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A smart fluorescent probe for simultaneous detection of GSH and Cys in human plasma and cells

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Abnormal levels of glutathione (GSH) and cysteine (Cys) are associated with some diseases, thus monitoring the dynamic changes and the quantification of GSH/Cys are of great significance in clinical diagnosis. Herein, a smart fluorescent probe was developed for the detection of Cys and GSH, which was constructed with a far-red emitting indole-BODIPY and 7-nitrobenzofurazan (NBD) linked via an ether bond. Upon substitution of the ether with nucleophilic thiolate of GSH/Cys, the probe released indole-BODIPY fluorophore, which gave rise to a significant fluorescence "turn-on" signal at 635 nm. Moreover, Cys induced an intramolecular rearrangement reaction on electrophilic site of NBD, resulting in another emission band at 540 nm. Therefore, this probe can be used to simultaneously detect Cys and GSH in different emission channel. Such capability of the probe has been demonstrated for the measurement of Cys and GSH in human plasmas with fast response and low detection limit (0.64 μM). Furthermore, fluorescence imaging of HeLa cell indicated that the probe was cell membrane permeable and could be used for visualizing GSH and Cys in living cells.

Introduction

Glutathione (GSH) and cysteine (Cys), the most abundant small molecular bio-thiols in living organisms, [1] play important roles in some physiological processes including maintenance of intracellular redox activities, xenobiotic metabolism, intracellular signal transduction and liver damage.[2,3] However, abnormal levels of these species are associated with many diseases such as cancer, AIDS, osteoporosis, Alzheimer's disease, inflammatory bowel and cardiovascular diseases.[3-5] Therefore, monitoring the dynamic changes and the quantification of GSH/Cys are of great significance in the area of clinical diagnosis.

Fluorescent probe provides a useful tool for monitoring the levels of intracellular thiols because of its simplicity as well as high selectivity and sensitivity.[6,7] Up to date, a large number of fluorescent probes for bio-thiols have been developed on the basis of Michael addition,[8-10] cleavage of sulfonamide and sulfonate esters, [11-13] cleavage of S–S bonds,[14] and so on.[15, 16] Nevertheless, these approaches only determine the total concentrations of thiols because they utilize the strong nucleophicility property of SH group. As we known, GSH and Cys usually coexist in organisms, but they play different physiological functions and are related to different diseases. To clarify the complicated relationship between these bio-thiols in various physiological processes, it is highly desirable to develop fluorescent probes that exhibit distinct signals in response to GSH and Cys. Recently, some sensing strategies for differentiating Cys from GSH have been proposed including cyclization with aldehydes,[17-19] conjugate addition–cyclization with acrylates,[20-23] native chemical ligation,[24-27] and aromatic substitution-rearrangement.[28-33] Among these approaches, a promising strategy reported by Yang is attractive due to the unique sensing manner for discriminating Cys from GSH.[28, 29] However, the emission spectra of products significantly overlapped after treatment with GSH and Cys, so it cannot simultaneously detect GSH and Cys in the practical samples. Guo et al tactfully designed a chlorinated coumarin-hemicyanine dye for simultaneous detection of Cys and GSH with different emission channels.[34] Unfortunately, the excitation wavelength located at 360 nm and the emission wavelength was 420 nm after addition of Cys, which might suffer from the interference from plasma.

Herein, we report a far-red emitting fluorescent probe for simultaneous detection of GSH and Cys with different emission as shown in Scheme 1. Indole-BODIPY is attached to 7-nitrobenzofurazan moiety (NBD) by an ether linker (Scheme 2), which serves as a reactive site for Cys and GSH. This probe is non-fluorescent due to photo-induced electron transfer from NBD to indole-BODIPY. We envisioned that the ether bond would be substituted by nucleophilic thiolate, and indole-BODIPY fluorophore was released to give rise to a "turn-on" fluorescence signal at 635 nm (shown in Scheme 1). Moreover, Cys would induce an intramolecular rearrangement cascade reaction on electrophilic site of NBD, thus another emission band originated from NBD could be observed at 540 nm. Such

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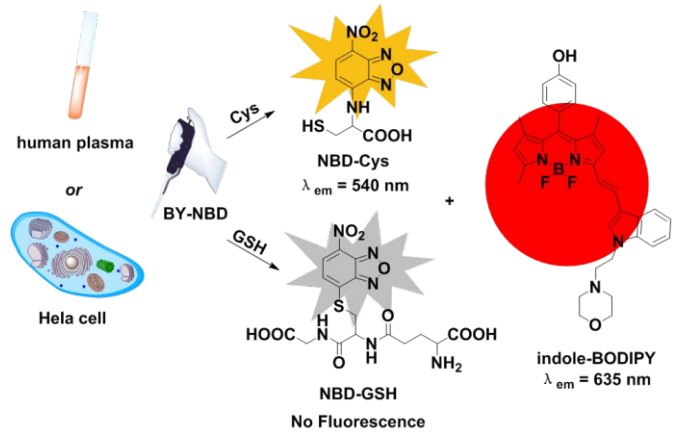
† Electronic Supplementary Information (ESI) available: ^{1}H NMR, ^{13}C NMR MS and emission spectra of the fluorescent probes. See DOI: 10.1039/x0xx00000x



Scheme 1 The proposed sensing mechanism for differentiating Cys from GSH and simultaneous detection of them.

a different emission mode can be used to differentiate Cys from GSH. The concentration of Cys can be determined by plotting the fluorescence intensity at 540 nm *versus* the concentration of Cys. Furthermore, the concentration of GSH can be calculated by subtracting the fluorescence intensity (I_{635}) corresponding to Cys. Thus, this probe can be used to simultaneously determine the concentrations of Cys and GSH. To improve the water solubility of the probe, a morpholine group was employed.

Experimental

Materials

Unless otherwise stated, all chemicals and solvents were of analytical grade and used without further purification. Twice-distilled water was used for preparing all test solutions. 4,4-difluoro-8-(4-hydroxyphenyl)-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene, 1-(2-morpholinoethyl)-1*H*-indole-3-carbaldehyde were synthesized according to literatures.[35,36]

Equipment and methods

The ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AV-400 spectrometer with tetramethylsilane (TMS) as the internal standard. The chemical shift was recorded in ppm and the following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, m = multiplet. Mass spectra were measured by a HP-1100 LC-MS spectrometer. UV-vis spectra were recorded on a Hitachi UV 3310 spectrometer. Fluorescence spectra were recorded on a Hitachi FL-4500 fluorometer. Fluorescent images were acquired on a Nikon A1 confocal laser-scanning microscope with a 100 objective lens. Numerical Aperture of the objective: 100x/1.4 Oil (DIC N2), OFN 25, Plan Apo VC, Nikon Company. Immersion oil, type: NF, nd= 1.515 (23 ^{o}C). Microscope: Ti Microscope, Lightpath: L100, Condnser: 3 (DIC N2), Zoom: 1.00x, Nikon Company. Human plasma was supplied by three healthy volunteers who have taken a physical examination in the Tianjin Cancer Institute & Hospital. All experiments were performed in compliance with the relevant laws and was approved by the committee of Tianjin Cancer Institute & Hospital.

Synthesis of indole-BODIPY

4,4-difluoro-8-(4-hydroxyphenyl)-1,3,5,7-tetramethyl-4-bora-3a,4a-diaza-s-indacene (170.0 mg, 0.50 mmol), 1-(2-morpholinoethyl)-1*H*-indole-3-carbaldehyde (168.0 mg, 0.65 mmol) were dissolved in 30 mL of toluene in a three-neck round bottom flask. Piperidine (0.05 mL) and acetic acid (0.05 mL) were added to the reaction mixture in nitrogen atmosphere, and the reaction mixture was heated to 110 ℃ for 16 h. After the reaction was completed, the solvent was removed under reduced pressure. The resulting residue was purified by silica gel column chromatography (CH_2Cl_2/C_2H_5OH = 70/1) to yield indole-BODIPY as dark blue solid (179.9 mg, 62%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 9.82 (s, 1H), 7.93 (s, 2H), 7.79 (d, *J* = 16.4 Hz, 1H), 7.62 (d, *J* = 7.2 Hz, 1H), 7.46 (d, *J* = 16.4 Hz, 1H), 7.28 (m, 2H), 7.15 (d, *J* = 8.2 Hz, 2H), 6.99 (s, 1H), 6.9[illegible] (d, *J* = 8.2 Hz, 2H), 6.10 (s, 1H), 4.38 (t, *J* = 5.7 Hz, 2H), 3.56 (s, 4H), 2.70 (s, 2H), 2.48 (s, 6H), 1.50 (s, 3H), 1.42 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 158.47, 155.61, 151.25, 143.37, 139.94, 139.55, 137.75, 133.39, 132.80, 131.22, 129.87, 125.95, 125.19, 123.12, 121.52, 120.48, 120.18, 118.27, 116.39, 113.78, 113.59, 111.46, 66.64, 57.99, 53.73, 43.49, 15.01, 14.73, 14.47. HR-MS (ESI): calcd for $C_{34}H_{35}BF_2N_4O_2$+H 581.2905; Found 581.2906.

Synthesis of the probe

4-Chloro-7-nitrobenzofurazan (60 mg, 0.30 mmol), indole-BODIPY (116 mg, 0.20 mmol) and triethylamine (42 μL, 0.30 mmol) were placed in a round bottom flask with 20 mL of CH_2Cl_2. The reaction mixture was stirred at room temperature for 12 h. Then, it was diluted with CH_2Cl_2 (50 mL), and washed with water (50 mL × 3) for three times. The organic layer was separated, and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure, and the resulting residue was purified by silica gel column chromatography (CH_2Cl_2/C_2H_5OH = 60/1) to yield the desired compound **BY-NBD** as a dark blue solid (49 mg, 33%). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.78 (d, *J* = 8.3 Hz, 1H), 7.96 (s, 2H), 7.85 (d, *J* = 16.2 Hz, 1H), 7.63 (d, *J* = 4.1 Hz, 6H), 7.48 (d, *J* = 16.3 Hz, 1H), 7.31 – 7.27 (m, 2H), 7.08 (s, 1H), 6.80 (d, *J* = 8.3 Hz, 1H), 6.17 (s, 1H), 4.39 (t, *J* = 6.3 Hz, 2H), 3.58 – 3.55 (m, 4H), 2.71 (t, *J* = 6.2 Hz, 2H), 2.47 (s, 6H), 1.57 (s, 3H), 1.49 (s, 3H). ^{13}C NMR (100 MHz, DMSO-d_6) δ 156.23 , 154.00 , 144.89 , 143.29 , 139.66 , 137.75 , 137.33 , 136.05 , 133.43 , 131.51 , 131.08 , 130.62 , 125.97 , 122.03 , 121.63 , 120.82 , 120.17 , 118.84 , 113.87 , 111.49 , 110.63 , 66.54 , 57.72 , 53.59 , 29.29 , 15.18 , 14.75 , 14.61 . HR-MS (ESI): calcd for $C_{40}H_{37}N_7O_5BF_2$+H 744.2924; Found 744.2916.

Optical responses of the probe toward various analytes

Stock solution of the probe (1 mM) was prepared in HPLC grade ethanol. Stock solutions of analytes (5 mM) were prepared in twice-distilled water. For optical measurements, the probe was diluted to 10 μM in EtOH/PBS solution (v/v = 1/9, pH 7.4), and 3.0 mL of the resulting solution was placed in a

quartz cell of 1 cm optical path length each time. All spectroscopic experiments were carried out at room temperature.

Cell culture and fluorescence imaging

HeLa cells (Perking Union Medical College, China) were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (Invitrogen Corp., Carlsbad, CA) and penicillin (100 units/mL)-streptomycin (100 μg/mL) liquid (Invitrogen Corp., Carlsbad, CA) at 37 ℃ in a humidified incubator containing 5% CO_2 in air. The cells were incubated for 2 days before dye loading on an uncoated 35 mm diameter glass-bottomed dish (D110100, Matsunami, Japan). Then, the cells were incubated with DMEM containing 10% FBS and 10 μM probe (2% DMSO, v/v) at 37 ℃ for 30 min, washed twice with PBS, and mounted on the microscope stage. Fluorescent images were captured using a Nikon A1 Application.

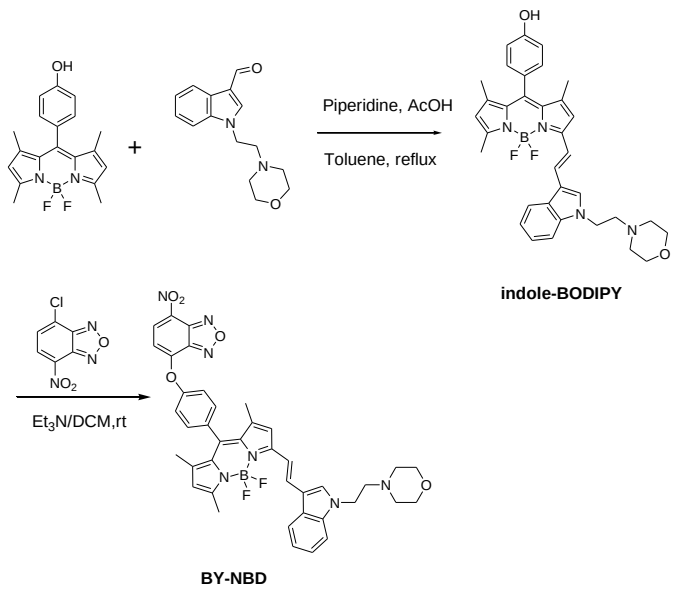
Results and discussion

Synthesis

The probe **BY-NBD** was readily synthesized in two steps, as shown in Scheme 2. BODIPY was condensed with 1-(2-morpholinoethyl)-1*H*-indole -3-carbaldehyde in the presence of piperidine and HOAc, affording the indole-BODIPY in 62% yield. Then, indole-BODIPY reacted with 4-chloro-7-nitrobenzofurazan to produce the target compound **BY-NBD**. The structures of compound **BY-NBD** and indole-BODIPY were fully characterized by ^{1}H NMR, ^{13}C NMR and HR-MS (Fig. S1-6, in the ESI†).

The optical response to Cys and GSH

As shown in Fig. 1, the probe displays a broad absorption band centred at 620 nm. Upon addition of 640 μM GSH, the



Scheme 2 Synthesis of the probe **BY-NBD**

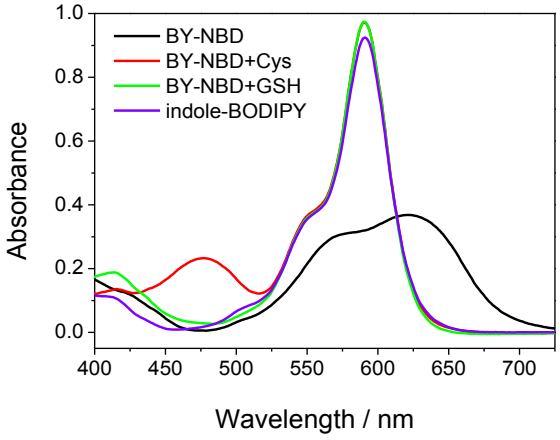


Fig. 1 UV-vis absorption spectra of **BY-NBD** (10 μM, C_2H_5OH/PBS = 1:9, pH 7.4) in the presence of Cys (640 μM) and GSH (640 μM) at 25 °C for 30 min.

maximum absorption band became much sharper and blue-shifted to 590 nm. The newly formed absorption band was similar to that of indole-BODIPY (Fig. 1), and high-resolution mass spectrum confirmed indole-BODIPY as the major product (m/z = 581.2891 for $[M]^+$, Fig. S7, in the ESI†). When the probe was treated with 640 μM Cys, two well-resolved peak at 590 nm and 476 nm appeared in the UV-vis absorption spectra. The absorption band centred at 590 nm can be assigned to the characteristic absorption band of indole-BODIPY (shown in Fig. 1), implying that indole-BODIPY was released from **BY-NBD** after treatment with Cys. This hypothesis was confirmed by high-resolution mass spectroscopy, where a dominant peak at an m/z value of 581.2891 (calcd 581.2905) was corresponding to $[M]^+$ (Fig. S7 in the ESI†). Another absorption band (λ_{abs} = 476 nm) of **BY-NBD** in the presence of Cys was attributed to the formation of NBD-Cys via

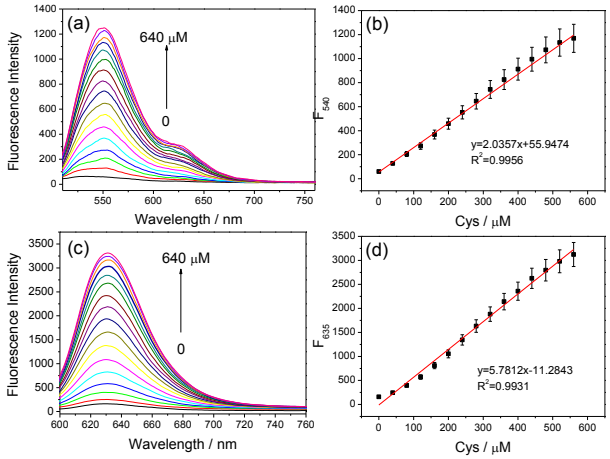


Fig. 2 (a) Fluorescence spectra changes of **BY-NBD** (10 μM in C_2H_5OH/PBS = 1:9, pH 7.4) in the presence of Cys (0 – 640μM). λ_{ex} = 470 nm, slits: 2.5/5 nm. (b) The linear relationship between fluorescence intensity of **BY-NBD** at 540 nm and the concentration of Cys (0 – 560 μM). (c) Fluorescence spectra changes of **BY-NBD** (10 μM) in the presence of Cys (0 – 640 μM). λ_{ex} = 580 nm, slits: 2.5/5 nm. (d) The linear relationship between fluorescence intensity of **BY-NBD** at 635 nm and the concentration of Cys (0 – 560 μM). Each spectrum was recorded after incubation with the analyte for 30 min at 25 °C. Error bars are ± SD, n = 3.

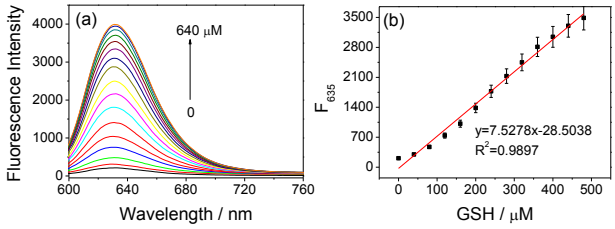


Fig. 3 (a) Fluorescence spectra titrations of **BY-NBD** (10 μM) with GSH (0 – 640 μM) in PBS aqueous solution (C_2H_5OH/PBS = 1:9, pH 7.4, 10 mM). λ_{ex} = 580 nm, slits: 2.5/5 nm. (b) The linear relationship between fluorescence intensity of **BY-NBD** at 635 nm and the concentration of GSH (0 – 480 μM). Each spectrum of the probe was recorded after incubation with the analytes for 30 min at 25 °C. Error bars are ± SD, n = 3.

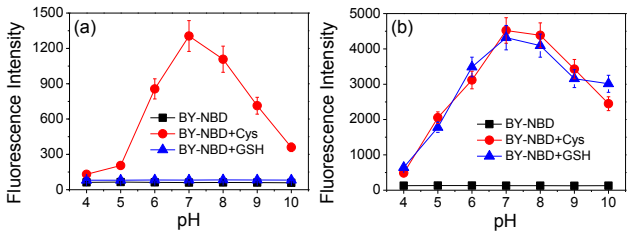


Fig. 4 pH-dependent fluorescence responses of **BY-NBD** (10 μM, C_2H_5OH/PBS = 1:9, pH 7.4) to Cys (640 μM) and GSH (640 μM). (a) λ_{ex} = 470 nm, λ_{em} = 540 nm, slits: 2.5/5 nm. (b) λ_{ex} = 580 nm, λ_{em} = 635 nm, slits: 2.5/5 nm. Each spectrum of the probe was recorded after incubation with the analytes for 30 min at RT. Error bars are ± SD, n = 3.

nucleophilic aromatic substitution- rearrangement,[29,34] which was verified by mass spectrum (m/z = 285.0296 $[M]^+$; Fig. S8, in the ESI†). While, the probe **BY-NBD** only exhibited a characteristic absorption band of indole-BODIPY in the presence of GSH. By examining the mass spectrum (Fig. S9, in the ESI†), it could be observed that the NBD moiety was attached to GSH via a sulfur atom. Such a dramatic difference in the absorption spectra can be used to discriminate Cys from GSH.

The probe **BY-NBD** itself is almost non-fluorescent due to photo-induced electron transfer (PET). Upon addition of increasing amount of GSH, the fluorescence intensity of probe **BY-NBD** at 635 nm increased progressively (~ 30 folds enhancement) until the concentration of GSH was 64.0 equiv. with respect to the probe (Fig. 2a). The fluorescence OFF–ON switch might be triggered by removal of NBD moiety, thus giving rise to the characteristic emission of indole-BODIPY (Φ = 0.29 in ethanol), which was consistent with the absorption spectra (Fig.1) and high-resolution mass spectral (Fig. S7, in the ESI†). By contrast, the probe **BY-NBD** displayed two emission band at 540 nm and 635 nm in the presence of 64 equiv. of Cys. Thus, Cys can be easily identified by the simultaneous appearance of two well-resolved emission bands, whereas GSH can be identified by the generation of single emission at 635 nm. Such different emission behaviors can be attributed to different product from the probe **BY-NBD** treated with Cys and GSH. After treatment with Cys, the probe **BY-NBD** generated indole-BODIPY and NBD-Cys, which were responsible to the emissions at 635 nm and 540 nm, respectively. Whereas, GSH induced the probe **BY-NBD** to produce indole-BODIPY and a non-fluorescent product NBD-GSH, thereby only a single emission band at 635 nm was detected. These different emission patterns provide the basis to conveniently discriminate Cys from GSH.

To test the ability of **BY-NBD** for quantitative detection of Cys and GSH in aqueous solution, the probe **BY-NBD** (10 μM) was treated with different concentrations of Cys/GSH, and the fluorescence spectra were recorded. As shown in Fig. 2b, the fluorescence intensity of probe at 540 nm was linearly related to the concentration of Cys ranging from 0 to 560 μM (R^2 = 0.9956), and the detection of limit was determined to be 0.79 μM based on signal to noise ratio (S/N =3), which was much lower than that in the physiological condition (130 – 290 μM in the plasma).[37-38] A similar linear relationship was obtained by plotting the fluorescence intensity of probe **BY-NBD** at 635 nm and the concentrations of Cys. GSH induced a significant fluorescence enhancement at 635 nm under the same condition. For GSH, the fluorescence intensity at 635 nm was linearly related to the concentration of GSH ranging from 0 to 480 μM (R^2 = 0.9897), and the detection of limit was determined to be 0.64 μM based on signal to noise ratio (S/N =3). Therefore, Cys can be accurately determined at 540 nm even though Cys and GSH coexists in the tested system. Importantly, both Cys and GSH induced the probe to produce an identical fluorescence maxima at 635 nm, which indicated that the quantification of GSH might be achieved by subtracting the fluorescence contribution of Cys at 635 nm.

Time-resolved fluorescence response and pH-dependent fluorescence response to Cys and GSH

The pH-dependent fluorescence responses of the probe to Cys/GSH were also investigated, as shown in Fig. 4. The fluorescence intensity of the probe remains unchanged at pH 4.0 to 10.0, suggesting that this probe is very stable at a wide range of pH values. Upon addition of 640 μM Cys, the fluorescence of the probe at 540 nm and 635 nm increased drastically at pH values ranging from 6.0 to 10.0. Whereas, GSH just led to a significant fluorescence enhancement at 635 nm. Time-dependent fluorescence response of the probe (10 μM) to Cys/GSH (640 μM) was measured at 25°C in C_2H_5OH/PBS buffer (v/v = 1:9, pH 7.4, 10 mM,) (Fig. 5a and 5b). Upon addition of 640 μM Cys/GSH, the fluorescence

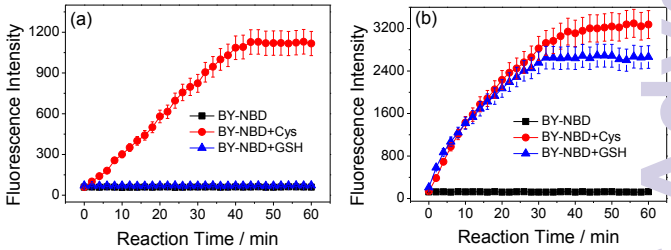


Fig. 5 Time-dependent fluorescence changes of **BY-NBD** (10 μM, pH 7.4) in the presence of Cys (640 μM) and GSH (640 μM), respectively. (a) The fluorescence intensity was recorded at 540 nm. λ_{ex} = 470 nm, slits: 2.5/5 nm. (b) The fluorescence intensity was recorded at 635 nm. λ_{ex} = 580 nm, slits: 2.5/5 nm. Each spectrum of the probe was recorded after incubation with the analytes for 30 min at 25 °C. Error bars are ± SD, n = 3.

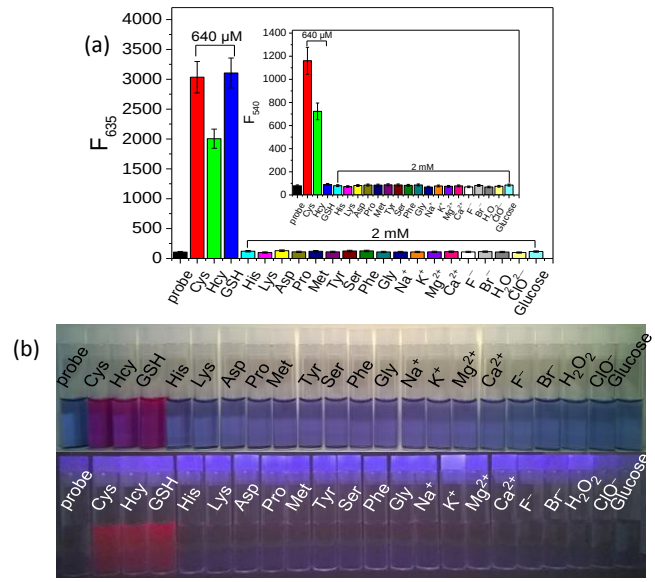


Fig. 6 (a) Fluorescence responses of **BY-NBD** (10 μM) to various biologically relevant species (2 mM for His, Lys, Asp, Pro, Met, Tyr, Ser, Phe, Gly, Na^+, K^+, Mg^{2+}, Ca^{2+}, F^-, Br^-, H_2O_2, ClO^-, Glucose, 640 μM for GSH, Hcy, Cys) in C_2H_5OH/PBS (v/v = 1:9, pH 7.4, 10 mM). Spectra were recorded after incubation with the relevant species for 30 min. λ_{ex} = 580 nm, λ_{em} = 635 nm. Slits: 2.5/5 nm. Inset (a): λ_{ex} = 470 nm, λ_{em} = 540 nm. Error bars are ± SD, n = 3. (b) Colour changes and fluorescence photographs of **BY-NBD** (10 μM) in the presence of Cys, Hcy or GSH (640 μM) and some relevant species (2 mM) in PBS aqueous solution (C_2H_5OH/PBS = 1:9, pH 7.4, 10 mM).

intensity of the probe at 635 nm increased promptly and nearly reached a plateau within 30 min, suggesting that **BY-NBD** could serve as a "fast response" fluorescent probe for Cys/GSH, which might be suitable for real-time sensing of Cys/GSH in living cells.

The selectivity experiments

To evaluate the selectivity of probe **BY-NBD** for GSH and Cys, various biologically relevant species were examined including amino acids, glucose, some representative anions and reactive oxygen species. As shown in Fig. 6, the addition of 64 equiv. of GSH/Cys led to a significant fluorescence turn-on emission at 635 nm, while the probe showed negligible response to glucose, some representative anions (F^-, Br^-) and reactive oxygen species (H_2O_2, ClO^-) at the biologically relevant concentrations. Although the probe also displayed fluorescence turn-on response to homocysteine (Hcy) at 540 and 635 nm, the level of Hcy (12 – 15 μM) in living organism is much lower that that of Cys (130 – 290 μM). [37-40] These results suggest that the probe has good selectivity for GSH/Cys over other biological species, thus can be used for the detection of GSH/Cys in complex biological environment.

Determination of Cys and GSH in human plasma

Next, we used human plasma to evaluate whether our probe could detect Cys and GSH in practical biological samples. The detection was performed in PBS solution containing 25% human plasma. Fig. S10 shows the fluorescence spectra of the diluted plasma in the absence and presence of probe. The diluted plasma itself did not display background emission

Table 1 Determination of Cys and GSH in human plasmas

	Analyte in plasmas	Added (μM)	Found[a] (μM)	Recovery[a] (%)	GSH(μM)
Sample 1	Cys	0	141 ± 7.2	—	46 ± 2.3
		50	187 ± 8.8	93 ± 4.4	—
		100	244 ± 8.0	103 ± 2.0	—
Sample 2	Cys	0	166 ± 4.2	—	33 ± 2.5
		50	214 ± 8.1	97 ± 9.0	—
		100	277 ± 7.6	111 ± 1.1	—
Sample 3	Cys	0	130 ± 2.1	—	32 ± 2.2
		50	176 ± 7.8	92 ± 2.5	—
		100	233 ± 4.4	102 ± 1.1	—

[a] Mean of three determinations ± standard deviation.

Sample 1: male (age:24), Sample 2: female (age:24), Sample 3: female (age:73).

upon excitation at 580 nm. When the plasma was mixed with probe **BY-NBD** (10 μM), two strong emission bands located at 540 nm and 635 nm were observed, as shown in Fig. S10. The average concentrations of Cys and GSH in human plasmas were determined to be 166 μM and 33 μM, respectively, which was in good agreement with the reported results (130 to 290 μM Cys, and 5 to 50 μM GSH in human plasma). [37-40] Furthermore, different concentrations of Cys were added to the plasma, and the recovery was determined by the present method. As shown in Table 1, the recoveries of Cys ranged from 97 to 111% with a relative error of less than 5%, suggesting that the probe **BY-NBD** could quantitatively detect Cys and GSH in practical biological samples with good performance.

Cell imaging

Furthermore, we tentatively used the probe **BY-NBD** to monitor the levels of Cys/GSH in living cells. As shown in Fig. 7a, b and c, HeLa cells exhibits a clear cell profile with red colour (from red channel) and yellow colour (from yellow channel) after incubation with **BY-NBD** (10 μM) for 30 min. The result suggests that this probe has permeated into cells and reacted with endogenous thiols to produce discernible fluorescence responses. To verify this hypothesis, control experiments were performed. In the control experiment, HeLa cells were pre-treated with an intracellular thiol scavenger N-ethylmaleimide (NEM) for 30 min and then incubated with probe **BY-NBD** for 30 min. As shown in Fig. 7d, e and f, HeLa cells showed non-fluorescence after they were pre-incubated with NEM (2 mM) for 30 min. When the cells were further incubated with GSH (2 mM) for 30 min, strong red fluorescence was observed from the red channel (Fig. 7i), but non-fluorescence could be detected from yellow channel (Fig. 7h). According to our previous study, the yellow fluorescence was induced by Cys via an intramolecular rearrangement cascade reaction on electrophilic site of NBD, but GSH cannot cause this reaction to happen. Hence, the probe just produced red fluorescence in the presence of GSH. These results indicate that the probe **BY-NBD** can be utilized as a tool to simultaneously monitor the level of Cys and GSH in living cells

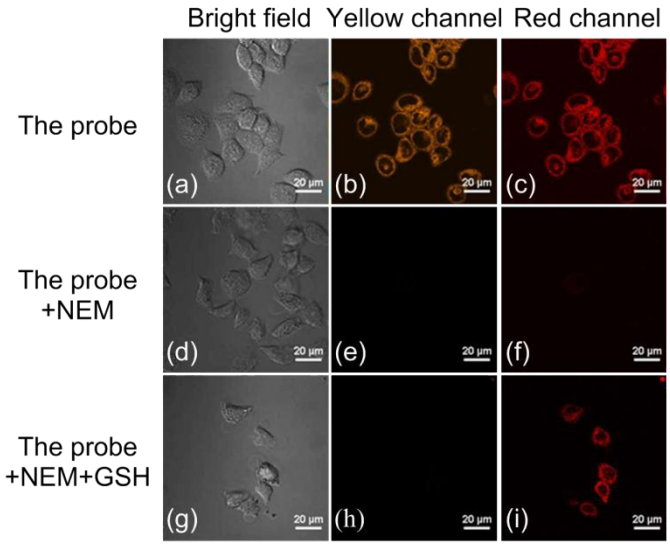


Fig. 7. Confocal fluorescence images of HeLa cells: (a-c) The cells incubated with **BY-NBD** (10 μM) for 30 min. (d-f) The cells were pre-incubated with 2 mM N-ethylmaleimide (NEM) for 30 min and then treated with **BY-NBD** (10 μM) for 30 min. (g-i) The cells were pre-incubated with 2 mM of NEM for 30 min, then treated with **BY-NBD** (10 μM) for 30 min and then treated with 2 mM of GSH for 30 min. Scale bar: 20 μm. Object lens: 100x.

Conclusion

In summary, we have successfully developed a novel fluorescent probe based on the conjugate of indole-BODIPY and 7-nitrobenzofurazan. This probe displays fast and dual-emission response to Cys through nucleophilic aromatic substitution-rearrangement, while it only shows fluorescence "turn-on" response to GSH at single emission band, which can be used to discriminate Cys from GSH. This probe has some advantages including long emission wavelength (λ_{em} = 635 nm), good selectivity, and capability for simultaneous detection of Cys and GSH. Fluorescence imaging of HeLa cells indicated that the probe was cell membrane permeable and could be used for monitoring the levels of GSH and Cys in living cells. This study not only develops a fluorescent probe that can sense GSH and Cys simultaneously, but also provides a strategy for multicomponent analysis.

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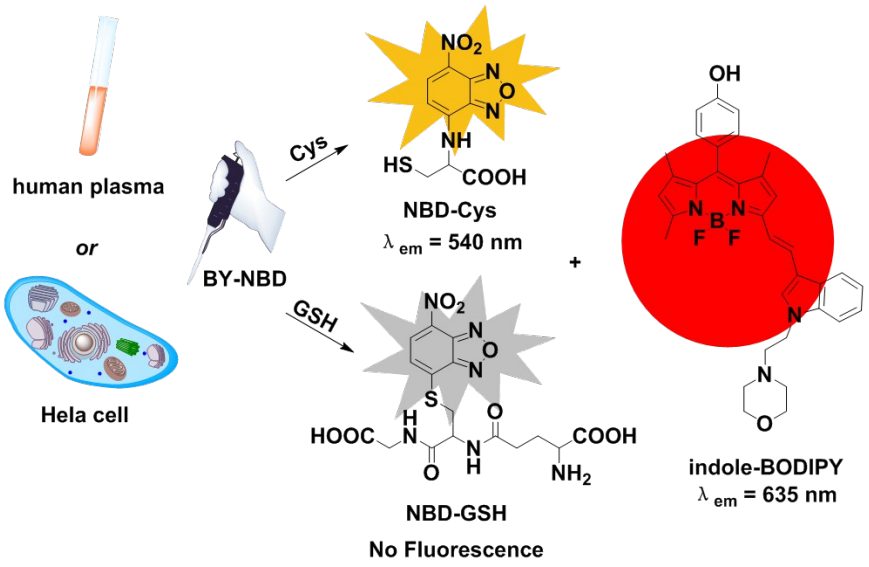

A smart fluorescent probe was developed to simultaneously detect Cys and GSH in different emission channel with low detection limit.