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Graph Abstract

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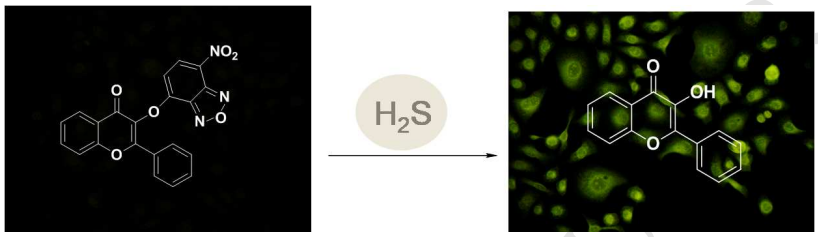

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A highly selective and sensitive 3-hydroxyflavone-based colorimetric and fluorescent probe for hydrogen sulfide with a large Stokes shift

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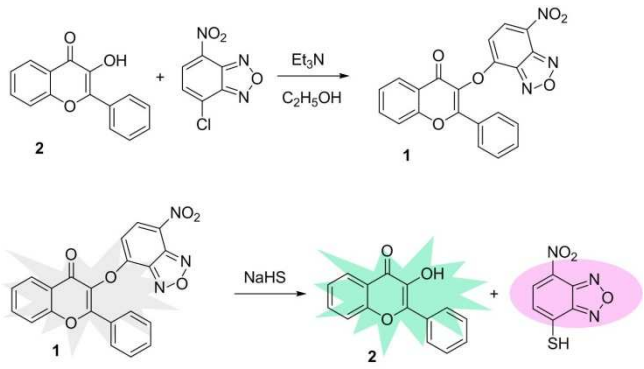
ABSTRACT

A 3-hydroxyflavone-based colorimetric and fluorescence probe for hydrogen sulfide (H_2S) has been developed with high sensitivity and excellent selectivity. This probe was designed based on the mechanism that H_2S selectively cleaved the NBD (7-nitro-2,1,3-benzoxadiazole) ether moiety in this probe and therefore release the fluorophore, 3-hydroxyflavone. The addition of H_2S to the solution of probe **1** resulted in a green fluorescence and an obvious color change from colorless to pink, indicating that this probe can serve as a colorimetric and fluorescent dual probe for H_2S. Furthermore, this probe displays a rapid response and reaches to a plateau within 5 min. It also exhibits a 146 nm Stokes shift and a low detection limit (20 nM, based on S/N = 3) in detecting H_2S. Importantly, practical utility of this probe for the selective detection of H_2S in living cells has been successfully demonstrated.

1. Introduction

Hydrogen sulfide (H_2S) with unpleasant smell is well known as toxic gas which affects the nervous, respiratory, and cardiovascular system of mammals.[1] Recently, it has been demonstrated that H_2S was the third gas signaling molecule after NO and CO, and endogenous H_2S can be formed from cysteine and homocysteine by enzymes catalyzation. It plays vital roles in many physiological and pathological processes because of its remarkable cardio-, retina- and neuron-protective and other biological properties.[2-3] It was reported that the toxic gas can induce the inhibition of cytochrome c oxidization in the mitochondrial electron transport chain. Furthermore, the abnormal levels of H_2S are closely associated with many diseases such as atherosclerosis, Alzheimer's disease, ischemic stroke and Down syndrome.[4-7] Therefore, the selective and sensitive detection of a trace amount of hydrogen sulfide to understand its physiological properties and functions mechanism has gained increasing attention.

Owing to the advantages of sensitivity, rapid response, non-invasiveness and ease of operation,[8-22] fluorescent probes has been a powerful tool to detect and image H_2S in biological systems. The design of fluorescent probes for H_2S is mainly based on the reduction of azide to amine,[23-26] nucleophilic reactions,[27-28] reduction of hydroxyl amine[29], disulfide exchange[30-32], high affinity of S^{2-} to Cu^{2+},[33-34] the thiolysis of 2,4-dinitrophenyl ethers and NBD (7-nitro-2,1,3-benzoxadiazole).[35-38] Due to their low cost, naked-eye mode and simple pretreatment, colorimetric and fluorescent probes have received extensive research attention. However, most of the current fluorescent probes are still only respond to H_2S with the changes in fluorescence intensity. If a probe with color change and fluorescence enhancement could be developed, the biological roles of H_2S would be expected to be clarified greatly.



Scheme 1 Synthetic route to probe **1** and the sensing reaction with NaHS.

3-Hydroxyflavone processes many excellent optical properties

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such as emission in green, good photostability, relatively high quantum yield. Importantly, it features an ESIPT process with a large Stokes shift upon being excited, which was desirable for the design of fluorescent probe.[39] In this work, we designed and synthesized a 3-hydroxyflavone-based dual probe (probe **1**) for the selective detection of H_2S. The preparation of probe **1** was outlined in Scheme 1. We reasoned that probe **1** was essentially none-fluorescent due to the photoinduced electron transfer (PET) process caused by the NBD (7-nitro-2,1,3-benzoxadiazole) ether moiety. We anticipated that H_2S would selectively cleaved the NBD moiety in probe **1** and subsequenlty dye **2** was released, in which process the solution of probe **1** displayed a green fluorescence and an obvious colour change from colourless to pink. As a consequence, probe **1** can serve a colorimetric and fluorescent dual probe for real-time detection of H_2S.

Results and discussion

1.1. Absorption properties

NaHS was used as a standard H_2S source to investigate the absorption spectra changes of probe **1** (10 μM) in PBS buffer (50 mM, pH=7.40, 20% acetonitrile, v/v). As shown in Fig. 1, with the increasing addition of H_2S (0-50 equiv.), the absorption peaks of probe **1** at 370 nm gradually decreased accompanying with a new peak at 540 nm appeared with an isosbestic point at 416 nm (red-shifted 170 nm). This large red-shift may be due to H_2S-specific cleavage of the NBD ether group in probe **1** to yield 7-nitrobenzo-2, 1, 3-oxadiazole-4-thiol (NBD-SH). Upon treatment with 50 equiv. H_2S, the solution of probe **1** instantaneously displayed a pink color, indicating the probe was able to detect H_2S with naked-eye.

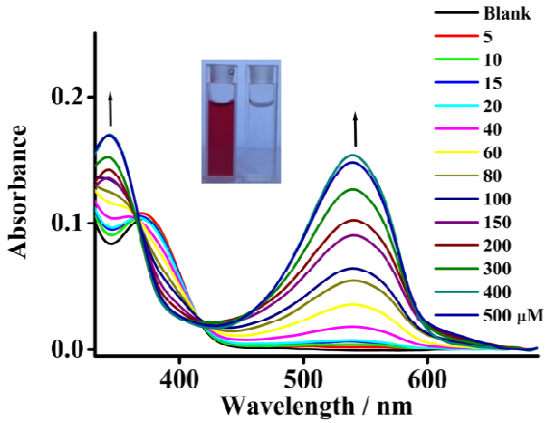

Fig. 1 The absorption spectra of probe **1** (10.0 μM) upon the addition of NaHS (0.0 – 500.0 μM) in PBS buffer. Inset: The visual color changes of probe **1** (10.0 μM) in the absence (right) and presence (left) of NaHS under natural light.

1.2. Fluorescence properties and linearity

The changes in the fluorescence spectra of probe **1** (10 μM) in the absence or presence of H_2S (0-200 μM) was investigated in PBS buffer and the results were displayed in Fig. 2. The probe **1** was essentially non-fluorescent in the absence of H_2S due to the PET process. However, the addition of H_2S caused a dramatic change in the fluorescence spectra. A strong new emission peak at 516 nm appeared, and an enhancement of the fluorescence intensity (25-fold) was observed with a concomitant appearance of green emission color under illumination with a 365 nm UV lamp. This may attribute to H_2S-induced the cleavage of the NBD ether group in probe **1** and thereby eliminating the PET process

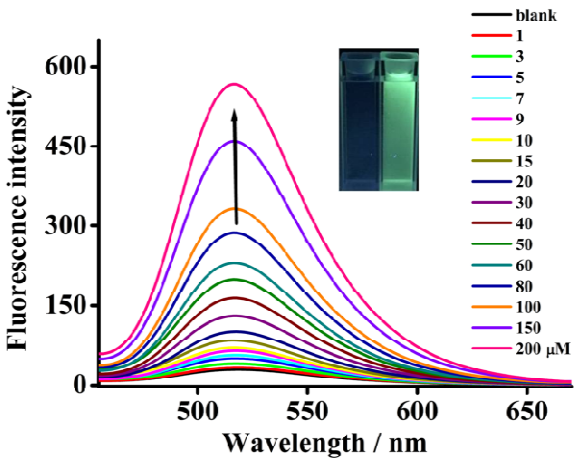

Fig. 2 Fluorescence response (λ_{ex}^{max} = 346 nm, λ_{em}^{max} = 516 nm) of probe **1** (10.0 μM) upon the addition of NaHS (0.0 – 200.0 μM) in PBS buffer. Inset: Fluorescence images of probe **1** (10.0 μM) in the absence (left) and presence (right) of NaHS under a 365 nm UV lamp.

to generate the 3-hydroxyflavone **2**. Moreover, the reaction mechanism was strongly supported by the mass spectrum analysis on the reaction product of probe **1** with H_2S. The MS spectrum of the reaction product displayed a peak at m/z = 239.1 (Fig. S3), which was identical to the exact weight of reference dye **2** ($[M + H]^+$ = 239. 1). Furthermore, a good linear relationship was obtained between the fluorescence intensity and the concentration of H_2S from 1.0 to 10.0 μM (Fig. 3). The linear equation was found to be y = 4.0022 + 28.8395x (R = 0.9987) and the detection limit was 20 nM based on S/N = 3. These results suggested that probe **1** could quantitatively detect H_2S.

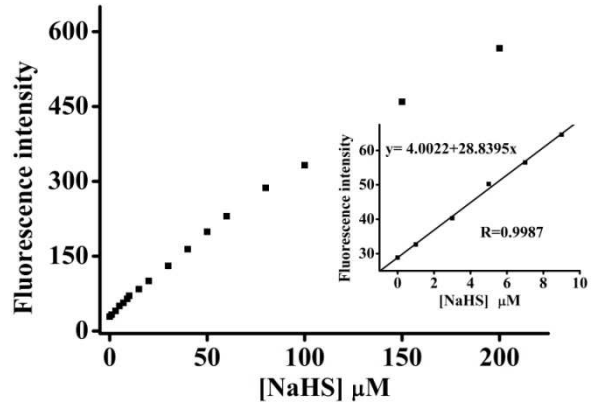

Fig. 3 Fluorescence intensity of probe **1** (10.0 μM) at 516 nm as a function of NaHS concentration in PBS buffer. Inset: The linear relationship between fluorescence intensity and NaHS at low concentrations.

1.3. Time-dependent fluorescence spectra

The time-dependent fluorescence spectra of probe **1** with H_2S were investigated by monitoring the fluorescence intensity at 516 nm as a function of time. As shown in Fig. 4, fluorescence intensity increased with the increase of reaction time after the addition of different concentration of H_2S (0.7, 2, 5, 10, 20 equiv.) to the solution of probe **1**. Upon the addition of 20 equiv. of H_2S, the fluorescent enhancement instantaneously initiated and reached to plateau within 5 min, indicating that probe **1** was capable of rapidly detecting H_2S in aqueous media.

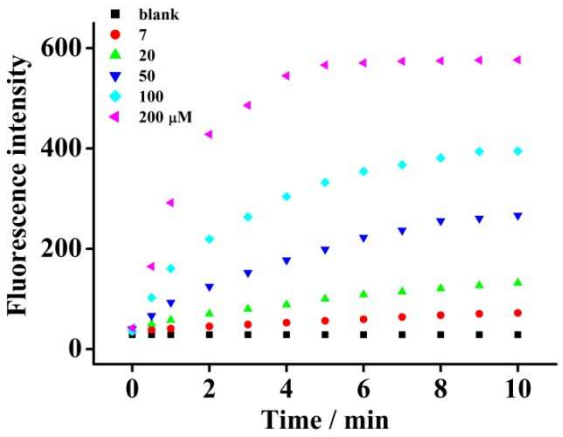

Fig. 4 Time-dependent fluorescence intensity of probe **1** (10.0 μM) at 516 nm with different concentration of NaHS (7, 20, 50, 100, 200 μM) in PBS buffer.

1.4. Selectivity and competition studies

Next, we evaluated the selectivity of probe **1** (10.0 μM) for H_2S over other relevant analytes including F^-, NO_3^-, OAc^-, $S_2O_3^{2-}$, Cl^-, NO_2^-, HCO_3^-, SCN^-, citrate, Cys, GSH, $H_2PO_4^-$, SO_4^{2-}, Ca^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, H_2O_2, ClO^-, CN^-, $S_2O_4^{2-}$, N_3^-. As shown in Fig. 5, only H_2S induced a prominent fluorescence enhancement of the probe at 516 nm, whereas no obvious fluorescence changes were observed for other relevant analytes. It's noteworthy that this probe was able to detect H_2S over Cys and GSH which was the main competitive species in biological systems. However, there are still many challenges in optical analysis of H_2S in real biological systems.[40] Moreover, the fluorescence responses of probe **1** to H_2S in the presence of typical competition analytes were studied to confirm the effective application of the probe **1**. The test result was displayed in Fig. 6. All of competing analytes had little interference in the detection of H_2S. This suggested that probe **1** was a highly selective H_2S probe over other relevant analytes.

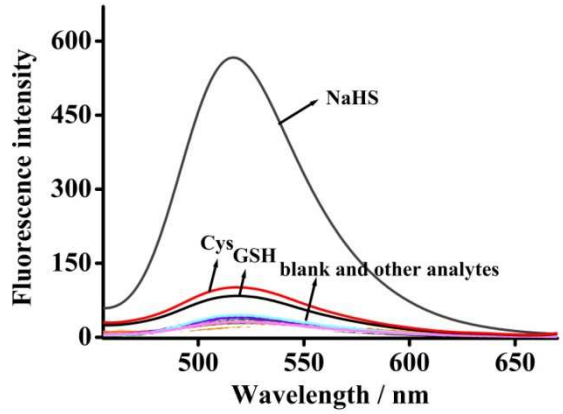

Fig. 5 Fluorescence response (λ_{ex}^{max} = 346 nm, λ_{em}^{max} = 516 nm) of probe **1** (10.0 μM) upon the addition of different tested analytes (NaHS: 200.0 μM, other tested analytes: 500.0 μM (F^-, NO_3^-, OAc^-, $S_2O_3^{2-}$, Cl^-, NO_2^-, HCO_3^-, SCN^-, citrate, Cys, GSH, $H_2PO_4^-$, SO_4^{2-}, Ca^{2+}, Cu^{2+}, Mg^{2+}, Zn^{2+}, H_2O_2, ClO^-, CN^-, $S_2O_4^{2-}$, N_3^-) in PBS buffer.

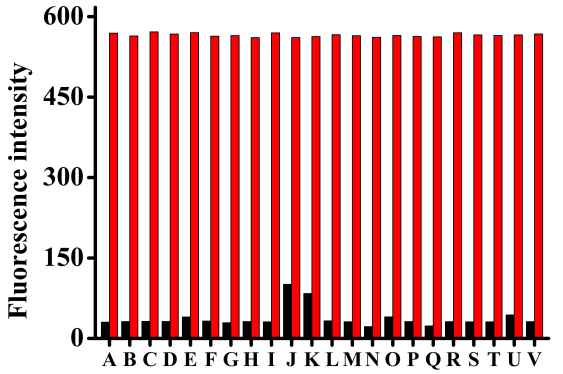

Fig. 6 The fluorescence response of probe **1** (10.0 μM) in response to the tested analytes (black bars) and NaHS (200.0 μM) with the competition analytes (500.0 μM A. F^-, B. NO_3^-, C. OAc^-, D. $S_2O_3^{2-}$, E. Cl^-, F. NO_2^-, G. HCO_3^-, H. SCN^-, I. citrate, J. Cys, K. GSH, L. $H_2PO_4^-$, M. SO_4^{2-}, N. Ca^{2+}, O. Cu^{2+}, P. Mg^{2+}, Q. Zn^{2+}, R. H_2O_2, S. ClO^-, T. CN^-, U. $S_2O_4^{2-}$, V. N_3^-) in PBS buffer.

1.5. Fluorescent imaging of living A431 cells

To explore the application ability of the probe **1** towards H_2S in biological samples, the living A431 cells were determined to monitor the behavior of the probe **1** recognized with H_2S using fluorescence microscopy. It can be seen in Fig. 7 that only very weak fluorescence was observed when cells were incubated with probe **1** for 30 min at 37℃. In contrast, an intense yellow fluorescence was produced when the cells were pre-treated with 100 μM H_2S for 30 min at 37℃ and then incubated with 5.0 μM probe **1** for another 30 min. The above results demonstrate that probe **1** could be used as a reliability and practicality possible sensor to image H_2S in living cells.

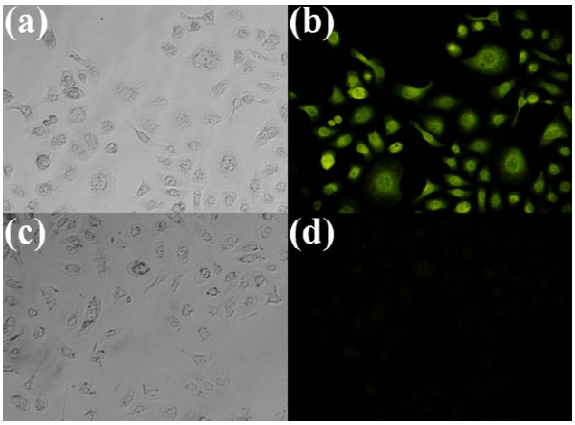

Fig. 7 Fluorescence images of A431 cells incubated with 5.0 μM probe **1** for 30 min at 37℃ (bottom row) and pre-treated with 100.0 μM NaHS, followed by incubation with probe **1** for 30 min at 37℃ (top row). (a, c) Bright field images; (b, d) Fluorescence images.

2. Conclusions

In conclusion, we have developed a 3-hydroxyflavone-based fluorescent probe, **1**, for the detection of H_2S with high sensitivity and selectivity. This probe can serve as a colorimetric and fluorescent dual probe for H_2S due to the colour and fluorescence induced by the interaction of probe **1** with H_2S. It also displays a rapid response, a low detection limit (20 nM), and a large Stokes shift (146 nm). Importantly, the probe was

successfully demonstrated to the practical utility in the imaging of H_2S in living A431 cells.

3. Experimental section

3.1. Instruments and Materials

Unless otherwise stated, solvents were purified by standard methods prior to use. All reagents were purchased from commercial suppliers and used as received. Twice-distilled water was used throughout all experiments. NMR spectra were recorded on a BRUKER 500 or 600 spectrometer, All accurate mass spectrometric experiments were performed on a micrOTOF-Q II mass spectrometer (BrukerDaltonik, Germany). UV-Vis absorption spectra were measured using a Shimadzu UV-2450 spectrophotometer. Uncorrected emission spectra were recorded at room temperature on a HITACHI F4600 fluorescence spectrophotometer with both the excitation and emission slit widths set at 5.0 nm. Cell imaging was performed with an Olympus 71X microscope. TLC analysis was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200–300), both of which were obtained from Qingdao Ocean Chemicals.

*3.2. Synthesis of Probe **1***

A mixture of 3-hydroxyflavone **2** [41] (238 mg, 1.0 mmol) and NBD-Cl (199 mg, 1.0 mmol) was dissolved in 20 mL ethanol. Triethylamine (121 mg, 1.2 mmol) was added to the reaction mixture, and the resultant reaction mixture was stirred at 45℃ overnight. The obtained solid was isolated by filtration, washed with 10 mL ethanol and yielding a brown solid **1** (248 mg, 62%). ^{1}H NMR (500 MHz, DMSO) δ 8.61 (d, J = 8.4 Hz, 1H), 8.12 (dd, J = 7.9, 1.3 Hz, 1H), 7.95 (ddd, J = 15.8, 11.6, 7.4 Hz, 4H), 7.65 – 7.50 (m, 4H), 7.34 (d, J = 8.4 Hz, 1H). ^{13}C NMR (150 MHz, DMSO) 171.37, 157.71, 155.82, 151.37, 145.18, 144.81, 135.49, 135.44, 135.25, 132.45, 131.48, 129.51, 128.82, 126.33, 125.66, 123.81, 119.35, 110.71. HRMS (EI) m/z calcd for $[C_{21}H_{11}N_3O_6 + Na]^+$: 424.0648, Found : 424.0549.

3.3. Imaging of A431 cells

A431 cells were seeded in a 12-well plate in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin, incubated under the atmosphere of 5% CO_2 and 95% air at 37 ℃ for 24 h. Immediately before the experiments, the cells were washed with PBS buffer. the cells were pre-incubated with NaHS (100.0 μM) for 30 min. After washing with PBS three times, A431 cells were then incubated with the probe (5.0 μM) for 30 min at 37 °C. For the control experiment, A431 cells were incubated with the probe (5.0 μM) for 30 min at 37 °C, and Fluorescence imaging was performed after washing the cells three times with PBS buffer. The fluorescence images were obtained using Olympus 71X microscope for collecting experiment images.

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