] BMF,[26] coumarin,[27-28] cresyl violet,[29] genetically encoded fluorescent protein,[30] pyrene,[31] DCDHF,[32] DCMC[33] and phenanthroimidazole.[34] By attaching a sub-cellular targetable group, fluorescent probes can image H₂S in specific regions of cells, like mitochondria[35] and lysosomes.[36] However, these fluorescent probes work with one photo microscopy (OPM) that requires short excitation wavelength, which has the disadvantages such as photo-bleaching, photo-damage, shallow penetration depth, and cellular auto-fluorescence. Two-photo microscopy (TPM), a new technique that utilizes two photos of lower energy for excitation, has become a vital tool in biology. Compared to traditional fluorescence microscopy, TPM offers intrinsic 3D resolution combined with reduced phototoxicity, increased specimen penetration, and negligible background fluorescence.[37-40]. Unfortunately, two-photon
fluorescent probes for H$_2$S are still rare.[26, 41]

In this work, we introduced dinitrophenyl ether group into 4 position of 1,8-naphthalimide, which acted as the H$_2$S reactive site,[42] and easily obtained the two-photo fluorescent probe NI-NHS.[36] The synthesis of NI-NHS is shown in Scheme 1, which is quite straightforward started from the cheap commercial available material 4-bromo-1,8-naphthalic anhydride. The probe NI-NHS was finally obtained in good yield and characterized by $^1$H-NMR, $^{13}$C-NMR and HRMS. The experimental details are given in supporting materials.

Scheme 1

2 Experimental

2.1 Materials and instruments

Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification.$^1$H-NMR and $^{13}$C-NMR spectra were recorded on a VARIAN INOVA-400 spectrometer, using TMS as an internal standard. Mass spectrometry data were obtained with a HP1100LC/MSD mass spectrometer and a LC/Q-TOF MS spectrometer. UV-visible spectra were collected on a Perkin Elmer Lambda 35 UV/VIS spectrophotometer. Fluorescence measurements were performed on a VAEIAN CARY Eclipse fluorescence spectrophotometer (Serial No. FL0812-M018).

2.2 Synthesis

2.2.1 Synthesis of 4-Bromo-N-butyl-1,8-naphthalimide (3).

4-bromo-1,8-naphthalic anhydride (2) (5g, 0.018mol) and n-butylamine (1.05ml, 0.036mol) were
dissolved in 100 mL ethanol, and the solution was refluxed for 8 hours. After cooling to room
temperature, the yellowish sediments were collected by filtration and then dried overnight at room
temperature in a vacuum oven to give 3 (5.5 g, yield: 91.7%).\(^1\)HNMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.63 (d, 
\(J = 7.3\) Hz, 1H), 8.53 (d, \(J = 8.5\) Hz, 1H), 8.38 (d, \(J = 7.9\) Hz, 1H), 8.01 (d, \(J = 7.9\) Hz, 1H), 7.82 (t, 
1H), 4.26 – 4.07 (m, 2H), 1.71 (m, 2H), 1.52 – 1.37 (m, 2H), 0.98 (t, 3H). HRMS (ESI) calcd for 
\(\text{C}_{16}\text{H}_{14}\text{BrNO}_2\) [MH\(^+\)] 331.0208, found 331.0205.

2.2.2 Synthesis of N- butyl -4-methoxy-1,8-naphthalimide (4).

A mixture of compound 3 (1.66 g, 5 mmol) and K\(_2\)CO\(_3\) (4.15 g, 25 mmol) in 30 mL CH\(_3\)OH was
refluxed for 24 h. The precipitate was filtered and washed with water (30 mL \(\times\) 3). Compound 4 was obtained as yellow needles (1.1 g, yield: 78%).\(^1\)H NMR (400 MHz, CDCl\(_3\)) \(\delta\) 8.59 
(d, \(J = 7.3\) Hz, 1H), 8.55 (d, \(J = 2.0\) Hz, 1H), 8.53 (d, \(J = 2.1\) Hz, 1H), 7.69 (t, \(J = 8.2\) Hz, 1H), 7.03 (d, \(J = 
8.3\) Hz, 1H), 4.20 – 4.14 (m, 2H), 4.12 (s, 3H), 1.79 – 1.66 (m, 2H), 1.46 (m, 2H), 0.98 (t, 3H). HRMS 
(ESI) calcd for \(\text{C}_{17}\text{H}_{17}\text{NO}_3\) [MH\(^+\)] 283.1208, found 283.1205.

2.2.3 Synthesis of N- butyl -4-hydroxy-1,8-naphthalimide (1).

A mixture of compound 4 (1 g, 3.5 mmol) and 50 mL concentrated HI (57%) was refluxed for 6 h.
After cooling and adjusting pH to neutral, the precipitate was filtered to give compound 1 as
yellow needles (0.81 g, yield: 86.2%).\(^1\)HNMR (400 MHz, DMSO) \(\delta\) 11.85 (s, 1H), 8.50 (d, \(J = 
8.3\) Hz, 1H), 8.43 (d, \(J = 7.2\) Hz, 1H), 8.33 (d, \(J = 8.2\) Hz, 1H), 7.73 (t, \(J = 7.8\) Hz, 1H), 7.14 (d, \(J = 
8.2\) Hz, 1H), 4.01 (t, \(J = 7.3\) Hz, 2H), 2.53 (s, 1H), 1.68 – 1.52 (m, 2H), 1.42 – 1.25 (m, 2H), 
0.93 (t, 3H). HRMS (ESI) calcd for \(\text{C}_{16}\text{H}_{15}\text{NO}_3\) [MH\(^+\)] 269.1052, found 269.1051.

2.2.4 Synthesis of NI-NHS

Compound 1 (1 g, 3.7 mmol), 1-bromine-2,4-dinitrobenzene (1.5 g, 6.14 mmol) and K\(_2\)CO\(_3\)
(0.848 g, 6.14 mmol) were dissolved in anhydrous DMF (10 mL). The reaction mixture was then heated at 90°C for 4 hours under N₂ atmosphere. Cooling to room temperature, the reaction mixture was poured into ice water (100 mL). The crude product was extracted with ethyl acetate (3×25 mL) and dried over MgSO₄, and purified by flash column chromatography (ethyl acetate/CH₂Cl₂ = 1/1) to obtain the compound Ni-NHS as a white solid (0.91 g, 56.2%).

^1^HNMR (400 MHz, CDCl₃) δ 8.98 (d, J = 8.0, 1H), 8.71 (d, J = 8.0, 1H), 8.59 (d, J = 8.0 Hz, 1H), 8.48 (d, J = 8.4 Hz, 1H), 8.44 (d, J = 8.0 Hz, 1H), 7.85 (m, J = 8.4 Hz, 1H), 7.23 (m, 2H), 4.28 – 4.14 (m, 2H), 1.73 (m, 2H), 1.46 (m, 2H), 0.99 (t, 3H). ^1^C NMR (100 MHz, CDCl₃) δ 163.80, 163.18, 155.30, 153.97, 143.2, 140.65, 132.48, 131.89, 129.88, 129.27, 127.96, 127.61, 124.20, 123.18, 122.45, 121.06, 120.39, 114.46, 104.40, 30.20, 20.38, 13.84. HRMS (ESI) calef for C_{22}H_{17}N_{3}O_{7} [MH⁺] 435.1066, found 435.1069. Anal Calc for C_{22}H_{17}N_{3}O_{7}: C 60.66; H 3.94; N 9.64; O 25.72. Found C 60.62; H 3.95; N 9.66; O 25.73.

2.3 Culture of MCF-7 cells and fluorescent imaging

MCF-7 (human breast carcinoma) was cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% FBS (fetal bovine serum) in an atmosphere of 5% CO₂ and 95% air at 37 °C. The cells were seeded in 24-well flat-bottomed plates and then incubated for 24 h at 37 °C under 5% CO₂. Ni-NHS (5 µM) was then added to the cells and incubation for another 30 min followed. The cells were washed three times with phosphate-buffered saline (PBS). Fluorescence imaging was observed under a confocal microscopy (Olympus FV1000) with a 60×objective lens.

3 Results and discussion
3.1 *The spectroscopic properties of NI-NHS with H₂S in aqueous solutions*

The absorption and fluorescence titration experiments of NI-NHS with H₂S were recorded in aqueous solution (CH₃CN:PBS = 1:9, pH = 7.4, 10 μM NI-NHS) (Fig.1). In the absence of H₂S, NI-NHS presented a major absorption band at 358 nm. On addition of 0-20 equiv of H₂S to the solution of NI-NHS, the absorbance at 358 nm decreased sharply to its limiting value, while a new absorption band centered at 438 nm developed which induced the colour change from colourless to yellow (Fig. S1). The free NI-NHS displayed quite weak fluorescence. Importantly, with the addition of NaHS, the fluorescence intensity of NI-NHS increased significantly at 550 nm (37 fold) due to the thiolysis of the dinitrophenyl ether by H₂S. The MS and HPLC analysis confirmed that the fluorescence emission and enhancement was due to the formation of compound 1 (Fig. S2-3). The detection limit was calculated to be 0.18 μM (S/N=3) (Fig. S4).

![Fig. 1](image)

The influence of pH on the fluorescence of NI-NHS was determined by fluorescence titration (Fig. S5). The fluorescence at 550 nm of NI-NHS remains unaffected between pH 9-6.5, then gradually decreases from pH 6.5 to pH 3, and below pH 3 slight changes in fluorescence were finally obtained leading to a sigmoid curve. The studies of pH effect suggest that the compound NI-NHS is applicable in neutral medium like cells.

3.2 *The time-dependent spectroscopic properties of NI-NHS with H₂S in aqueous solutions*

The time-dependent fluorescence responses were next detected with the addition of 20 equiv H₂S and the results showed that the reaction was completed within 20 min (Fig. 2). Obviously, the
background fluorescence of **NI-NHS** is very weak, and a high fluorescence increase is observed within several minutes which responds the reaction of **NI-NHS** with H₂S (Figure 2b), then the timescale allows **NI-NHS** to sense H₂S in real-time intracellular imaging.

![Fig. 2](image1)

**3.3 The selectivity of **NI-NHS** for H₂S**

The probe **NI-NHS** (5 μM) was treated with various biologically relevant species to examine the selectivity. As shown in Fig. 3, **NI-NHS** showed selective response for H₂S over reactive oxygen species (ROS), reactive nitrogen species (RNS) and anions. Only ascorbic acid glutathione and cysteine gave limited increase in the fluorescence intensity. However, the intensity of the fluorescence increase was far weaker than that caused by H₂S. Thus, the probe **NI-NHS** has a very high selectivity for H₂S.

![Fig. 3](image2)

**3.4 The spectroscopic properties of **NI-NHS** with H₂S in bovine serum**

The tests in buffer solutions have shown the potential utility of **NI-NHS** in biological samples. We first checked the fluorescence response of **NI-NHS** with H₂S in bovine serum. The background fluorescence of bovine serum sample is relatively weak. With the addition of NaHS, the fluorescence intensity of emission of bovine serum sample with **NI-NHS** increases significantly. It should be noted that the fluorescence enhancement is observed immediately with the addition of NaHS and reaches the maximum value in minutes (Fig. 4a). The
concentration-dependent fluorescence responses of **NI-NHS** with NaHS were next detected, and this produced a linear relationship of the fluorescence intensity of **NI-NHS** versus hydrogen sulphide concentration. As seen in Fig. 4b, an excellent linear correlation between the added NaHS concentration and the fluorescence intensity of **NI-NHS** at 550 nm was observed. The fast responses and excellent linear relationship provided a real-time quantitative detection method for hydrogen sulfide in biological samples.

![Fig. 4](image)

**3.5 Two-photon cell imaging of H₂S**

We next tested the ability of **NI-NHS** to be used to visualize H₂S in live cells. OPM was first used. MCF-7 cells were incubated with **NI-NHS** (5 μM) for 30 min and exhibited no fluorescence (Fig. 5a). Then the cells were incubated with 50 μM NaHS, a concentration of H₂S comparable with physiological H₂S levels, and after 15 minutes displayed enhanced green fluorescence (Fig. 5b). We then tested the two-photon imaging ability of **NI-NHS** to detect H₂S in MCF-7 cells. Notably, the background fluorescence of **NI-NHS** is very weak without H₂S (Fig. 6a). Then the cells were incubated with 50 μM NaHS for 15 min. Upon TP excitation at 810 nm, the fluorescence intensity significantly increased (Fig. 6b). The cytotoxicity of **NI-NHS** was examined toward MCF-7 cells by a MTT assay (Figure S6). The results showed that >90% MCF-7 cells survived after 12 h (5.0 μM **NI-NHS** incubation), and after 24 h the cell viability remained at ~80%, demonstrating that **NI-NHS** was of low toxicity toward cultured cell lines. These experiments indicate that **NI-NHS** can act as a two-photo fluorescent probe to detect H₂S in
living cells.

Fig.5

Fig.6

4 Conclusion

In summary, we developed a 1,8-naphthalimide-derived compound \textbf{NI-NHS} as a two-photo fluorescent probe for H$_2$S based on thiolysis of dinitrophenyl ether. Due to the rapid reaction of \textbf{NI-NHS} with H$_2$S, a large fluorescence increase was obtained with emission centered at 550 nm in aqueous solution. Concomitantly, the solution color changed from colourless to yellow. The probe displayed a high selectivity for H$_2$S over competitive reactive sulfur, oxygen, and nitrogen species. This probe is applicable to detect H$_2$S in bovine serum and living cells in TPM mode.

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Supplementary data

Supplementary data related to this article can be found at doi:10.1016/j.dyepig.XXXX.XX.XXX.

References and notes


Fig. 3

Fig. 4

Fig. 5
Scheme 1

Fig. 6