BODIPY-Based Fluorometric Sensor for the Simultaneous Determination of Cys, Hcy, and GSH in Human Serum

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S *Supporting Information*

ABSTRACT: Cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) are interconnected and play essential roles for regulating the redox balance of biological processes. However, finding a simple and effective method for the simultaneous determination for these three biothiols in biological systems is always a challenge. In this work, we report a method for the simultaneous quantitative determination of three biothiols in a mixture using a monochlorinated boron dipyrromethene (BODIPY)-based fluorometric sensor. At a specified period of time, after reacting with excess sensor, Hcy and GSH form predominantly sulfur-substituted BODIPY, while Cys generates sulfur-amino-diBODIPY due to a fast substitution−rearrangement−substitution reaction. A significant difference in polarities of these respective major products simplifies their separation by TLC, thus leading to the simultaneous determination of Cys, Hcy, and GSH readily. The sensor was successfully applied for the simultaneous quantitative detection of three biothiols in human serum, and the results were in good agreement with those obtained via high performance liquid chromatography (HPLC).

KEYWORDS: *BODIPY, cysteine, homocysteine, glutathione, serum*
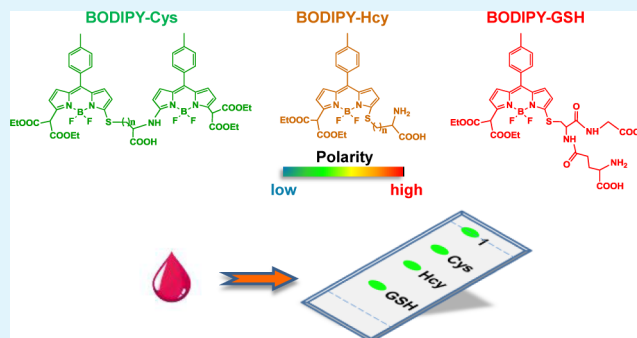
1. INTRODUCTION

Biological thiols, such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH), play essential roles for regulating the redox balance of biological processes. Cys is a metabolic product of Hcy and a precursor of the antioxidant GSH.[1] Cys deficiency can induce various diseases, e.g., slow growth, hair depigmentation, liver damage, skin lesions, etc.[2] Increased Hcy is an independent risk factor for Alzheimer's and cardiovascular diseases. Moreover, the Hcy content in blood is related to homocystinuria, renal diseases, psychiatric disorders, birth defects, and pregnancy complications.[3] An alteration of GSH homeostasis is associated with neurodegenerative diseases, aging, cystic fibrosis, HIV infection, and cancer.[4] These three biothiols interconnect and play different roles in biological systems. To elucidate the complicated relationship between Cys, Hcy, and GSH and investigate the function of each biothiol in various physiological processes, the simultaneous quantitative detection of these three biothiols is very necessary. While HPLC affords the simultaneous quantitative detection of the three biothiols in a mixture, it requires skilled staff, is labor intensive, and has low throughput; thus, the development of a

simple and effective method for the simultaneous quantitative determination for Cys, Hcy, and GSH in biological systems, such as human blood, is highly desirable.

Fluorescent sensors are powerful tools for the detection of mercapto biological molecules due to their simplicity and high sensitivity. In recent years, numerous fluorescent sensors have been developed for distinguishing biothiols from other amino acids by utilizing the strong nucleophilicity of the thiol group, including Michael addition, cleavage of disulfide and sulfonamide, etc.[5−12] The discrimination between three thiol-containing molecules is, however, problematic simply because of their similar structures and reactivities (Scheme 1).

Lately, considerable efforts have been devoted to develop fluorescent sensors which have selective response to Cys,[13−20] Hcy,[21−24] or GSH.[25−28] Unfortunately, sensors capable of simultaneous quantitative determination of Cys, Hcy, and GSH in a mixture are extremely rare. Very recently, the simultaneous

Scheme 1. Structures for Cys, Hcy, and GSH

Cysteine (Cys)

Homocysteine (Hcy)

Glutathione (GSH)
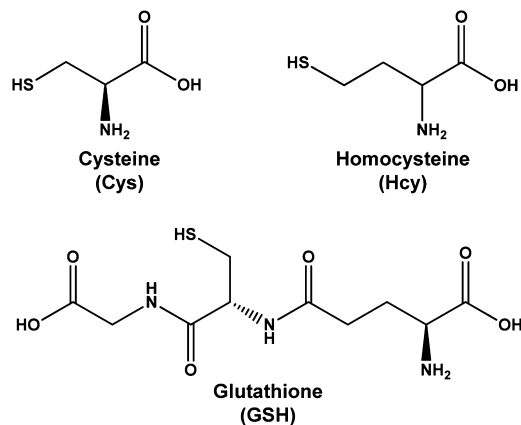
detections of Cys/Hcy and GSH were reported using fluorescent sensors with either different emission channels or a dual emission.[29−32] Although these sensors have been successfully applied for the detection of Cys/Hcy and GSH in biological systems, the simultaneous quantitative determination of all three biothiols in the mixture is still challenging as biothiols generally coexist and react competitively with sensors (see Supporting Information (SI): the determination of Cys/Hcy/GSH using various sensors). Additionally, the large difference in concentrations in biological samples (e.g., plasma Cys concentrations are typically 20−30 times that of Hcy)[33] have also increased the difficulty of quantitative determination.

Herein we report an inexpensive and effective strategy for the simultaneous quantitation of Cys, Hcy, and GSH in a mixture by using monochlorinated BODIPY-based fluorometric sensor **1**. At a specified period of time, after reacting with excess sensor **1**, Hcy and GSH form predominantly sulfur-substituted BODIPY, while Cys generates sulfur-amino-diBODIPY due to a fast substitution−rearrangement−substitution reaction. By taking advantage of the significant difference in polarities of the respective products, the separation could be completed on a regular TLC plate rather than by more expensive HPLC. Combined with digital imaging, the simultaneous quantitation of Cys, Hcy, and GSH could be readily obtained. For the first time, the simultaneous quantitative detection of three biothiols in human serum was realized via such a simple and convenient strategy, and the results were in good agreement with those obtained via HPLC.

2. EXPERIMENTAL SECTION

Materials and Instrumentation. All the reagents and solvents were of commercial quality and used without further purification. Silica gel 60 TLC plates were purchased from Merck Company. Fluorescence spectra were obtained on a PerkinElmer LS 55 fluorescence spectrometer. Absorption spectra were determined on a Persee TU-1901 UV−vis spectrophotometer. The images of the TLC plates were taken using a Nikon D7000 digital camera. The emission spectrum of the UV light source was acquired utilizing an Ocean Optics USB 4000 spectrometer coupled with a reflection probe. ^{1}H and ^{13}C NMR spectra were recorded on an Avance Bruker 400 M spectrometer and referenced to solvent signals. Mass spectra were obtained on a Bruker Apex IV Fourier transform mass spectrometer and on a Thermo LTQ Orbitrap XL mass spectrometer. The purification of sensor **1** after column chromatography was performed on a Waters AutoPurification System, and its purity analysis was performed on an Alliance 9 2695 PDA 2998. The HPLC

determination of biothiols was carried out using an Agilent HPLC system model 1200.

Synthesis and Purification of Sensor 1. BODIPY-Cl₂ (see SI, Scheme S1) was prepared according to the reported literature methods.[34,35] BODIPY-Cl₂ (100 mg, 0.28 mmol) was dissolved in 20 mL of dry acetonitrile, and sodium hydride (57−63% oil dispersion, 12 mg, 0.3 mmol) was added. Diethyl malonate (86 μL, 0.57 mmol) in dry acetonitrile (10 mL) was added dropwise with stirring. After being stirred at room temperature for 2 h, the reaction was quenched with water and extracted with dichloromethane. The organic layer was dried over MgSO₄, concentrated, and purified by column chromatography over silica (dichloromethane/petroleum ether = 1/1 as eluent) to give sensor **1** (92 mg, 69%) as an orange solid. ^{1}H NMR (400 MHz, CDCl₃): δ 7.42 (d, 2H, J = 8.0 Hz), 7.33 (d, 2H, J = 8.0 Hz), 6.92 (d, 1H, J = 4.0 Hz), 6.86 (d, 1H, J = 4.4 Hz), 6.72 (d, 1H, J = 4.0 Hz), 6.41 (d, 1H, J = 4.4 Hz), 5.49 (s, 1H), 4.28 (q, 4H, J = 7.2 Hz), 2.47 (s, 3H), 1.30 (t, 6H, J = 7.2 Hz). Waters AutoPurification System coupled with a UV−vis detector was used for the further purification of sensor **1**. The separation was performed using a homemade C18ME preparative column (20 × 250 mm, 10 μm particles) and an isocratic elution method with 75% acetonitrile as the mobile phase. The detection wavelength was 510 nm, and the flow rate was 20 mL/min. After the further purification of sensor **1**, Alliance 9 2695 PDA 2998 was utilized to test its purity, and the chromatogram is presented in SI, Figure S1.

Detection of Cys/Hcy/GSH in Human Serum in Our Proposed Method. Human serum samples were obtained from healthy volunteers at a local hospital and stored at −20 °C until further analysis. After being thawed at room temperature, 100 μL of human serum was transferred to a 1.5 mL centrifuge tube. Ten microliters of 0.2 M HCl (Cys/Hcy/GSH mixture in 0.2 M HCl for plotting standard curves) was added, followed by the addition of 4 μL of 0.4 M triphenylphosphine (PPh₃, reducing reagent) in water/acetonitrile (2:8 v/v, containing 2 M HCl). The mixture was vortex-mixed for 15 min and then incubated for 15 min at room temperature. A 200 μL amount of methanol was added to precipitate the PPh₃ and proteins followed by centrifugation (15 000g, 20 °C, 10 min). The supernatant was mixed with 500 μL of acetonitrile to precipitate residual proteins followed by further centrifugation (15 000g, 20 °C, 5 min). The obtained supernatant was dried under the flow of high-purity N₂. A 400 μL amount of HEPES buffer (20 mM, pH 7.4, containing 2 mM EDTA) was added, and then the tube was centrifuged at 15 000g for 5 min at 20 °C. The final supernatant was stored at 4 °C for further analysis. A 50 μL amount of the reduced serum sample was mixed with the same volume of 300 μM sensor **1**. The reaction was performed at 35 °C in a water bath for 1 h. The final reaction mixture was separated on TLC plate using n-butanol−water−acetic acid−methanol mixture (7:1.5:1:0.5, v/v/v/v) as mobile phase. The fluorescent images of the TLC plates were captured using a digital camera. To conveniently filter the influence of the excitation light source, 370 nm was chosen as the excitation wavelength (see SI, Figure S2). A homemade device was developed for convenient image capture (see SI, Figure S3).

HPLC Method for the Detection of Cys/Hcy/GSH in Human Serum. With the aim of getting more convincing data to verify TLC results obtained in our proposed method, a completely different way to prepare the human serum for HPLC analysis was employed according to previous reports with some modifications.[36] Briefly, 50 μL of human serum sample was thawed at room temperature and transferred to a 1.5 mL centrifuge tube. Then 7.5 μL of a 66.7 g/L tris(2-carboxyethyl)phosphine solution (TCEP, reducing reagent) at nearly neutral pH (ca. pH 6) was added to the sample. The resulting mixture was vigorously vortex-mixed at intervals and incubated for 30 min at room temperature. Afterward, 250 μL of acetonitrile was introduced to precipitate the proteins. The obtained slurry was blow-dried by a pure N₂ flow. Then 225 μL of a 0.125 M borate buffer solution (pH 9.5, with 4 mM EDTA) was added to the tube, vortex-mixed, and centrifuged at 15 000g for 5 min. Eventually, 90 μL of the supernatant was carefully taken out and mixed with 10 μL of a 5 g/L ammonium 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid (SBD-F) solution. The derivatization reaction was performed at 60 °C in a water bath for 1 h.

Scheme 2. Proposed Reaction Mechanism of Excess Sensor 1 with Cys, Hcy, and GSH
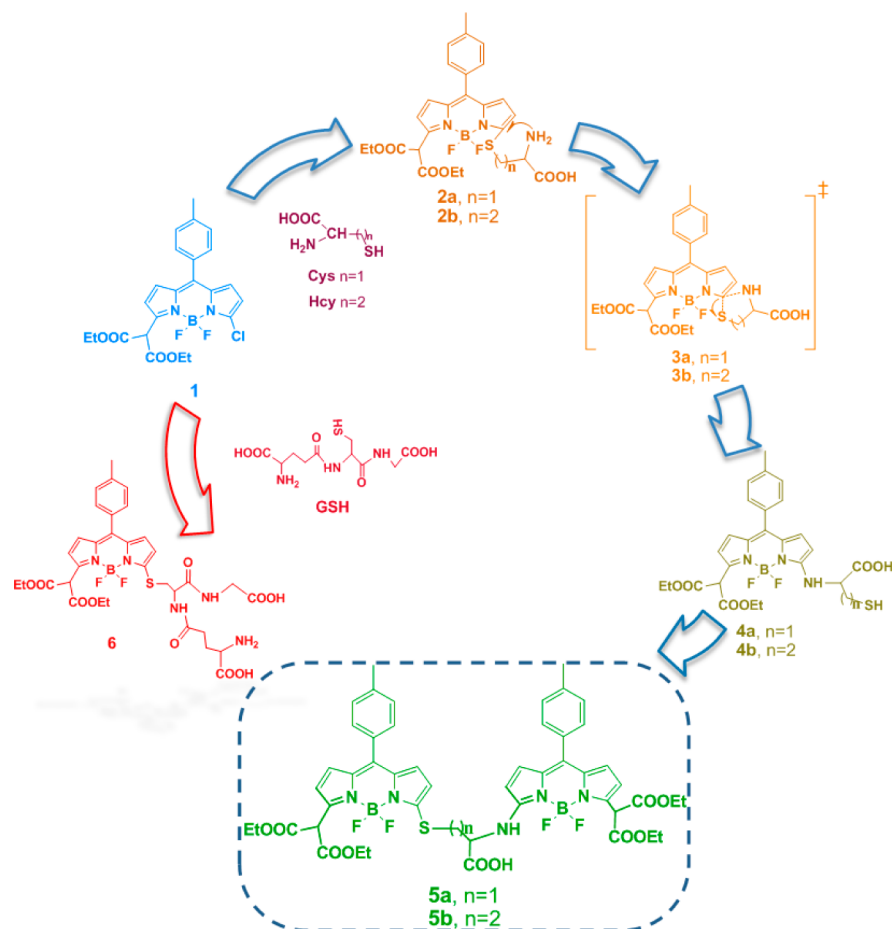
The final solution was filtered with a 0.22 μm Millipore membrane and kept at 4 °C for further HPLC analysis. The reaction mechanism of aminothiols with SBD-F is expressed in SI, Scheme S2. The simultaneous determination of total Cys, Hcy, and GSH in human serum was performed using an Agilent HPLC system (model 1200) coupled with a fluorescence detector. The fluorescence signals were measured with excitation at 385 nm and emission at 515 nm. The separation of the three biothiols was carried out using a conventional Kromasil C18 column (4.6 × 250 mm, 5 μm particles) and a gradient elution method. Two mobile phases consisted of A: 0.1 M acetate buffer (pH 4.5)−methanol [97:3 (v/v)] and B: methanol. For the first 8 min, 100% A at a flow rate of 0.8 mL/min was set, and then 80%:20% A:B (changed linearly over 1 min) at a flow rate of 1.0 mL/min for 6 min followed, and finally 5 min was given for column re-equilibration at 100% A at 0.8 mL/min. The three biothiols were baseline separated, and the chromatogram is presented in Figure S4 in SI.

3. RESULTS AND DISCUSSION

BODIPY derivatives are good candidates as fluorescent sensors in biological systems due to their high molar absorption coefficients and quantum yields, inertness under physiologically relevant conditions, and resistance to photobleaching.[37−40] We have previously reported the first ratiometric fluorescent sensor for the selective detection of GSH based on monochlorinated BODIPY derivatives.[26] Free thiol displaces the chlorine by thiol−halogen nucleophilic substitution, resulting in sulfur-substituted BODIPY. The amino groups of Cys/Hcy but not GSH further displace the thiolate to form amino-substituted BODIPY. The substitution−rearrangement mechanism enables

the discrimination of GSH over Cys/Hcy. The simultaneous determination of three biothiols, however, still remains a big challenge. As we mentioned, the biothiols react with **1** to produce sulfur-substituted BODIPY (**2a**, **2b**, and **6**) (see Scheme 2), and subsequently **2a**/**2b** but not **6** rearrange through five-/six-membered transition states (**3a**/**3b**) to form amino-substituted BODIPY (**4a**/**4b**). The intramolecular rearrangement for **2a** to produce **4a** would occur more rapidly than **4b** for **2b** due to the formation of **4a** via the favorable five-membered cyclic transition state. Thus, at an early stage, the reaction of sensor **1** with Cys would produce **4a** after the quick rearrangement, while Hcy would remain at stage **2b**. As for GSH, the major product would be sulfur-substituted BODIPY **6**. If the reaction is maintained at this stage,[26] it would be difficult to distinguish among Cys, Hcy, and GSH because products **2b** and **6** are expected to have similar photophysical properties, while **4a** and **2b** have analogous structures, resulting in comparable polarities.

To achieve the simultaneous determination of the three target biothiols, excess **1** is required because the three biothiols react competitively with sensor **1**. Scheme 2 shows the proposed reaction mechanism of excess sensor **1** with Cys, Hcy, and GSH. The exposed thiol group of amino-substituted BODIPY (**4a**/**4b**) may further react with excess sensor **1** to ultimately form sulfur-amino-diBODIPY (**5a**/**5b**). Accordingly, within a given period of time, the reaction of excess sensor **1** with Cys, Hcy, and GSH would produce **5a**, **2b**, and **6**, respectively. Since the structures of **5a**, **2b**, and **6** differ so greatly, there is a large variance in their respective polarities.

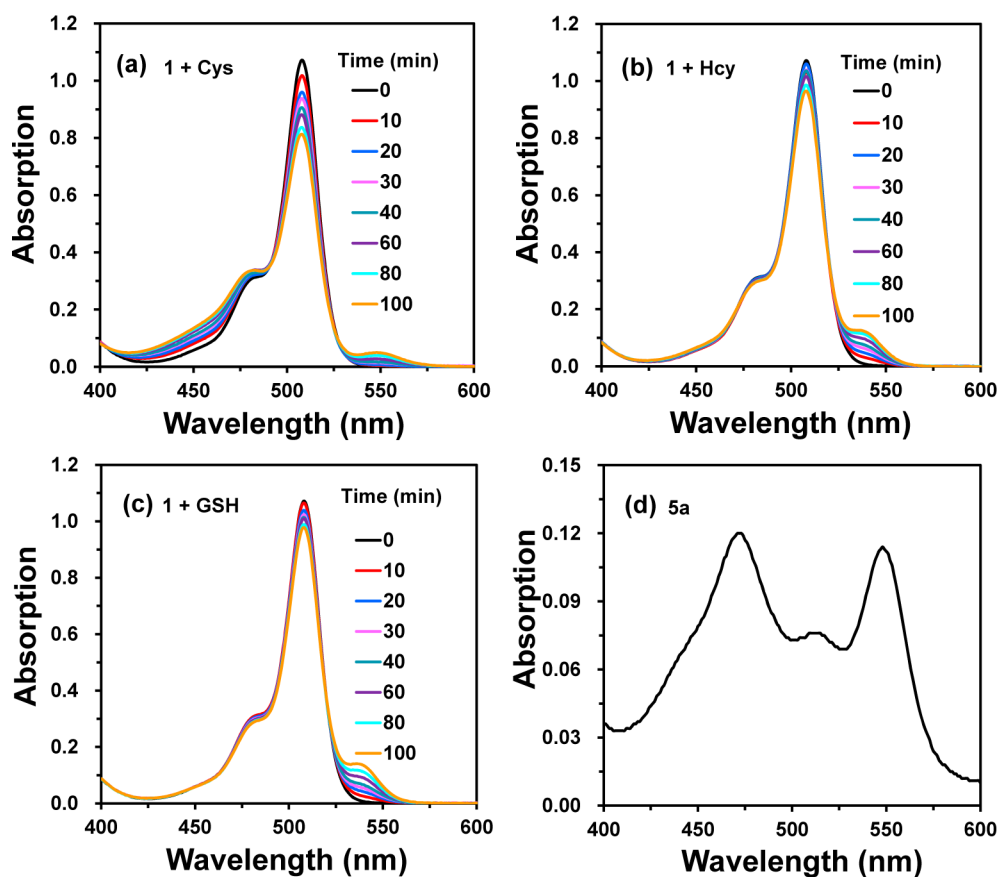**Figure 1.** (a−c) Time-dependent absorption spectra of excess sensor **1** (300 μM) with Cys, Hcy, or GSH (100 μM). Each spectrum was acquired in acetonitrile/HEPES buffer (50:50 v/v, 20 mM, pH 7.4) at 35 °C and the solutions were diluted 20 times with the acetonitrile/HEPES buffer before measurement. (d) Absorption spectrum of separated **5a** in acetonitrile/HEPES buffer (v:v = 5:5, 20 mM, pH = 7.4).

Instead of using HPLC column separation, this large polarity difference enables simple separation on inexpensive and convenient TLC plates. Combined with digital imaging, it provides us simultaneous quantitative detection of Cys, Hcy, and GSH in a mixture.

To verify our presumption that the reaction of excess sensor **1** with Cys, Hcy, and GSH would produce **5a**, **2b**, and **6**, respectively, within a given period of time, we first examined the reaction of Cys/Hcy/GSH (100 μM) with excess sensor **1** (300 μM) through time-dependent UV−vis spectroscopy in acetonitrile/HEPES buffer (50:50 v/v, 20 mM, pH 7.4) at 35 °C. As shown in Figure 1a−c, sensor **1** shows an absorption maximum at 508 nm. According to our previous work, the sulfur-substituted BODIPY results in a red shift in absorption, while amino-substituted BODIPY induces a blue shift.[26] Upon addition of GSH to **1**, a decrease in the absorption at 508 nm was accompanied by the appearance of a new band at 535 nm, which is attributed to sulfur-substituted BODIPY **6**. For Hcy, similar phenomena were observed, and the absorption at 535 nm was assigned to sulfur-substituted BODIPY **2b**. Alternatively, in the presence of Cys, two absorption bands at 450 and 548 nm emerged. The blue-shifted absorption at ~450 nm was attributed to amino-substituted BODIPY, while the red-shifted absorption at 548 nm was likely caused by sulfur-substituted BODIPY, including sulfur-substituted BODIPY **2a** or sulfur-amino-diBODIPY **5a**. Since only a blue-shifted absorption was observed upon the addition of excess Cys to sensor **1** in 100 min (see SI, Figure S5), sulfur-substituted BODIPY **2a** was minimal. Thus, the absorption at 548 nm

could be assigned to sulfur-amino-diBODIPY **5a**. After a column separation, the formation of sulfur-amino-diBODIPY **5a** was further evidenced in the mass spectrum, where a prominent peak at m/z 1020.3 corresponding to $[5a + Na]^+$ was clearly observed (see SI, Figure S6). As shown in Figure 1d, the separated **5a** shows adsorption peaks at 550 and 474 nm, which correspond to the new absorption band in Figure 1a.

Taken together, the above time-dependent absorption spectra show that the reaction of excess sensor **1** with the three biothiols mainly produced sulfur-amino-diBODIPY **5a** (for Cys, amino-substituted BODIPY **4a** may also exist), sulfur-substituted BODIPY **2b** (for Hcy), and sulfur-substituted BODIPY **6** (for GSH), respectively, in 100 min, which is in accordance with our hypothesis.

The polarity of reaction products of BODIPY sensor **1** with biothiols would be higher than that of sensor **1** itself as a result of the addition of high polarity groups, such as amino groups and carboxyl groups, from biothiols. Obviously, the higher proportion of polarity group the molecule contains, the stronger its polarity is. Judging from the structure of the main products **5a**, **2b** and **6**, a clear polarity difference, which is **6** > **2b** > **5a**, could be easily obtained, as shown in Figure 2. Theoretically, **2b** would finally generate sulfur-amino-diBODIPY **5b** after a long reaction period, which would disturb the discrimination between Cys and Hcy.

Due to the large difference in polarities of the respective products (**6**, **2b**, **5a**) at different reaction stages after reacting with sensor **1**, instead of HPLC, commercially available silica gel 60 TLC plates were used to separate the mixture of excess **1**
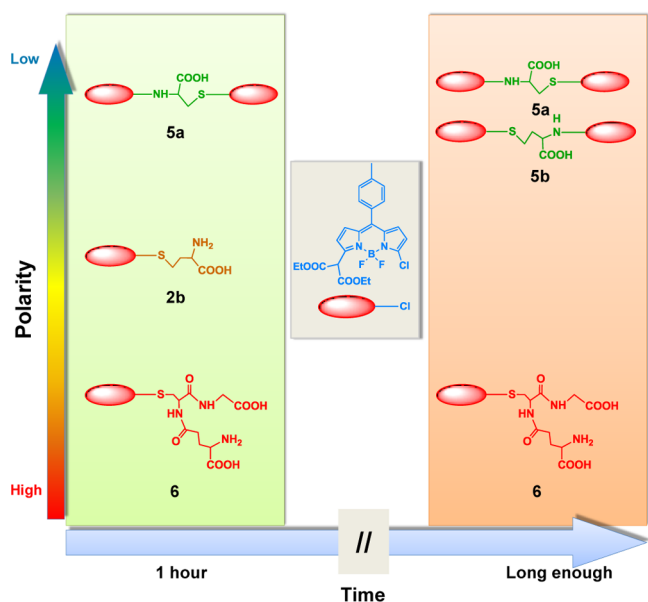
Figure 2. Difference in polarities of the respective products at different reaction stages after reacting with sensor **1**.

and the reaction products of **1** with Cys/Hcy/GSH. In this case, due to the strong affinity to Si−OH groups on the plate, the stronger the molecular polarity is, the lower R_f value is. Therefore, the R_f value of sensor **1** and products **5a**, **2b**, and **6** should theoretically be **1** > **5a** > **2b** > **6**.

To prove this deduction, excess sensor **1** was used to separately react with Cys, Hcy, and GSH. Fluorometric methods were utilized to take advantage of the high fluorescent quantum yield of BODIPY derivatives. Figure 3a1 shows the fluorescent spots of sensor **1** after developing on the TLC plate. With the exception of the strong fluorescent spot of the sensor itself, there exist two additional weak fluorescent spots, which

were most likely caused by hydrolysis of sensor **1**. For the reaction mixture of Hcy with excess sensor **1** (Figure 3a3), only one resultant spot, spot B, was observed, which was induced by **2b** (its mass spectrum is shown in SI, Figure S7). For the mixture of GSH with excess sensor **1** (Figure 3a4), spot C was attributed to product **6**; this assumption was verified by the mass spectrum (see SI, Figure S8). However, for the reaction mixture of Cys with excess sensor **1** (Figure 3a2), more than one resultant spot was observed. According to our previous hypothesized mechanism, the expected products of Cys and excess **1** might be **4a** and **5a** along with some minor products. We have chosen to only focus on the main spot A, which clearly showed fluorescence intensity stronger than that of other spots. Since the polarity of **5a** is the weakest among all products **2b**, **4a**, **5a**, and **6**, we deduced that spot A was product **5a** due to its large R_f value. The mass spectrum of this spot further verified this deduction (see SI, Figure S9). Therefore, **1**, **5a**, **2b**, and **6** were well separated with an apparent difference in R_f value, and the order of their R_f values was in agreement with our deduction.

The reduced human serum was also evaluated. Following 1 h of reaction time, the mixture of excess **1** and reduced serum was separated (see SI, Figure S10) and the corresponding fluorescent spots for Cys, Hcy, and GSH were observed, as shown in Figure 3b1. Furthermore, the relative intensities for Cys/Hcy/GSH were clearly enhanced after the addition of extra corresponding biothiol (Figure 3b2−b4). This clearly suggests a successful separation of Cys, Hcy, and GSH in serum samples based on the difference in polarities of their respective major products after reacting with excess sensor **1**.

Encouraged by these results, we attempted to evaluate the capability of a proposed strategy for the simultaneous quantitative detection of Cys, Hcy, and GSH in human serum. Initially, the repeatability and the reliability of the experimental device were evaluated. By using rhodamine B as a standard sample, less than 0.95% fluorescent brightness
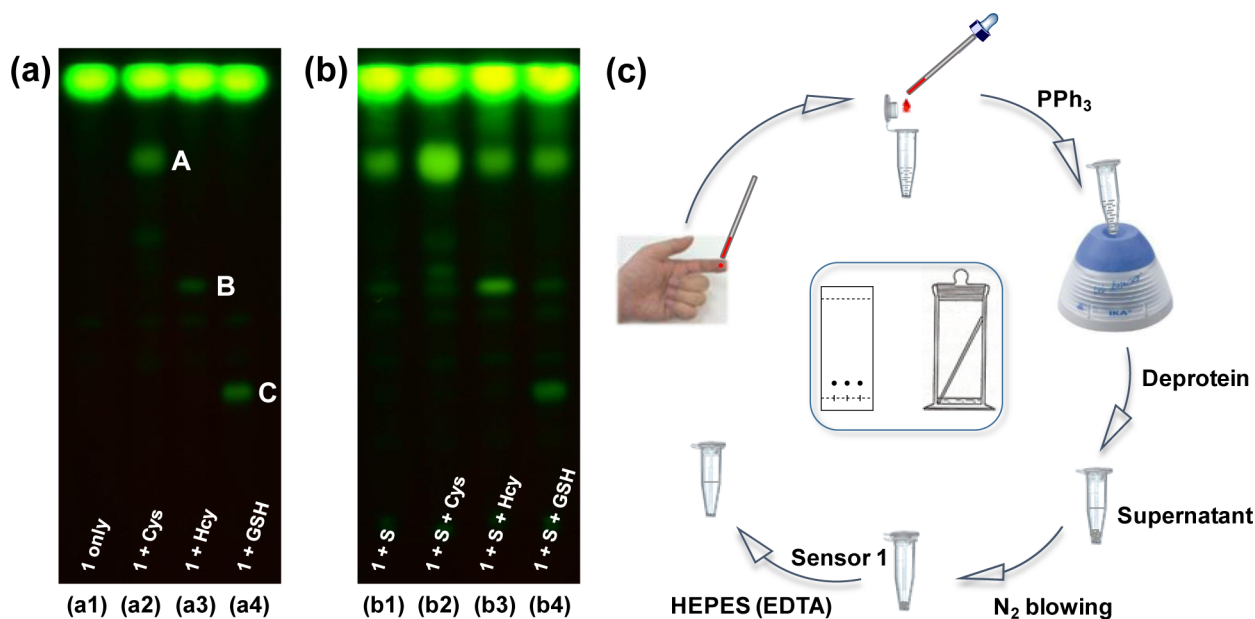

Figure 3. (a) Fluorescence images of (a1) 300 μM sensor **1**, (a2−a4) reaction mixture of 300 μM sensor **1** with 25 μM Cys (a2), 5 μM Hcy (a3), 5 μM GSH (a4). (b) Fluorescence images of reaction mixture of 300 μM sensor **1** with (b1) reduced serum sample (S), (b2−b4) reduced serum sample (S) after addition of 25 μM Cys (b2), 5 μM Hcy (b3), 5 μM GSH (b4). (c) The preparation of serum sample. Parameters of the digital camera: exposure time 1/2 s; F 10; ISO 500. For display purposes, the blue channel was removed from the fluorescence images.
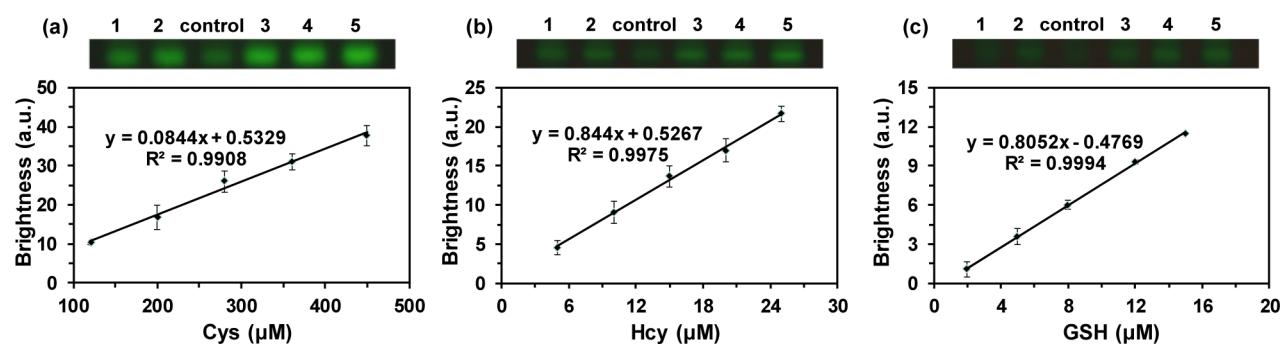
Figure 4. (a) Fluorescent images of **5a** on TLC plates and linearity between fluorescent brightness and concentration of Cys. (b) Fluorescent images of **2b** on TLC plates and linearity between fluorescent brightness and concentration of Hcy. (c) Fluorescent images of **6** on TLC plates and linearity between fluorescent brightness and concentration of GSH. Parameters of the digital camera: exposure time 1/2 s; F 10; ISO 250 for Cys, 500 for Hcy, and 800 for GSH. For display purposes, the blue channel was removed from the fluorescence images, and the brightness and contrast were increased 40% and 20%, respectively.

Table 1. Concentration Values Obtained from the Proposed and HPLC Methods for Cys/Hcy/GSH Analysis in Serum Samples

	Cys (μmol/L)		Hcy (μmol/L)		GSH (μmol/L)	
no.	this work[a]	HPLC[b]	this work[a]	HPLC[b]	this work[a]	HPLC[b]
1	242.9 ± 6.5	249.9 ± 1.6	12.9 ± 0.7	12.3 ± 0.7	4.7 ± 0.4	3.4 ± 0.3
2	195.1 ± 4.3	205.4 ± 14.2	12.5 ± 0.5	11.7 ± 0.5	5.4 ± 0.6	5.6 ± 0.0
3	235.8 ± 1.4	227.1 ± 2.9	13.1 ± 0.2	13.0 ± 0.2	5.7 ± 0.3	5.0 ± 0.5
4	271.3 ± 14.1	246.9 ± 5.3	12.8 ± 2.2	12.8 ± 0.1	4.9 ± 0.1	4.5 ± 0.2
5	230.7 ± 13.7	225.7 ± 0.4	12.6 ± 0.4	12.8 ± 0.4	4.9 ± 0.5	5.3 ± 0.0
6	278.8 ± 4.8	265.0 ± 4.6	14.5 ± 1.5	13.5 ± 0.1	5.0 ± 0.3	5.5 ± 0.2
7	236.6 ± 22.2	216.4 ± 4.3	12.9 ± 1.2	12.0 ± 0.1	4.9 ± 0.3	4.0 ± 0.0
8	256.3 ± 18.4	231.7 ± 0.2	12.6 ± 1.8	13.5 ± 0.6	4.9 ± 0.3	4.4 ± 0.6
9	209.3 ± 16.0	206.2 ± 3.3	11.7 ± 1.0	11.7 ± 0.1	4.5 ± 0.1	4.5 ± 0.2
10	234.2 ± 6.6	245.6 ± 0.4	12.4 ± 0.3	12.0 ± 0.1	4.9 ± 0.1	4.9 ± 0.3

[a]Results are reported as mean ± SD (standard deviation) for triplicate trials. [b]Values are reported as mean ± SD (standard deviation) for duplicate trials.

deviation of eight parallel trials offered proof that our experimental setup was suitable for such analysis (see SI, Figures S11, S12). The linear relationship between the brightness of the fluorescent spots and the biothiols' initial concentrations in a complex mixture was theoretically deduced in advance. A large excess of the sensor was required for the quantitative analysis to be carried out correctly (see SI, deduction section). Therefore, to ensure that sensor **1** was in large excess, the reduced serum samples were diluted eight times using acetonitrile/HEPES buffer with 2 mM EDTA when reacting with sensor **1** (300 μM). During the standard curve preparation procedure, to avoid the influence of the serum matrix, aquilots of human serum sample spiked with a mixture of the three biothiols in gradually increasing concentrations (see SI, Table S1) were prepared according to the same procedure with the target serum samples. The difference in fluorescent spot brightness between serum sample itself and serum sample spiked with biothiols was used to respectively generate the standard curves for biothiols and **5a**, **2b**, and **6**. As shown in Figure 4, in accordance with our deduction, a good linear relationship between the brightness of the fluorescent spot for product **5a** and the Cys concentrations (120−450 μM) was easily obtained. Similar results were also observed for Hcy and GSH, and the brightness of fluorescent spots for products **2b** and **6** was linearly proportional to the concentration of Hcy from 5 to 25 μM (Figure 4b) and GSH from 2 to 15 μM (Figure 4c), respectively.

On the basis of the above results, 10 human serum samples were prepared following the previously described procedure and then analyzed according to the proposed strategy. Meanwhile, the same serum samples were also analyzed using a standard HPLC method as a reference. The levels of Cys, Hcy, and GSH in 10 human serum samples determined through two methods (this work and HPLC) are given in Table 1. As expected, the results from the strategy we proposed in this work are in close agreement with HPLC results. Furthermore, the concentrations of Cys, Hcy, and GSH in these 10 human serum samples also agree with those reported in the literature.[41−43]

4. CONCLUSIONS

We have developed a facile strategy for the simultaneous determination of Cys, Hcy, and GSH in a mixture using monochlorinated BODIPY-based fluorometric sensor **1**. After reacting with excess sensor **1**, GSH and Hcy predictably form sulfur-substituted BODIPY within a specified period of time. However, due to a fast substitution−rearrangement−substitution reaction, Cys mainly generates sulfur-amino-diBODIPY. The significant differences in polarities of these respective major products simplify their separation by TLC, thus leading to the simultaneous determination of Cys, Hcy, and GSH. The strategy has been successfully applied for the detection of three biothiols in human serum, and the results were in good agreement with those obtained via HPLC. This method is

simple and accurate, and multiple samples could be analyzed at one time, which enables wide application prospects.

■ ASSOCIATED CONTENT

⑤ Supporting Information
Experimental setup, deductions, mass spectra of products **2b**, **5a**, and **6** after the reaction between sensor **1** and Cys/Hcy/GSH, UV−vis spectra of sensor **1** after addition of excess biothiols, fluorescence excitation and emission spectra of sensor **1**, and HPLC method for the detection of biothiols in serum samples. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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