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A fluorescent probe for discrimination of cysteine/homocysteine, glutathione and hydrogen polysulfides

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Abstract

Biothiols of cysteine (Cys), homocysteine (Hcy), glutathione (GSH) and hydrogen polysulfides (H_2S_n, $n>1$) play vital roles in physiological and pathological processes. In this manuscript, a fluorescent probe (compound **1**) for simultaneous detection and discrimination of Cys/Hcy, GSH and H_2S_n was developed, employing fluorophore of 1-(benzo[d]thiazol-2-yl)naphthalen-2-oxy linked with 7-nitrobenzo-2-oxa-1,3-diazole (NBD) as a response unit and a quencher. Compound **1** exhibited different ratiometric colorimetric responses toward GSH at 430 nm and 375 nm from Cys/Hcy at 480 nm and 375 nm, while it displayed a different colorimetric response at 561 nm toward H_2S_n. Meanwhile it could act as a naked-eye probe toward GSH, Cys/Hcy and H_2S_n with corresponding solution color of light yellow, orange and purple, respectively. Upon excitation at 370 nm, compound **1** responded to all these biothiols with 'turn-on' blue fluorescence, whereas upon excitation at 470 nm, it could respond to Cys/Hcy with 'turn-on' green fluorescence. Data of mass spectra, lifetimes and ^{1}H NMR spectra interpreted the aromatic nucleophilic substitutions of compound **1** by GSH, Cys/Hcy and H_2S_n to produce fluorophore1-(benzo[d]thiazol-2-yl)naphthalen-2-ol (compound **2**) and corresponding NBD derivatives which could be discriminated through their absorption and fluorescence signals as well as solution colors. Furthermore, compound **1** was applied to image Cys/Hcy and H_2S_n/GSH in 786-O cells.

Keywords Fluorescent probe · Biothiols · Discrimination · Ratiometric colorimetric responses · Aromatic nucleophilic substitutions

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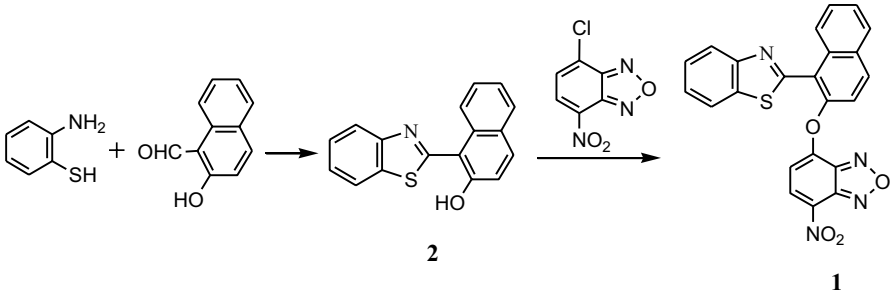
Introduction

Reactive sulfur species (RSS) are essential in many respects of biological redox homeostasis, cell signaling and the regulation of metabolism. Important RSS include cysteine (Cys), homocysteine (Hcy), glutathione (GSH), hydrogen sulfide (H_2S), and hydrogen polysulfides (H_2S_n, $n > 1$) et al. [1–3], endogenously distributed in living cells. Among these RSS, H_2S has been recognized as the third critical gaseous molecule besides carbon monoxide (CO) and nitrogen (II) oxide (NO) in biological systems. Explorations in recent years have demonstrated that both the endogenously produced H_2S and the exogenously administrated H_2S would cause protective effects during the course of many pathologies [4–6]. H_2S_n ($n > 1$) have recently aroused a lot of interest, because they were found to act as the real regulators rather than H_2S in cellular signaling transduction and H_2S may be the final product of H_2S_n in physiological activity [7–10]. H_2S_n and H_2S are redox partners and possibly coexist in biological systems, and they work together to regulate sulfur redox balance [11]. An active species of H_2S_n was reported to be hydrogen disulfide (H_2S_2) [12].

Thus, the development of novel techniques to monitor H_2S_n in living systems is essential for good investigation of their contribution to physiology and pathology. The usage of fluorescence spectroscopy in the analysis of H_2S_n is interesting to analyze their pathological/toxicological effects in living systems [13]. Based on the high nucleophilicity and reducing properties of H_2S_n, many research groups have developed fluorescent selective probes for H_2S_2/S_2^{2-} in recent years [14–22].

RSS of Cys and Hcy are well-known precursors of H_2S, which can be endogenously produced by enzymes such as cystathionine β-synthase and cystathionine γ-lyase, respectively, exhibiting vital functions in the regulation of matrix degradation and cell motility [23–25]. Peptides such as GSH play important roles in combating oxidative stress and defending against free radicals to protect thiol proteins and enzymes from oxidation in biological systems [26, 27]. Due to similar chemical structures and reaction activities of these biothiols, it is very challenging to distinguish Cys/Hcy, GSH and H_2S_n from one another. Although fluorescent probes for selectively detecting Cys/Hcy, GSH or H_2S_n have been developed, till now, there are rare reports about distinguishing Cys/Hcy, GSH and H_2S_n from one another by use of a single fluorescent probe [28–30].

Several reported fluorescent probes that could distinguish Cys/Hcy from GSH or H_2S were fluorophores linked with 7-nitrobenzo-2-oxa-1,3-diazole (NBD) ether moities as electrophilic reactive sites for Cys/Hcy, GSH or H_2S to generate different NBD-biothiols and leaving groups in the thiol-induced aromatic nucleophilic substitution as well as fluorescence quenchers for fluorophores to provide a low fluorescence background with the photo-induced electron transfer (PET) process [31–42]. However, detecting for hydrogen polysulfides were not involved in these NBD ether-based fluorescent probes. In this manuscript, a fluorescent probe (compound **1**) for simultaneous detection and discrimination of Cys/Hcy, GSH and H_2S_n was developed by introducing a fluorophore of 1-(benzo[d]thiazol-2-yl)naphthalen-2-ol (compound **2**) with a NBD moiety via an ether linker (Scheme 1).



Scheme 1 Synthesis route of probe **1**

Experimental

Reagents and apparatus

Dimethyl sulfoxide (DMSO) of spectroscopic grade and deionized water (distilled) were used throughout the experiment. All chemicals of analytical grade for syntheses were purchased from commercial suppliers and were used without further purification. ^{1}H NMR and ^{13}CNMR spectra were recorded with a 400 MHz Varian spectrometer. Mass spectra were measured on a Waters SYNAPT G2-Si mass spectrometer with ion source of MALDI and α-cyano-4-hydroxycinnamic acid as the main matrix. Absorption spectra were obtained on a TU1901 Ultraviolet–visible spectrophotometer. The fluorescence spectra were measured with a Cary Eclipse fluorescence spectrometer. The absolute fluorescence quantum yields were measured with a Hamamatsu Absolute PL Quantum Yield Spectrometer C11347. The lifetimes were recorded with a Hamamatsu Compact fluorescence lifetime spectrometer C11367. Cell imaging experiments were conducted on an Olympus FV3000 laser scanning confocal microscope. Measurements of pH were made on a pH S-3C pH meter.

Syntheses

1-(Benzo[d]thiazol-2-yl)naphthalen-2-ol (compound **2**) was synthesized according to the reported literature [43]. Compound **2** (0.1390 g, 0.5 mmol), NBD-Cl (0.12 g, 0.6 mmol) and two drops of Et_3N in CH_2Cl_2 (20 ml) were stirred at room temperature for 24 h. Then, the mixture solution was evaporated under reduced pressure and purified by silica chromatography (eluted with petroleum/ethyl acetate = 4/1, v/v) to afford compound **1** (0.088 g, 40%). ^{1}H NMR ($CDCl_3$, δ, ppm), 8.34 (d, J = 8.0 Hz, 1H), 8.17 (d, J = 4.0 Hz, 1H), 8.13 (d, J = 4.0 Hz, 1H), 8.01–8.04 (m, 2H), 7.88 (d, J = 4.0 Hz, 1H), 7.62–7.66 (m, 2H), 7.51(t, J = 4.0 Hz, 1H), 7.42–7.46 (m, 2H), 6.60 (d, J = 8.0 Hz, 1H) (see the Supporting information, Fig. S1). ^{13}CNMR ($CDCl_3$, δ, ppm), 160.15, 153.46, 153.03, 148.44, 144.84, 144.05, 136.05, 133.41, 132.96, 132.29, 131.10, 128.71, 128.44, 126.43, 125.87, 123.68, 123.34, 121.57, 119.57,

108.90 (Fig. S2). HR-ESI–MS, calculated for $C_{23}H_{12}N_4O_4S$: 440.0579, found: 441.0657 [M + H^+], 463.0476 [M + Na^+] (Fig. S3).

Preparation of solutions

Sodium polysulfide solution was prepared from sodium sulfide and sulfur in deionized water based on the literature [44].

The stock solutions of each anion (5 mM) of S^{2-}, SO_3^{2-}, HSO_3^-, $S_2O_5^{2-}$, SO_4^{2-}, HSO_4^-, NO_3^-, F^-, Cl^-, I^-, CO_3^{2-}, HCO_3^-, OAc^-, HPO_4^{2-}, NO_2^- and S_2^{2-} were prepared in ultrapure water from their sodium or potassium salts. Amino acid solutions (5 mM) of alanine (Ala), arginine (Arg), cysteine (Cys), glutamic acid (Glu), glutamine (Gln), glutathione (GSH), glycine (Gly), histidine (His), homocysteine (Hcy), lysine (Lys), methionine (Met), phenylalanine (Phe), serine (Ser) and tyrosine (Tyr) were prepared in ultrapure water. Stock solution of compound **1** (1 mM) was prepared in DMSO. 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer solutions (10 mM) were prepared by dissolving HEPES in deionized water, and the value of pH of the solution was adjusted by NaOH or HCl. For a typical optical measurement, a solution of **1** (20 μM) was prepared in HEPES buffer (10 mM, pH 7.0, with 30% DMSO, v/v). Then 3 mL of this solution was put into a quartz cuvette with an optical path of 1 cm. The stock solution of each analyte was added into the quartz cuvette via a microsyringe, and the solution was mixed before measurement of the spectra. All the measurement experiments were performed at 298 K.

Cell culture and fluorescence imaging experiments

786-O cells were incubated in 90% of RPMI-1640 (GIBCO, stock No. 31800022) supplemented with 10% of fetal bovine serum at 37 °C in an atmosphere of 5% CO_2 and 95% air. For cell imaging, cells were pre-treated with probe **1** (10 μM) for 30 min and then with 100 μM analytes for another 30 min. And cells incubated only with probe **1** (10 μM) for 30 min were used as the control experiment. All the fluorescence images were collected after cells were washed twice with PBS buffer.

Results and discussion

Rational design of compound 1

Due to the strong electron-withdrawing ability of nitro group on NBD moiety, the PET process in compound **1** could be prohibited, resulting in no emission [45]. Interaction of compound **1** with Cys/Hcy may break the ether bond based on aromatic thiolysis of NBD, releasing the fluorophore 1-(benzo[d]thiazol-2-yl)naphthalen-2-ol (compound **2**) with the excited-state intramolecular proton transfer (ESIPT) properties and NBD-*S*-Cys/NBD-*S*-Hcy, followed by amino-substituted NBD chromophores NBD-*N*-Cys/NBD-*N*-Hcy after sequential nucleophilic substitution

and intramolecular rearrangement, which will lead to fluorescence 'turn-on' in blue and green spectral region, respectively, upon exciting with different wavelengths. The thiol group in GSH could induce the break of ether group and form compound **2** too and NBD-*S*-GSH. However, NBD-*S*-GSH would not undergo intramolecular rearrangement and not emit fluorescence. H_2S_n ($n > 1$) could also react with compound **1** due to its strong nucleophilicity. The ether bond of compound **1** would also be broken to form a NBD-$(S)_n$H chromophore with a visible purple color of the solution, which is non-emissive, and a free 1-(benzo[d]thiazol-2-yl)naphthalen-2-ol (compound **2**) with an emission band in blue region. Thus, compound **1** could simultaneously discriminate Cys/Hcy, GSH with different excitation wavelengths and H_2S_n with well-defined spectral signals.

Spectral responses of compound 1 toward Cys/Hcy, GSH and H_2S_n

The absorption responses of compound **1** (20 μM) to various anions (100 μM) and amino acids (100 μM) (10 mM, pH 7.0, with 30% DMSO, v/v) were measured (Fig. 1 and Fig. S4). Compound **1** showed a main absorption peak at 375 nm in HEPES buffer, attributing to the absorption from the 1-(benzo[d]thiazol-2-yl)naphthalen-2-oxy chromophore. In the presence of H_2S_n (5 eq.), a new strong absorption band around 561 nm could be observed, accompanied by a color change of the solution from colorless to purple (Fig. 2). However, in the presence of S^{2-} (5 eq.), there was a much weak absorption band around 561 nm. And addition of other anions, respectively, to compound **1** caused little change in the absorption spectra (Fig. S4a). Besides, the absorption spectra of compound **1** in the presence of Cys/Hcy exhibited a strong peak around 480 nm (Fig. 1), corresponding to a color change from colorless to orange**,** which is much different from the solution of **1**—H_2S_n (Fig. 2). The absorption band of compound **1** in the presence of GSH is rather weak

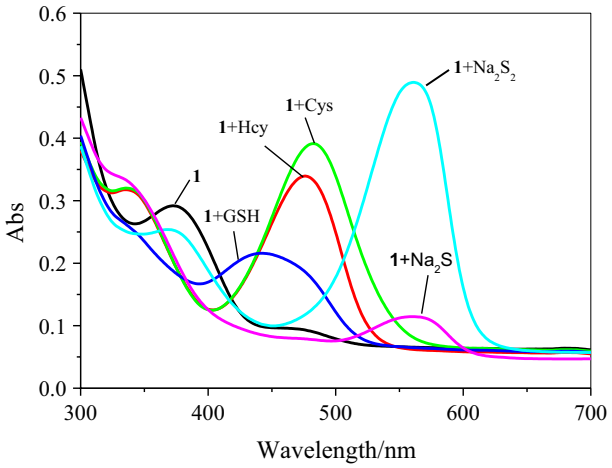


Fig. 1 Absorption spectra of **1** (20 μM) in the absence and presence of Cys, Hcy, GSH, Na_2S_2, Na_2S (100 μM) in HEPES buffer (10 mM, pH 7.0, with 30% DMSO, v/v). (Color figure online)

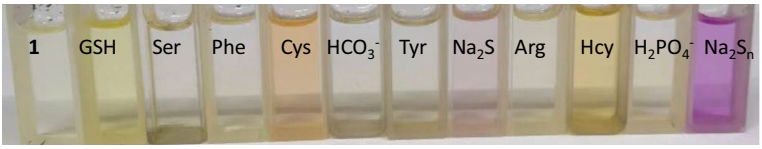


Fig. 2 Photograph of compound **1** in the presence of indicated analytes. (Color figure online)

around 430 nm, with a color change from colorless to yellow. However, addition of other amino acids, respectively, to compound **1** changed little of the absorption spectra (Fig. S4b). As different absorption peaks suggested the formation of different products after the reaction of **1** with these biothiols, the results of absorption measurements indicated that compound **1** could colorimetrically discriminate H_2S_n, Cys/Hcy and GSH from other amino acids.

To test the sensing ability of probe **1** toward H_2S_n, Cys/Hcy and GSH, different concentrations of these analytes were introduced and the response was evaluated by absorption spectra, respectively. As shown in Fig. 3, there were no absorption bands in 400–700 nm for free **1** in HEPES buffer (10 mM, pH 7.0, with 30% DMSO, v/v)**.** The new absorption peak at 561 nm appeared and the absorbance increased with the gradual addition of Na_2S_2 (Fig. 3a). It was shown that the absorbance at 561 nm was linearly proportional ($R^2 = 0.9933$, inset in Fig. 3a) to the concentrations of Na_2S_2 concentration (0–24 μM). The detection limit (3σ/K) was 1.1 μM. Upon titration with Cys/Hcy, the absorption of probe **1** displayed a decrease at 375 nm accompanied by a drastic increase at 480 nm, with three isobestic pints 310 nm, 360 nm and 410 nm (Fig. 3b and c). The absorbance ratios at 480 nm and 375 nm (A_{480}/A_{375}) exhibited a drastic change from 0.30 to 1.56 in the presence of 1 eq. Cys (or from 0.30 to 1.45 in the presence of 1 eq. Hcy). Ratiometric responses of A_{480}/A_{375} to the concentration of Cys/Hcy were observed, which exhibited good linear relationships between A_{480}/A_{375} and the concentration of Cys (0 – 20 μM, $R^2 = 0.9951$) or Hcy (0 – 30 μM, $R^2 = 0.9955$), respectively, and the detection limit was 0.56 μM (Cys) and 0.85 μM (Hcy), respectively. Upon titration with GSH, the absorbance of probe **1** displayed a decrease at 375 nm accompanying with an increase at 430 nm, with two isobestic points at 347 nm and 397 nm (Fig. 3d). The absorbance ratios at 430 nm and 375 nm (A_{430}/A_{375}) exhibited a moderate change from 0.30 to 1.10 in the presence of 1 eq. GSH. The linear relationship between A_{430}/A_{375} and GSH concentration (0 – 24 μM, $R^2 = 0.9977$) was observed, and a detection limit of 0.61 μM was obtained. Thus, the change in absorption bands and solution colors after the interaction of compound **1** with these biothiols make it possible to discern Cys/Hcy from H_2S_n and GSH with quantitative determination.

The fluorescence responses of compound **1** (20 μM) to anions (100 μM) and amino acids (100 μM) in HEPES buffer (10 mM, pH 7.0, with 30% DMSO, v/v) were studied upon exciting at 370 nm and 470 nm (Fig. 4 and Fig. S5), respectively. Compound **1** was almost nonfluorescent, and the absolute fluorescence quantum yield (Φ) is less than 0.001, indicating that the fluorescence of the fluorophore 1-(benzo[d]thiazol-2-yl)naphthalen-2-oxy was quenched by the linking NBD moiety. Upon addition of Na_2S_2, Cys, Hcy and GSH, respectively, to the solution of

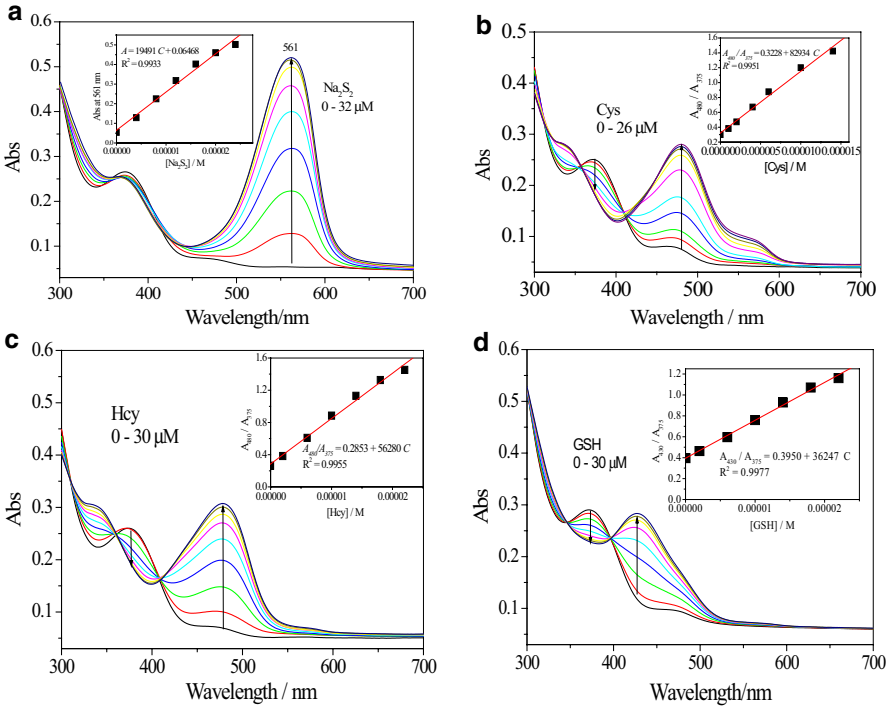


Fig. 3 Concentration-dependent absorption spectra of compound **1** (20 μM) upon titration with Na_2S_2 (**a**, inserted the linear relationship between absorbance at 561 nm and Na_2S_2 concentration), Cys (**b**, inserted the linear relationship between ratio of absorbance at 480 nm to absorbance at 375 nm and Cys concentration), Hcy (**c**, inserted the linear relationship between ratio of absorbance at 480 nm to absorbance at 375 nm and Hcy concentration), GSH (**d**, inserted the linear relationship between ratio of absorbance at 430 nm to absorbance at 375 nm and GSH concentration), respectively, in HEPES buffer (10 mM, pH 7.0, with 30% DMSO, v/v). (Color figure online)

compound **1**, when excited at 370 nm, very strong fluorescence in blue channel appeared at 468 nm (Fig. 4a, b), with the absolute fluorescence quantum yields of 0.060, 0.043, 0.048, and 0.053, respectively, indicating the cleavage of the ether linkage of probe **1** in the presence of these biothiols and producing of fluorophore 1-(benzo[d]thiazol-2-yl)naphthalen-2-ol (compound **2**). The emission shoulders near 418 nm and main peaks at 468 nm were attributed to the enol and keto form of compound **2** due to its ESIPT process. Besides, very strong fluorescence in green channel of probe **1** at 548 nm (excited at 470 nm) in the presence of Cys and Hcy was observed (Fig. 4c), with the absolute fluorescence quantum yields of 0.127 and 0.138, respectively. Thus, the emission shoulders in Fig. 4b near 540 nm for **1**-Cys and **1**-Hcy system could be the combination of emissions from two emissions. In contrast, when excited at 470 nm, there was very weak fluorescence of probe **1** upon interaction with GSH ($\Phi = 0.011$), and almost no fluorescence change upon its interaction with Na_2S_2 ($\Phi = 0.002$) or with Na_2S ($\Phi = 0.001$). The presence of other amino acids and anions caused almost no increase in fluorescence intensity of

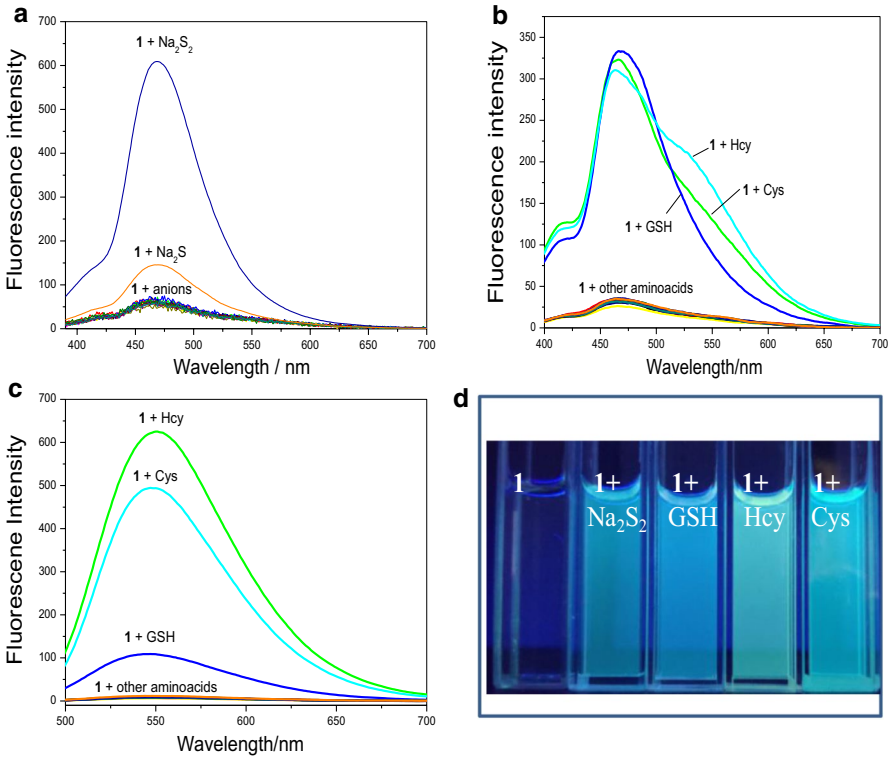


Fig. 4 Fluorescence spectra of **1** (20 μM) in the absence and presence of analytes (100 μM) in HEPES buffer (10 mM, pH 7.0, with 30% DMSO, v/v). **a** Anions excited at 370 nm. **b** amino acids, excited at 370 nm. **c** amino acids, excited at 470 nm. **d** Photograph of compound **1** in the presence of indicated analytes (λ_{ex} = 370 nm). (Color figure online)

1 when excited at either 370 nm or 470 nm. Therefore, the change in fluorescence bands after the interaction of compound **1** with these biothiols makes it possible to discern Cys/Hcy from H_2S_n and GSH under different excitation wavelengths.

The fluorescence of compound **1** was measured by the fluorescence titration experiment in order to identify its performance. As shown in Fig. 5, in a certain range, the fluorescence intensity of **1** increased gradually with increased concentrations of Cys/Hcy, GSH and H_2S_n, respectively, in the blue channel (Fig. 5a, c, e and f) when excited at 370 nm. Good linear relationships were obtained between the fluorescence intensities of compound **1** (20 μM) and the concentration of these thiols within a certain range (10 μM for Cys, 16 μM for Hcy, 25 μM for GSH, and 16 μM for Na_2S_n). The detection limits were 0.17 μM for Cys, 0.30 μM for Hcy, 0.32 μM for GSH, and 0.30 μM for H_2S_n when excited at 370 nm, calculated based on the equation DL = 3σ/*K*, where σ is the standard deviation of a blank measurement and *K* is the slope between fluorescence intensity at 468 nm and analyte concentration. Besides, the fluorescence intensity of **1** increased indicating gradually following increased concentrations of Cys/Hcy in the green channel (Fig. 5b and d) when

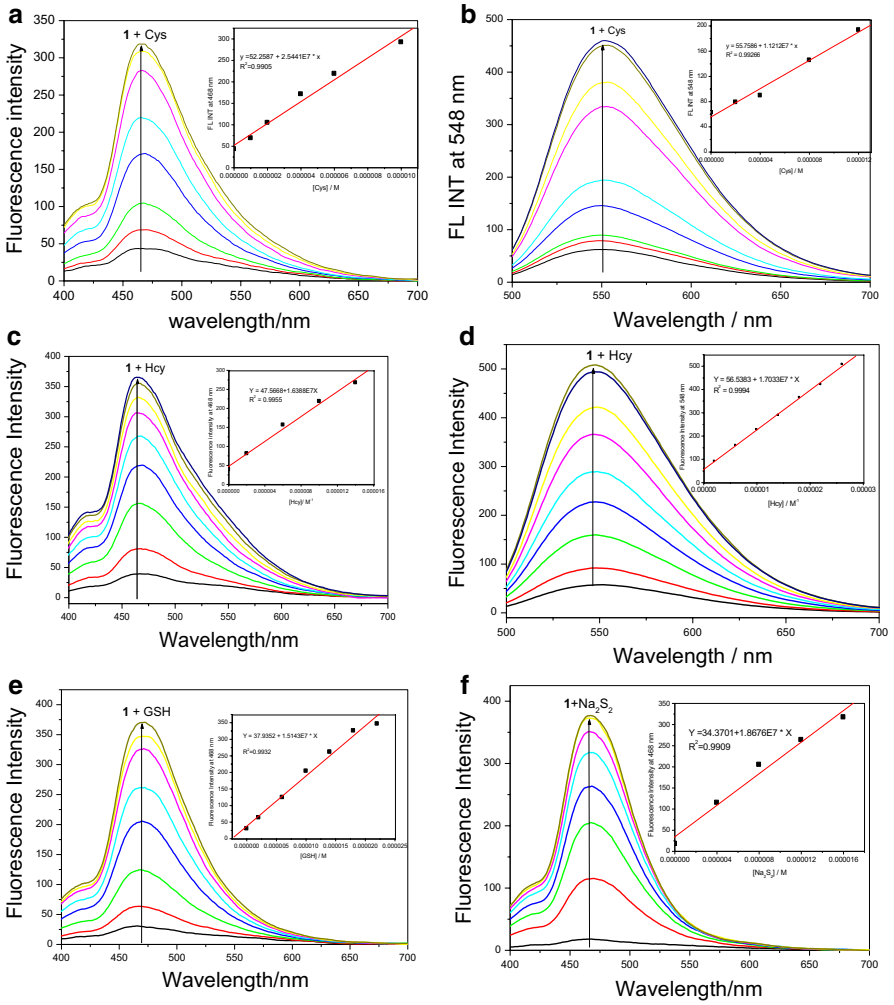


Fig. 5 Concentration-dependent fluorescence of **1** (20 μM) with Cys (**a**), Hcy (**c**), GSH (**e**), H_2S_n (**f**) upon exciting at 370 nm, and with Cys (**b**), Hcy (**d**) upon exciting at 470 nm. Insets: plot of the linear relationship between the fluorescence intensity at respective wavelengths (468 nm, 548 nm) and the low concentration of biothiols. (Color figure online)

excited at 470 nm. Good linear relationships were obtained between the fluorescence intensities of probe **1** and the concentration of these thiols within 12 μM. The results indicated that probe **1** could quantitatively detect these thiols at low concentrations.

The time- and pH-dependent experiments of the probe **1** to H_2S_n, Cys/Hcy and GSH, respectively, were carried out. The kinetic studies indicated that in the presence of Cys or H_2S_n, the fluorescence intensity of **1** at 468 nm showed the fastest growth rate and reached a plateau within 15 min (Fig. S5). In the first 15 min interval, the relative emission intensities (calculated according to the equation: Relative

Emission Intensity = F / F_0, where F was Emission intensity λ = 468 nm after addition of analyte, F_0 = Emission intensity of the probe **1** at λ = 468 nm without the addition of any analyte) were 9.98 and 7.72 for **1**-Na_2S_n and **1**-Cys systems, respectively, whereas the relative emission intensities increased 2.33 and 1.80 for **1**-Hcy and **1**-GSH systems, respectively. The response rate of **1** toward Cys, H_2S_n was faster than Hcy, GSH. The nucleophilicities of the biothiols could be attributed to different response rates [45]. The lowest pK_a value of H_2S_2 ($pK_a = 5.0$) among these analytes rendered H_2S_2 highly nucleophilic as a thiolate anion (HS_2^-) under neutral condition [46]. In addition, in the presence and absence of H_2S_n, Cys/Hcy and GSH, the fluorescence intensity at 468 nm remained stable over a pH range from 4.5 to 8.0 (Fig. S6), indicating that compound **1** could detect these thiols in the physiological pH range.

The fluorescence intensity at 468 nm of compound **1** with the addition of anions, Cys/Hcy, GSH, other amino acids and were monitored upon exciting at 370 nm in order to investigate the selectivity of compound **1** toward H_2S_n. As shown in Fig. 6, introduction of 1 equivalent of H_2S_n, Cys/Hcy and GSH to a solution of **1** resulted in an increase in fluorescence intensity, with about sixfold enhancements. Anions of sulfites (SO_3^{2-} and HSO_3^-) and pyrosulfites interfere the fluorescence response of **1** toward H_2S_n, possibly due to oxidation–reduction reactions of these anions with polysulfides. The fluorescent intensity of **1** toward other anions or amino acids was much lower than those of H_2S_n, Cys/Hcy and GSH, and showed almost no interfere upon detecting these biothiols. The presence of other amino acids would not interfere the detection of Hcy either (Fig. S7).

Possible interaction mechanisms

As shown in Scheme 2, electrophilic NBD derivative of compound **1** underwent nucleophilic substitution with biothiols Cys/Hcy/GSH to produce compound **2** (which can lead to the fluorescence enhancement at 468 nm upon excitation at 370 nm) and non-fluorescent thiolate derivatives (such as NBD-*S*-Cys, NBD-*S*-Hcy, or NBD-*S*-GSH, respectively), and then the amino moiety in NBD-*S*-Cys,

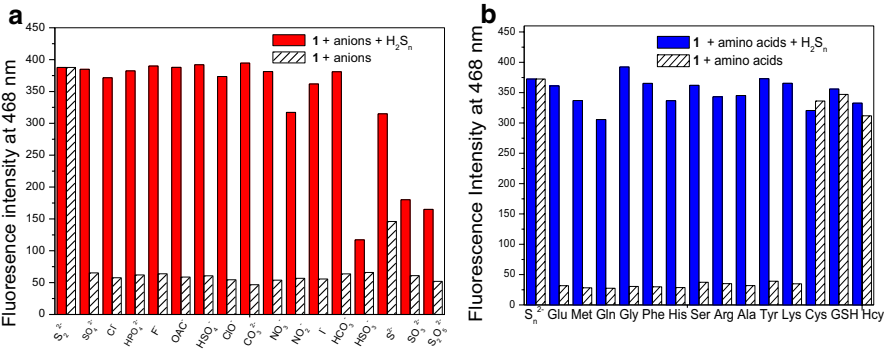
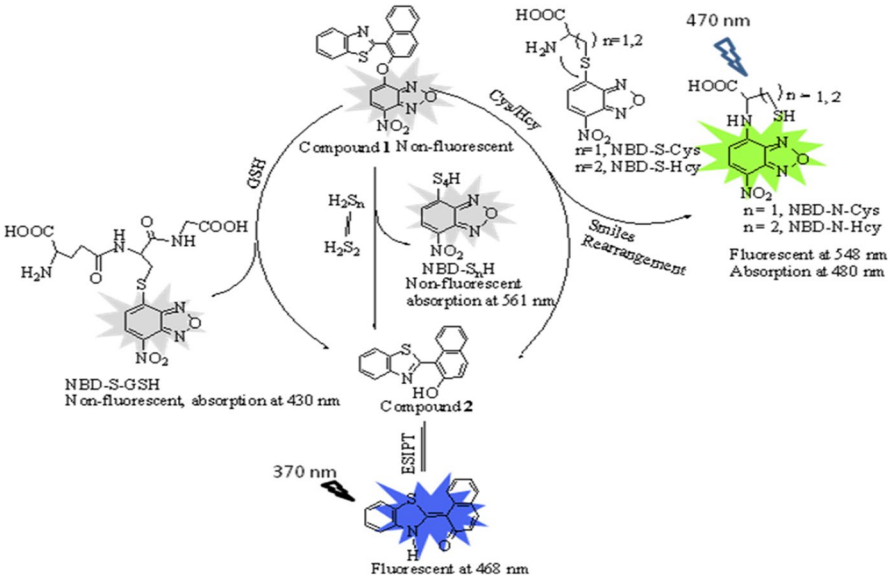


Fig. 6 The fluorescence intensity at 468 nm of **1** (20 μM) toward H_2S_n (20 μM) in the presence of anions (20 μM) (**a**) and amino acids (20 μM) (**b**), respectively. (Color figure online)



Scheme 2 Schematic illustration of the reaction of compound **1** with Cys/ Hcy, GSH and H_2S_n

NBD-*S*-Hcy can easily replace the thiolate via intramolecular nucleophilic aromatic (S_NAr) substitution reactions (Smiles rearrangement) to convert to fluorescent amino-substituted NBD derivatives (NBD-*N*-Cys and NBD-*N*-Hcy) through a five- or six-membered ring intermediate. Fluorescence efficiency of 4-substituted-7-nitro-2,1,3-benzoxadiazoles (NBD-X) markedly depends on the electron-donating ability of X through effective ICT process (e.g., relative fluorescence quantum yields in methanol: $X = NHCH_3$, 62.7; SCH_3, 0.6; OCH_3, 0.0) [47]. Due to the strong push–pull electron effects between amino and nitro groups of benzoxadiazole, NBD derivatives of NBD-*N*-Cys and NBD-*N*-Hcy could emit at 550 nm [48–50], whereas NBD-*S*-GSH could not further react due to the long distance of NH_2 group toward the thioether group within the molecules. H_2S_n could also nucleophilically attack the ether oxygen atom of compound **1** as Cys /Hcy and GSH did to generate compound **2** and derivatives of NBD-S_nH. NBD-S_nH could not emit but absorbed at 561 nm, a much different wavelength from NBD-*N*-Cys, NBD-*N*-Hcy and NBD-*S*-GSH. Kand et al. applied time-dependent density functional theory (TDDFT) calculations at the B3LYP/6-311G (d,p) level on optimized geometries in predicting the fluorescence properties of NBD-*S*-Cys and NBD-*N*-Cys, and the TDDFT calculations for NBD-*N*-Cys confirmed an allowed $S_0 \rightarrow S_1$ transition and S_1 as the emissive state, whereas the presence of a dark S_1 state and allowed $S_0 \rightarrow S_2$ transition confirmed NBD-*S*-Cys as a nonfluorescent species [51].

The sensing mechanism could be proved by mass spectrometry analysis of the reaction mixture of compound **1** with these biothiols. Peaks at m/z = 278.0 in the ESI–MS spectra of **1** in the presence of Cys, Hcy, GSH and H_2S_n, respectively (Fig. S8 ~ Fig. S11), corresponded to $[M+H]^+$ of compound **2**. Peaks at m/z = 284.3 (Fig. S8), 299.0 (Fig. S9), 471.0 (Fig. S10) and 316.0 (Fig. S11), corresponding

to [NBD-Cys]$^+$, [NBD-Hcy]$^+$, [NBD-GSH]$^+$ and [NBD-S_4H+Na]$^+$, respectively, were observed in the ESI–MS spectra, confirming the mechanisms proposed in Scheme 2. The assignment from the mass spectra was in accordance with the fluorescence spectra, where the probe with addition of all these biothiols provided a fluorescence band at 468 nm corresponding to compound **2** and Cys/Hcy led to a new fluorescence band peak at 550 nm corresponding to NBD-*N*-Cys and NBD-*N*-Hcy. These results support our proposed sensing mechanism, which is in good agreement with that reported in the literature [47–50].

In the same media, the lifetime of compound **2** was determined and compared with lifetimes of compound **1** in the presence of Cys, Hcy, GSH and H_2S_n, respectively (Fig. S12 and Table S1). The lifetime of compound **2** was found to be 4.33 ns when monitored at 468 nm. The solution of **1** displayed lifetimes of 4.34 ns monitored at 468 nm, 5.24 ns monitored at 550 nm for Cys, and 4.32 ns monitored at 468 nm, 4.72 ns monitored at 550 nm for Hcy. The mixture of **1** with GSH or H_2S_n showed a single lifetime monitored at 468 nm ($\tau = 4.38$ ns for GSH, $\tau = 4.35$ ns for H_2S_n). As the lifetimes measured at 468 nm of **1** in the presence of Cys, Hcy, GSH and H_2S_n were very close to the lifetime of compound **2**, the results proved that the reaction of **1** with Cys, Hcy, GSH and H_2S_n generated the same product of compound **2**.

^{1}H NMR comparison of **1** in DMSO-d_6 in the presence of Cys, Hcy, GSH and H_2S_n with compound **2** displayed that the signals belonging to the aromatic protons of compound **1** were shifted and most of the signals were close to signals of compound **2** (Fig. 7 and Fig. S13), indicating the reaction of **1** with these biothiols and

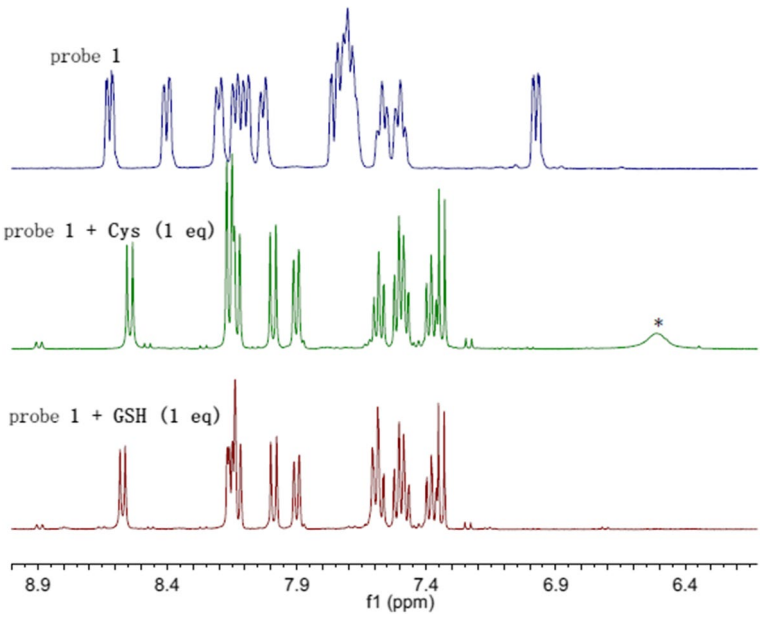


Fig. 7 ^{1}H NMR spectra of **1** in dmso-d_6 before and after addition of Cys (1 eq) and GSH (1 eq), respectively. (Color figure online)

the release of **2**. The peak at δ 6.50 ppm in the **1** + Cys mixture indicated the formation of NBD-*N*-Cys [50]. The rather weak peak at δ 6.36 ppm in the **1** + Hcy mixture indicated the slower conversion to NBD-*N*-Hcy, whereas no peaks appeared in this area in the **1** + GSH indicated no conversion to NBD-*N*-GSH from NBD-*S*-GSH (Fig. S13).

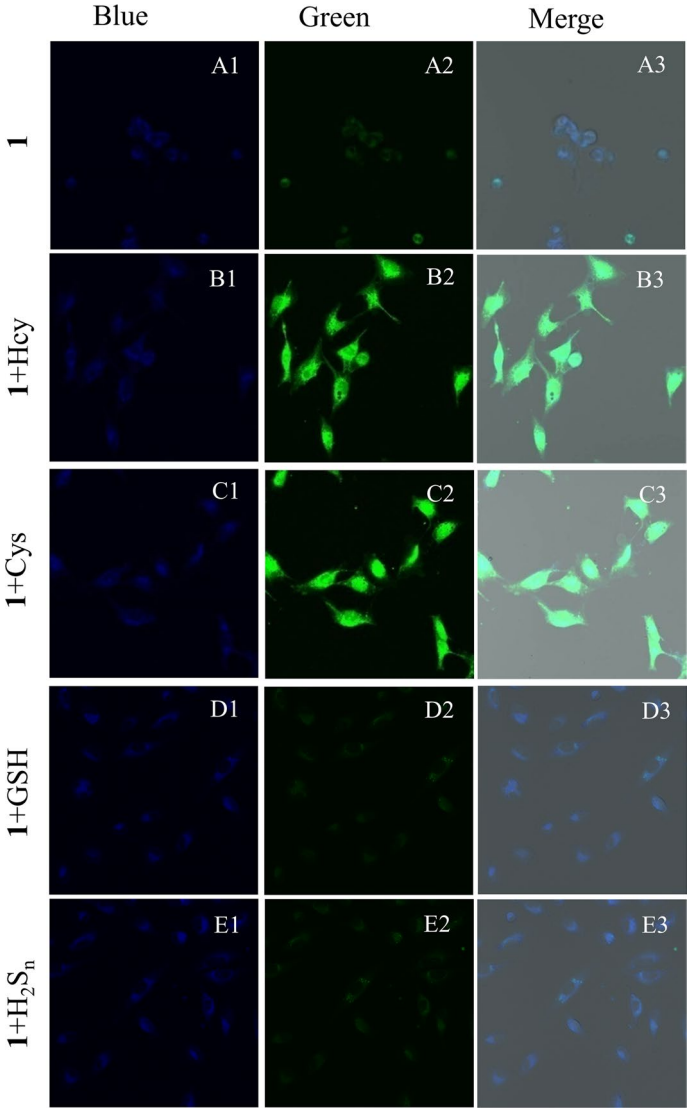


Fig. 8 Confocal microscopic images of compound **1** in presence of biothiols in living cells. 786-O cells incubated with compound **1** (10 μM) for 30 min (A1-A3). 786-O cells pre-incubated with compound **1** (10 μM) for 30 min and then, respectively, with 100 μM of Hcy (B1-B3), Cys (C1-C3), GSH (D1-D3), H_2S_n (E1-E3) for 30 min. Excitation wavelengths were 370 nm; emissions were collected at 450–490 nm for blue channels, and excitation wavelengths were 480 nm; emissions were collected at 530–560 nm for green channels. (Color figure online)

Cell imaging

As shown in Fig. 8, after the 786-O cells were pre-incubated with compound **1** (10 μM) for 30 min, very weak fluorescence appeared in blue and green channels, indicating compound **1** was slightly responsive to intracellular biothiols. When the pre-treated cells with compound **1** were incubated with 100 μM different analytes for another 30 min, much stronger fluorescence were observed in blue and green channels for Cys/Hcy, whereas for GSH/H_2S_n, only significant fluorescence increases in blue channels were observed. These results indicated that compound **1** was cell permeable and could be used for sensing intracellular Cys/Hcy and GSH/H_2S_n, simultaneously from different emission channels.

Conclusions

In conclusion, a simple fluorescent probe (compound **1**) bearing two fluorophores for simultaneous detection and discrimination of Cys/Hcy, GSH and H_2S_n was developed. The probe could selectively detect Cys/Hcy, GSH and H_2S_n from other amino acids by naked eyes. The interaction of compound **1** with these biothiols would form the fluorophore compound **2**, with turn on fluorescence at 468 nm, and form different NBD derivatives with different spectroscopic properties. The reaction of **1** with Cys/Hcy Probe **1** could be used to discriminate Cys/Hcy from GSH at excitation wavelength of 470 nm. Therefore, compound **1** could simultaneously discriminate Cys/Hcy, GSH with different excitation wavelengths and H_2S_n with well-defined absorption spectral signals. Preliminary cell imaging experiments indicated that compound **1** could simultaneously monitor intracellular Cys/Hcy and GSH/H_2S_n, from different emission channels in living 786-O cells.

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