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Determination of non-protein cysteine in human serum by a designed BODIPY-based fluorescent probe

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ABSTRACT

A novel fluorescent probe is designed and synthesized for the determination of cysteine in biological samples by incorporating 2,4-dinitrobenzenesulfonyl (DBS) group as a quencher into the BODIPY skeleton. The BODIPY-based probe itself shows weak fluorescence due to the strong intramolecular charge transfer process. Upon reaction with cysteine, however, the probe produces a rapid and large fluorescence enhancement through the removal of the DBS unit by nucleophilic aromatic substitution. This valuable property leads to the development of a new and simple method for cysteine assay. Under the optimized conditions, the fluorescence enhancement value is directly proportional to the concentration of cysteine in the range 2–12 μM, with a detection limit of 30 nM (S/N = 3). The applicability of the developed method has been successfully demonstrated on the determination of non-protein cysteine in human serum. Compared to most of the existing fluorescent probes proposed for cysteine, the BODIPY-based one exhibits an excellent overall performance in terms of selectivity, sensitivity and simplicity.

1. Introduction

Biological thiols, including cysteine, homocysteine, and reduced glutathione (GSH), play a key role in maintaining the internal redox homeostasis due to their high reactivity [1]. For instance, cysteine deficiency is involved in many syndromes, such as slow growth in children, hair depigmentation, edema, liver damage, and skin lesions [2]. The determination of cysteine is thus of great importance for various biochemical studies as well as the diagnosis of the related diseases. Although cysteine is the most abundant thiol in human serum [3–5], its simple detection is still a challenging subject because many coexisting substances may produce interference and spectroscopically inert cysteine usually requires time-consuming derivatization.

So far, a number of methods including high-performance liquid chromatography (HPLC) [6], capillary electrophoresis [7], electrochemical assay [8–10], UV/vis spectroscopy [11], chemiluminescence [3], mass spectrometry [12,13], and fluorescence spectroscopy [14–21] have been developed for cysteine detection. These methods are mostly based on the following three principles: nucleophilic addition of thiols to maleimidyl groups [14,22,23], redox reaction [3,8–10] and nucleophilic substitution [6,19] of thiol groups. Among them, nucleophilic aromatic substitution of cysteine has recently attracted attention because of its high specificity. By virtue of this substitution, Maeda et al. designed a thiol-reactive fluorescent probe, which can serve as an alternative to Ellman's reagent in thiol-quantification assays [18], though the application of the probe to real biological samples has not yet been demonstrated. The above existing observation activates us to develop a new fluorescent probe with improved analytical properties for the determination of cysteine in human serum.

In the construction of our probe, the key question that we confront is the choice of an appropriate fluorophore and quencher so that a latent fluorescent probe with low fluorescence background could be prepared to afford high sensitivity [24,25]. In this respect, BODIPY (boron dipyrromethene difluoride) bearing a phenol moiety would be a favorable candidate because of its high fluorescence quantum yield and excellent photo-stability to both solvent polarity and pH [26–28]. Moreover, the high reactivity of phenolic hydroxyl group would facilitate the introduction of a quencher such as 2,4-dinitrobenzenesulfonyl (DBS) unit via dehydrochlorination. As a result, the BODIPY-based fluorescent probe (**1**; Fig. 1) was successfully synthesized by treating 2,4-dinitrobenzenesulfonyl chloride with the phenol-appended BODIPY (**2**). The probe **1** itself exhibits weak fluorescence due to intramolecular charge transfer. Upon reaction with cysteine, however, a rapid and large fluorescence enhancement is produced through the removal of the DBS unit by nucleophilic substitution. Furthermore, the fluorescence enhancement shows high selectivity and sensitivity towards thiols. This behavior leads us to establish a new and simple method for the determination of cysteine in real biological samples such as human serum.

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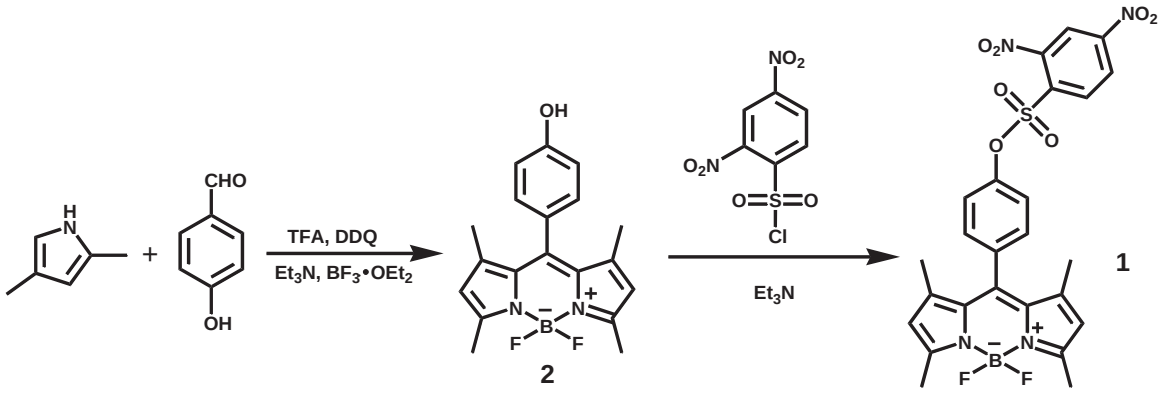


Fig. 1. Synthesis of **1**.

2. Experimental

2.1. Apparatus

Fluorescence measurements, unless otherwise noted, were performed on a Hitachi F-2500 spectrofluorimeter with both excitation and emission slit widths of 10 nm, and a 400 V PMT voltage. ^{1}H NMR spectra were measured on a Brucker DMX-400 spectrometer at 400 MHz in $CDCl_3$ with tetramethylsilane as the internal standard. Elemental analyses were performed on a Flash EA 1112 instrument. HPLC analyses were carried out with LC-20AT pumps, SPD-20A UV–vis detector (Shimadzu, Japan) and Inertsil ODS-SP column (5 μM, 4.6 mm × 250 mm, GL Sciences Inc.). A model HI-98128 pH-meter (Hanna Instruments Inc., USA) was employed for pH measurements.

2.2. Reagents

4-Hydroxybenzaldehyde, 2,4-dimethylpyrrole, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES), cysteine and GSH were purchased from Acros Organics. Homocysteine was from Sigma. 2,4-Dinitrobenzenesulfonyl chloride and BF_3 OEt_2 (boron trifluoride diethyl etherate) were purchased from Alfa Aesar. 2,3-Dichloro-5,6-dicyano-1,4-benzoquinone (DDQ) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from J & K Chemical. Trifluoroacetic acid (TFA) and triethylamine (Et_3N) were obtained from Beijing Chemical Plant. Other chemicals used were all of analytical grade. Distilled-deionized water was employed throughout.

Stock solutions of 2 mM cysteine, 2 mM homocysteine and 1 mM GSH were all prepared daily by dissolving requisite amount of them in 10 mL of 5% (w/v) sulfosalicylic acid under argon. A stock solution (5×10^{-4} M) of **1** was prepared by dissolving appropriate amount of **1** in dimethyl sulfoxide (DMSO). A HEPES buffer solution of pH 7.8 was employed.

2.3. Synthesis of *1*

Probe **1**, as shown in Fig. 1, can be prepared by treating 2,4-dinitrobenzenesulfonyl chloride with the phenol-appended BODIPY (**2**). First, the synthesis of **2** was performed as a one-pot reaction [29,30]: 2,4-dimethylpyrrole (5.26 mmol, 648 mg) and 4-hydroxybenzaldehyde (2.6 mmol, 533 mg) were dissolved in 200 mL methylene chloride, which was pre-dried over molecular sieves (4 Å) and was bubbled with argon gas for 30 min. Then, one drop of TFA was added and the solution was stirred at room temperature for 5 h, followed by adding dropwise a solution of DDQ (2.6 mmol, 640 mg) in absolute methylene chloride (50 mL).

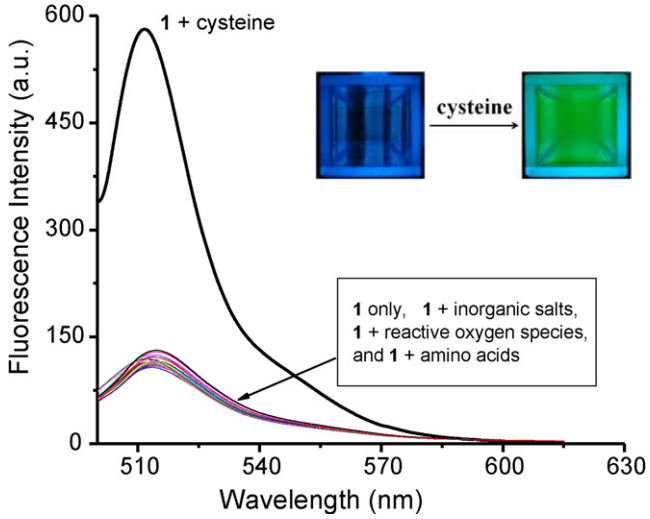


Fig. 2. Fluorescence emission spectra of **1** (5 μM) in 50 mM HEPES buffer (pH 7.8) containing 50% (v/v) DMSO upon addition of 50 μM of inorganic salts [K_2SO_4, NaCl, $CaCl_2$, $CuSO_4$, $ZnSO_4$, NaH_2PO_4, $MgCl_2$, $Pb(NO_3)_2$ or $FeCl_3$], reactive oxygen species (OCl^-, H_2O_2, $^•O_2^-$ or $^•OH$) or amino acids with various side-chain features (aspartic acid, phenylalanine, tryptophan, histidine, asparagine, tyrosine, glutamic acid, methionine, serine, arginine, glutamine, proline or threonine). The fluorescence spectra were recorded with λ_{ex} = 480 nm. Inset shows the image of fluorescence change of **1** (10 μM) before and after addition of 50 μM cysteine, which is photographed from the top of the quartz cell in a Hitachi F-2500 fluorimeter at λ_{ex} = 480 nm.

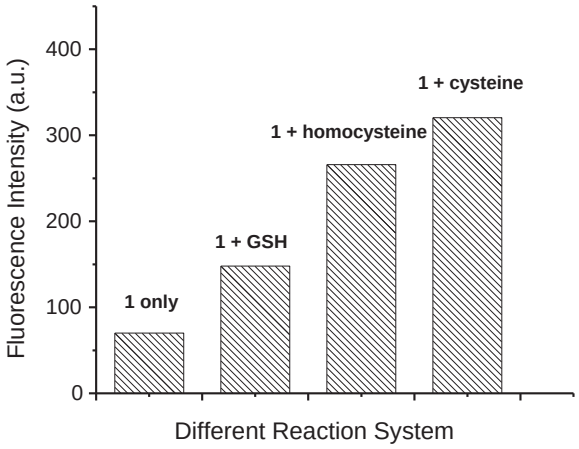


Fig. 3. Comparison of fluorescence intensities ($\lambda_{ex/em}$ = 480/512 nm) of 5 μM **1** (blank) and its reaction solutions with 10 μM of cysteine, homocysteine and GSH in 50 mM HEPES buffer (pH 7.8) containing 50% (v/v) DMSO.

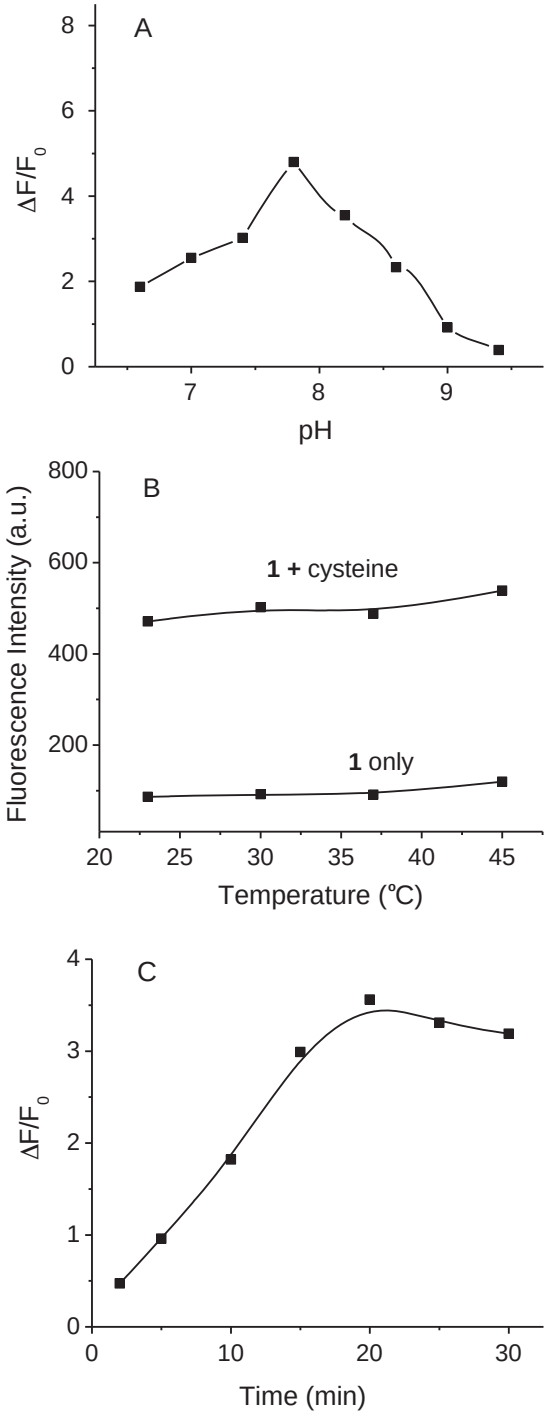


Fig. 4. Effects of pH (A), reaction temperature (B) and time (C) on the fluorescence ($\lambda_{ex/em}$ = 480/512 nm) of **1** (5 μM) reacting with cysteine (10 μM). Conditions: (A) the reaction was performed at room temperature for 20 min in 50 mM HEPES buffer containing 50% (v/v) DMSO with different pH values adjusted by NaOH; (B) the reaction was performed in 50 mM HEPES buffer (pH 7.8) containing 50% (v/v) DMSO at different temperatures for 20 min; (C) the reaction was conducted at room temperature for different times in 50 mM HEPES buffer (pH 7.8) containing 50% (v/v) DMSO.

After stirring for 30 min, 3 mL of Et_3N and 3 mL of BF_3 OEt_2 were added rapidly. The reaction solution was stirred for 90 min, and then washed three times with water. After dried over Na_2SO_4, the reaction solution was evaporated to remove the solvent, and the residue was purified by silica gel column chromatography with an eluent of 1:10 (v/v) methylene chloride/hexane, affording 51 mg of compound **2** as a red solid (yield 6%).

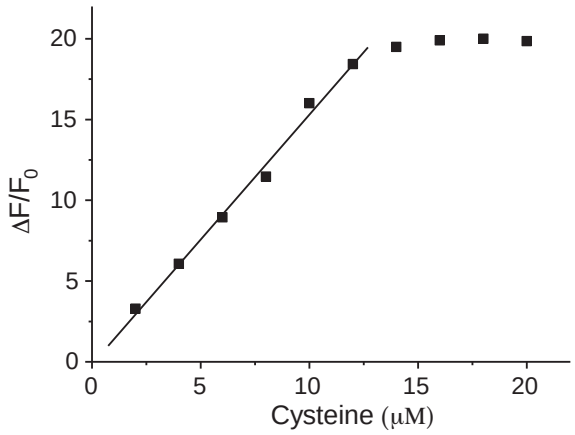


Fig. 5. The linear relationship between the relative fluorescence increase value $\Delta F/F_0$ at $\lambda_{ex/em}$ = 480/512 nm and the concentration of cysteine with the usage of 1 μM **1**.

Second, probe **1** was synthesized through the following procedure: the above compound **2** (0.15 mmol, 51 mg) and 2,4-dinitrobenzenesulfonyl chloride (0.6 mmol, 212 mg) were dissolved in 50 mL absolute methylene chloride. Then 500 μL of Et_3N was added and the reaction solution was stirred for 90 min at room temperature. The reaction solution was then evaporated to remove the solvent. The residue was washed three times with water, and purified by silica gel column chromatography with an eluent of 1:20 (v/v) methylene chloride/hexane. The obtained brownish red powder was dried under reduced pressure at 40 °C, affording 57 mg of **1** (yield 67%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.69 (s, 1H), 8.48–8.50 (d, 1H), 8.19–8.21 (d, 1H), 7.33–7.41 (q, 4H), 5.99 (s, 2H), 2.55 (s, 6H), 1.33 (s, 6H). Elemental analysis, calcd. for **1** ($C_{25}H_{21}BF_2N_4O_7S$): C 52.65, H 3.71, N 9.82%; found: C 52.45, H 3.77, N 9.55%.

2.4. General procedure for cysteine determination

In a typical procedure for cysteine determination, 100 μL stock solution of **1** was mixed with 5 mL DMSO and 4.5 mL HEPES buffer (0.1 M, pH 7.8) in a 10 mL tube under argon, followed by the addition of requisite volume of cysteine sample solution. The reaction solution was adjusted to 10 mL with water and mixed well. After 20 min at room temperature, a suitable volume of the reaction solution was transferred into a quartz cell of 1 cm optical length, and fluorescence spectra or/and intensities were measured at $\lambda_{ex/em}$ = 480/512 nm against a reagent blank without cysteine prepared under the same conditions.

2.5. DTNB-derivatization HPLC method for the determination of non-protein cysteine in human serum

HPLC analysis of cysteine was carried out following the known procedure [4,6]. Briefly, 0.45 mL of Tris–HCl buffer (0.5 M, pH 8.9), 130 μL standard solution of 0.75 mM cysteine, 120 μL of *p*-aminobenzoic acid (10 mg/mL, internal standard), and 250 μL of DTNB (10 mM) in K_2HPO_4 (0.5 M, pH 7.2), were successively mixed in a test tube. After 10 min, the mixture was acidified with 50 μL of H_3PO_4 (7 M), and filtered for measurements.

For analysis of non-protein cysteine in human serum, 200 μL of serum was deproteined by adding 200 μL of 3% (w/w) sulfosalicylic acid and then centrifuging at 10,000 × *g* for 15 min. The separated supernatant was diluted to 800 μL with Tris–HCl buffer (0.5 M, pH 8.9), followed by adding 140 μL of DTNB (10 mM) in K_2HPO_4 (0.5 M, pH 7.2) and 10 μL of *p*-aminobenzoic acid (10 mg/mL) as an internal standard. After derivatization for 10 min at room temperature, the

Table 1
Recovery of 2 μM cysteine in the presence of various substances.

Substance	Concentration (mM)	Molar ratio of the added substance to cysteine	Recovery (%)
NaCl	30	15,000	99.5
$KHCO_3$	30	15,000	101.9
$CaCl_2$	2	1000	102.6
$MgCl_2$	2	1000	102.5
$CuSO_4$	2	1000	103.9
$ZnSO_4$	1	500	98.7
NaH_2PO_4	2	1000	97.8
$Pb(NO_3)_2$	1	500	103.1
$FeCl_3$	0.02	10	103.0
Aspartic acid	2	1000	102.3
Phenylalanine	1	500	96.5
Tryptophan	0.6	300	101.8
Histidine	2	1000	102.7
Asparagine	1.6	800	103.3
Tyrosine	1.6	800	96.8
Glutamic acid	2	1000	103.7
Methionine	1.6	800	96.1
Serine	1	500	103.3
Arginine	2	1000	96.3
Glutamine	1.6	800	102.6
Proline	1	500	101.7
Threonine	1	500	99.3
Homocysteine	5×10^{-4}	0.25	106.8
GSH	1×10^{-3}	0.5	105.5

mixture was re-acidified with 50 μL of H_3PO_4 (7 M), filtered and analyzed by HPLC.

HPLC mobile phase employed solvent A (acetonitrile) and solvent B [aqueous solution with 0.9% (v/v) formic acid]. The flow rate of solvent A was first increased from 0 to 0.2 mL/min for 10 min, then to 0.32 mL/min for 5 min, and finally held stable for an additional 10 min; whereas that of solvent B was 0.4 mL/min invariably. The injection volume was 7.5 μL, and 326 nm was chosen as the detection wavelength.

3. Results and discussion

3.1. Fluorescence properties and selectivity studies

The design of probe **1** takes advantage of the properties of BODIPY, phenol and DBS moieties. First, like rhodamine [31–33], BODIPY is well recognized as a fluorophore with high molar absorptivity and photo-stability. Second, active phenol as a linker benefits the introduction of a quenching group. Finally, DBS with two strong electron-withdrawing nitro groups could act as an effective quencher, which is desired for a low background signal. Moreover, DBS as an easy leaving moiety under the attack of thiol may provide high selectivity for the resulting probe. Specifically, in this work the fluorescent probe **1** was prepared by esterification of the phenol-appended BODIPY with 2,4-dinitrobenzenesulfonyl chloride. As expected, probe **1** is a donor–acceptor molecule, and the internal charge transfer from the electron-donating BODIPY unit to the electron-accepting DBS unit largely decreases the probe's fluorescence (Φ = 0.03 in ethanol) compared with that of the BODIPY precursor (Φ = 0.55 in ethanol [28]). The introduction of cysteine into the solution of **1** gives rise to a great increase in fluorescence intensity (Fig. 2).

The reactions of **1** with various species were examined to test the selectivity. The examined substances included inorganic salts, reactive oxygen species, amino acids, and in particular coexisting thiols. As shown in Fig. 2, the fluorescence enhancement is observed only in the case of cysteine, instead of non-thiol species. Fig. 3 depicts the fluorescence response of **1** to thiols such as cysteine, homocysteine and GSH. As can be seen, all the three thiols generate fluorescence enhancements, but the fluorescence intensity

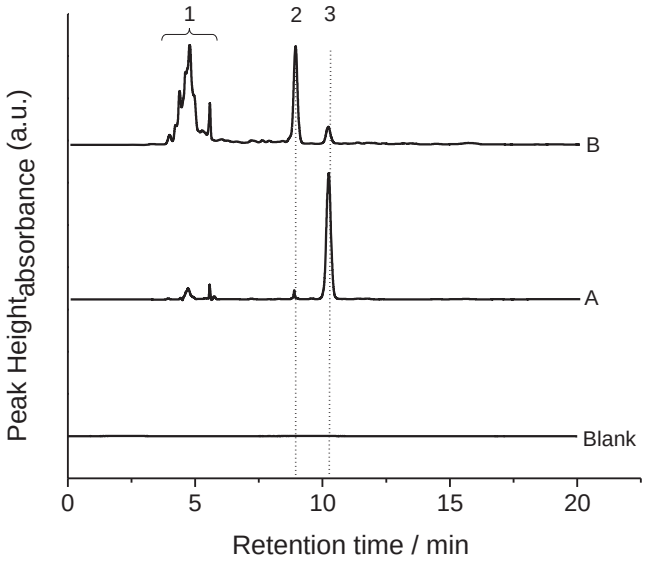


Fig. 6. Chromatograms of different reaction systems. (Blank) 100 μM cysteine; (A) 50 μM probe **1**; (B) the reaction products of 50 μM **1** with 100 μM cysteine following the general procedure given in Section 2. HPLC analyses were performed on an Inertsil ODS-SP (5 μM, 4.6 mm × 250 mm) column using a Shimadzu HPLC system that consists of two LC-20AT pumps and a SPD-20A UV–vis detector at 254 nm with methanol (flow rate, 0.6 mL/min) and water (flow rate, 0.1 mL/min) as eluents. The assignments of the peaks: (1) 4.5–5.5 min, some unidentified reaction products of **1**; (2) 8.78 min, compound **2**; (3) 10.13 min, probe **1**.

enhanced by cysteine is the strongest, which may be attributed to the steric hindrance effect of nucleophilic aromatic substitution [1,18,29]. Moreover, the concentration of cysteine in human serum is about one order of magnitude higher than those of the other thiols [3–5]. Therefore, the probe **1** might be used for selective detection of cysteine in human serum without any significant interference.

3.2. Optimization of experimental conditions

The reaction conditions (pH, temperature and time) of the present system were examined in details. Because of limited water solubility of **1**, the usage of 50% (v/v) DMSO as a cosolvent is required in this system. Fig. 4A shows the effect of pH on the relative fluorescence increase value $\Delta F/F_0$ ($\Delta F = F - F_0$, where F_0 and F are the fluorescence intensities before and after the addition of cysteine, respectively). As can be seen, pH 7.8 is suited for the present system because more acidic or alkaline media give lower sensitivity. Fig. 4B depicts the effect of reaction temperature from 23 to 45 °C, from which it is seen that either **1** or its reaction with cysteine is scarcely affected by temperature, though a slight increase in both background and response signals occurs at a higher temperature of 45 °C. Therefore, room temperature (about 25 °C) may be used for convenience. Fig. 4C shows the effect of reaction time on $\Delta F/F_0$,

Table 2
Determination of non-protein cysteine in human serum.[a]

Sample	Proposed method (μM)	DTNB-derivatization HPLC method (μM)
Human serum A	7.35 ± 0.05	7.28 ± 0.04
Human serum B	9.41 ± 0.23	9.25 ± 0.16
Human serum C	8.30 ± 0.02	8.23 ± 0.04
Human serum D	6.42 ± 0.11	6.37 ± 0.03

[a] Analytical result was expressed as: mean of three determinations ± S.D. Human sera from four healthy individuals were provided by Peking University First Hospital (Beijing).

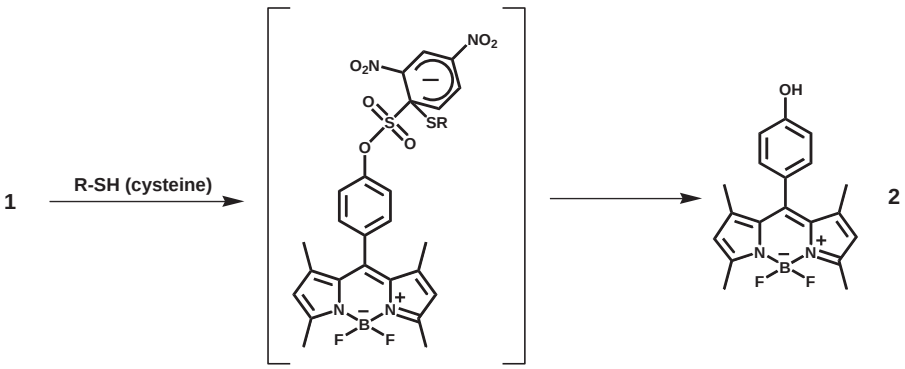
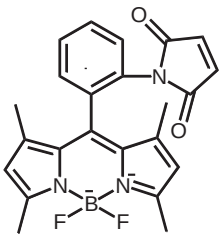
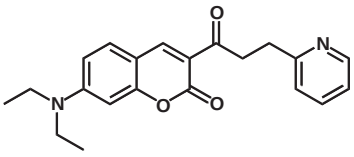
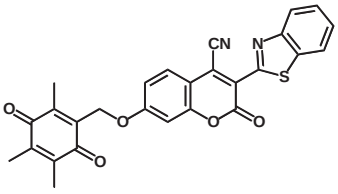
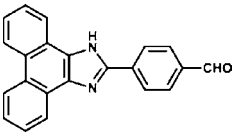
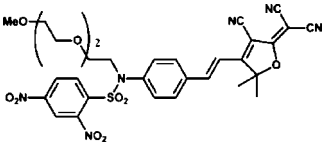
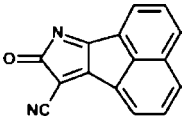
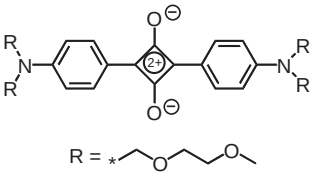


Fig. 7. Possible reaction mechanism of probe **1** with cysteine.

Table 3
Comparison of fluorescent probes proposed for the determination of thiols.

Probe	Selectivity	Linearity or/and detection limit	Application	Ref.
	Good	No data available	Labeling of bovine serum albumin	[14]
	Good	925 nM for cysteine	Detection of total thiols in new-born calf serum and human urine	[15]
	Good	1–100 μM for cysteine	No application	[16]
	Good	$(0.6–8.0) \times 10^{-4}$ M for cysteine	Detection of total thiols in new-born calf serum	[17]
	Good	No data available	Fluorescence imaging of thiols in cells	[19]
	Good	No data available	Fluorescence imaging of thiols in cells	[20]
	Good	200 nM	Determination of total thiols in human plasma	[21]
probe **1**	Good	2–12 μM/30 nM for cysteine	Determination of non-protein cysteine in human serum	This work

which reveals that a maximum value of $\Delta F/F_0$ can be achieved at the reaction time of 20 min. As a result, a reaction medium of pH 7.8, and a reaction time of 20 min at room temperature were chosen for the present system.

3.3. Linearity

As is known, the concentration of non-protein cysteine in human serum is at μM levels [4]. For accurate measurement, a calibration curve that corresponds to μM levels of cysteine should thus be constructed. On the other hand, it was observed that with the increase of the concentration of **1**, the background and response signals increased simultaneously. Therefore, a lower probe concentration of 1 μM that can produce a sufficient fluorescence signal was utilized to determine the linearity between $\Delta F/F_0$ and cysteine concentration. As shown in Fig. 5, a linear equation of $\Delta F/F_0 = 1.54 \times C\,(\mu M) - 0.06$ ($n = 6$, $R^2 = 0.995$) in the concentration range of 2–12 μM cysteine is obtained. The detection limit for cysteine is 30 nM based on 11 blank determinations (S/N = 3), featuring a high sensitivity.

3.4. Interference studies

The effects of various common substances present in human serum were studied on the detection of cysteine by analyzing artificial sample solutions containing 1 μM probe **1** and 2 μM cysteine. The tolerable concentration was determined by the criterion at which a substance gave a relative error of less than 10% in recovery of 2 μM cysteine. The results are summarized in Table 1, indicating that the fluorescence response of **1** to cysteine is of high selectivity.

3.5. Analysis of non-protein cysteine in human serum

The applicability of the proposed method was demonstrated by determining non-protein cysteine in human serum. Prior to determination, human serum was deproteined following the procedure given in Section 2. The results obtained are given in Table 2. The validity of the proposed method was further verified by the DTNB-derivatization HPLC method [4]. As shown in Table 2, the results obtained by the present method accord well with those by the DTNB-derivatization HPLC method, which are evaluated by *t*-test, proving that there is no significant difference between the two methods at the 95% confidence level [31].

3.6. Reaction mechanism

The reaction products of **1** with cysteine were subjected to HPLC analysis to explore the fluorescence response mechanism. As shown in curve A of Fig. 6, the probe **1** has a chromatographic peak at 10.13 min (peak 3), accompanied by tiny hydrolytic products at about 5 min (peak 1) and 8.78 min (peak 2). After reaction, the peak of probe **1** diminishes dramatically, whereas the peaks 1 and 2, representing the reaction products, increase greatly (curve B in Fig. 6). The product with the retention time of 8.78 min (peak 2 in curve B) is characterized to be compound **2** (see also Fig. 1) by electrospray ionization mass spectrometry (ESI-MS, *m*/*z*: 339.2 $[M-H]^+$). Based on the above findings, we propose that the fluorescence reaction of **1** with cysteine in this system might proceed through the route depicted in Fig. 7, that is, the strong nucleophilic substitution of cysteine leads to the cleavage of sulfonic acid ester bond of **1**, releasing the fluorophore (phenol-appended BODIPY) and thus accompanying large fluorescence enhancement.

3.7. Comparison with other fluorescent probes

DTNB is perhaps the most conventional chromogenic reagent used for pre-column derivatization of thiols in HPLC analysis. This method has been accepted as a standard for thiol assays in biological fluids like blood samples [34]. However, such detection is time-consuming and not very sensitive because it is based on the measurement of absorbance. To overcome this problem, various fluorescent probes have been developed for the sensitive and simple detection of thiols [14–21]. Table 3 shows the comparison of different fluorescence probes reported for cysteine. As is seen, all the probes exhibit high selectivity, but some still suffer from low sensitivity. Compared to most of the existing probes, however, **1** displays an improved overall performance in terms of both selectivity and sensitivity, which makes it of great potential use in simple and fast determination of cysteine in real biological samples.

4. Conclusions

Based on the specific nucleophilic aromatic substitution of thiols, probe **1** has been developed as a highly selective and sensitive fluorescence probe for cysteine detection. The probe reacts with cysteine producing a rapid and large fluorescence enhancement, which leads to the establishment of a new and simple method for cysteine assay. Furthermore, the proposed method has been applied to the determination of non-protein cysteine in human serum with satisfactory results.

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