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BODIPY-Based Ratiometric Fluorescent Sensor for Highly Selective Detection of Glutathione over Cysteine and Homocysteine

Li-Ya Niu,[†,‡] Ying-Shi Guan,[†,‡] Yu-Zhe Chen,[†] Li-Zhu Wu,[†] Chen-Ho Tung,[†] and Qing-Zheng Yang*[,†]

[†]Key Laboratory of Photochemical Conversion and Optoelectronic Materials, Technical Institute of Physics and Chemistry, Chinese Academy of Sciences, Beijing 100190, P. R. China

[‡]University of the Chinese Academy of Sciences, Beijing 100049, P. R. China

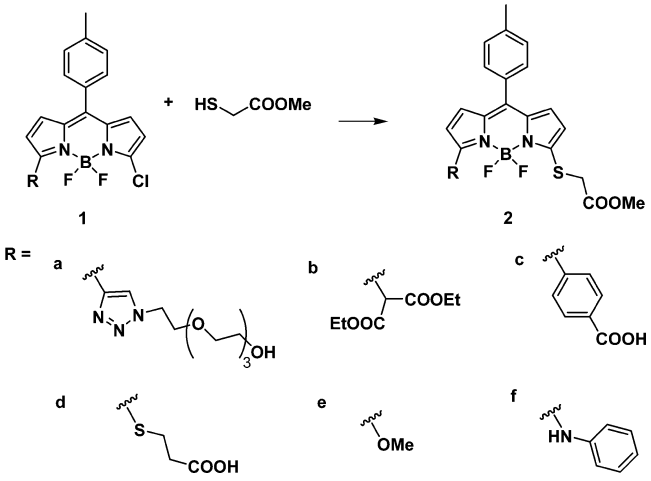
Supporting Information

ABSTRACT: We report a ratiometric fluorescent sensor based on monochlorinated BODIPY for highly selective detection of glutathione (GSH) over cysteine (Cys)/homocysteine (Hcy). The chlorine of the monochlorinated BODIPY can be rapidly replaced by thiolates of biothiols through thiol−halogen nucleophilic substitution. The amino groups of Cys/Hcy but not GSH further replace the thiolate to form amino-substituted BODIPY. The significantly different photophysical properties of sulfur- and amino-substituted BODIPY enable the discrimination of GSH over Cys and Hcy. The sensor was applied for detection of GSH in living cells.

Biological thiols, including glutathione (GSH), cysteine (Cys), and homocystein (Hcy), play crucial roles in maintaining the appropriate redox status of biological systems. GSH is the most abundant cellular thiol.[1] It is an essential endogenous antioxidant that plays a central role in cellular defense against toxins and free radicals. Abnormal levels of GSH can lead to cancer, aging, heart problems, and other ailments.[2] Its significant biological role explains the considerable contemporary effort devoted to the development of an efficient method for the detection and quantification of GSH under physiological conditions.[3,4]

Fluorescent sensors are well-suited for the detection of SH-containing biological molecules in vivo.[4] Most of the existing sensors utilize the strong nucleophilicity of the thiol group, operating by Michael addition,[5] cleavage of disulfide[6] and sulfonamide,[7] etc.[8] These sensors distinguish biothiols from other amino acids. However, it is still a challenge to discriminate among thiol-containing molecules with their similar structures and reactivities. By means of the cyclization of Cys/Hcy with aldehydes or acrylates, pioneered by the Strongin group, selective detection of Cys/Hcy over GSH was achieved.[9] On the other hand, the development of reaction-based fluorescent sensors for discrimination of GSH without interference from Cys/Hcy remains a tough task. Recently, on the basis of previous work,[9c] Strongin and co-workers reported a sensor for selective detection of GSH in cetyltrimethylammonium bromide (CTAB) media.[10] However, the need for a surfactant may limit its application in living systems. Herein we report a ratiometric fluorescent sensor for highly selective detection of GSH over Cys and Hcy based on monochlorinated boron dipyrromethene (BODIPY) derivatives. Free thiol displaces the chlorine by thiol−halogen nucleophilic substitution (Scheme 1),[11] resulting in a significant fluorescence red-shift. The amino groups of Cys/Hcy but not GSH further displace the sulfur to form amino-substituted BODIPY, which exhibits relatively weak, blue-shifted fluorescence. This allows GSH to be distinguished from Cys/Hcy.

Scheme 1. Reaction of 1 with Methyl Mercaptoacetate



BODIPY dyes have several attributes that make them good candidates as fluorescent sensors in biological systems, such as relatively high molar absorption coefficients and quantum yields, narrow and high-intensity emission peaks, relative inertness under physiologically relevant conditions, and resistance to photobleaching.[12] Their spectroscopic and photophysical properties can be finely tuned by substitution on the dipyrromethene core. Dehaen and co-workers originally performed systematic work on the reactivity of 3,5-dichlorinated BODIPY with carbon, nitrogen, oxygen, and sulfur directed toward nucleophilic aromatic substitution (S_NAr) and palladium-catalyzed cross-coupling.[13] Mono- and disubstituted products were prepared selectively by careful tuning of the reaction conditions. The monochlorinated BODIPY derivatives could be modified further by replacing the chlorine with a nucleophile, for example, a thiolate. The reaction is efficient and proceeds cleanly under mild conditions. Moreover, a thioether

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substituent red-shifts both the absorption and emission spectra.[13a] These results inspired us to design a ratiometric fluorescent sensor for detection of biothiols based on monochlorinated BODIPY. Ratiometric sensors have broader utility than fluorescent sensors whose response to the analyte is limited to an increase or a decrease in emission intensity because they measure the intensity ratio of fluorescence at two different wavelengths, which increases the dynamic range and provides a built-in correction for environmental effects.[14]

We synthesized the series of monochlorinated BODIPY derivatives **1a**–**f**. In preliminary experiments, we measured the time-dependent fluorescence of **1** in the presence of methyl mercaptoacetate as a model thiol in aqueous HEPES buffer (20 mM, pH 7.4) [Figures S1–S3 in the Supporting Information (SI)]. Compounds **1a**–**d** reacted readily with the thiol, and the absorbance and fluorescence of the resulting thioethers (**2a**–**d**) were red-shifted by ∼30 nm. The identity of product **2a** was confirmed by ^{1}H NMR spectroscopy and high-resolution mass spectrometry (see the SI). In contrast, almost no reaction of **1e** and **1f** with the thiol was observed in 3 h under identical conditions, probably because the electron-rich substituents decreased the reactivity of monochlorinated BODIPY toward nucleophilic aromatic substitution. For **1a** and **1b**, the reaction was almost complete within 2 min [the pseudo-first-order reaction rate constants were 4.6×10^{-2} s^{-1} ($t_{1/2}$ = 15 s) and 3.6×10^{-2} s^{-1} ($t_{1/2}$ = 19 s), respectively], indicating that they could be potential candidates for use as fluorescent probes for the detection of thiols under physiological conditions. The reaction of **1a** was accompanied by an increase in the fluorescence intensity, which is especially favorable for sensing (the intensity decreased for **1b**). Because of this, together with the better water solubility of **1a** versus **1b**, **1a** was chosen for further studies as a ratiometric fluorescent sensor for thiol detection.

The time-dependent fluorescence response of **1a** in the presence of GSH (1 mM) was measured at 37 °C in aqueous HEPES buffer (20 mM, pH 7.4) containing 5% acetonitrile (Figure 1a). As the reaction progressed, the emission band of free **1a** centered at 556 nm decreased, while a new emission peak at 588 nm increased. The fluorescence intensity ratio (I_{588}/I_{556}) was linearly proportional to the GSH concentration in the 0–60 μM range (R^2 = 0.993), indicating the suitability of **1a** for quantitative detection of GSH (Figure 1c,d). The detection limit was determined to be 8.6×10^{-8} M (S/N = 3).

We studied the fluorescence behavior of **1a** in the presence of Cys or Hcy under otherwise identical conditions (Figures S4 and S5). Upon the addition of Cys, a decrease in the fluorescence intensity at 556 nm was accompanied by the initial appearance of a new emission band at ∼590 nm that decreased rapidly. Finally, the emission maximum of **1a** was shifted to 564 nm. In the presence of Hcy, an emission peak centered at 587 nm increased gradually during the first 15 min of the reaction, similar to that observed with GSH. Subsequently, this peak decreased (Figure 2). The absorption and emission spectra of **1a** in the presence of GSH are significantly different from those in the presence of Cys and Hcy, indicating that **1a** can be used for selective detection of GSH over Cys and Hcy under physiological conditions.

To provide a better understanding of the mechanism of the different photophysical changes of **1a** in the presence of GSH, Cys, and Hcy, we also investigated the fluorescence responses of analogues **1b** and **1c** to the three biothiols (Figures S6–S8). They exhibited similar but delayed reactions because of their lower reactivities toward the thiols. In the presence of Cys, the

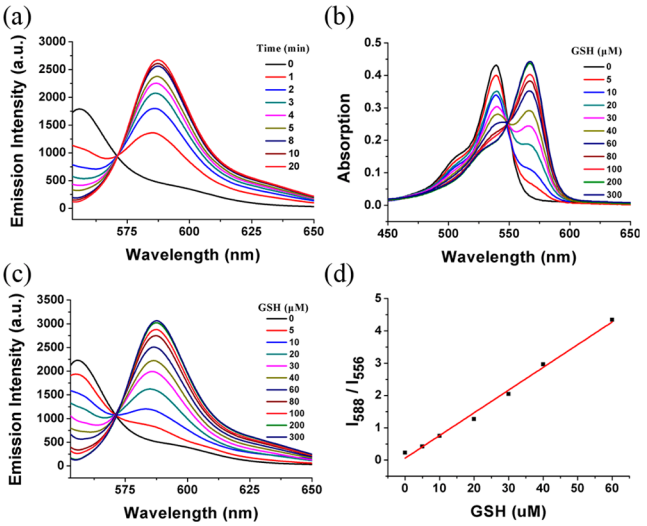

Figure 1. (a) Time-dependent fluorescence spectra of **1a** (10 μM) with 100 equiv of GSH. (b) Absorption and (c) emission spectra of **1a** (10 μM) after 1 h upon addition of increasing concentrations of GSH. (d) Ratio of the fluorescence intensities at 588 and 556 nm (I_{588}/I_{556}) as a function of the GSH concentration. Each spectrum was acquired in acetonitrile/HEPES buffer (5:95 v/v, 20 mM, pH 7.4) at 37 °C with λ_{ex} = 550 nm. The excitation wavelength of 550 nm corresponds to the isosbestic point of the two chromophores **1a** and **2a**.

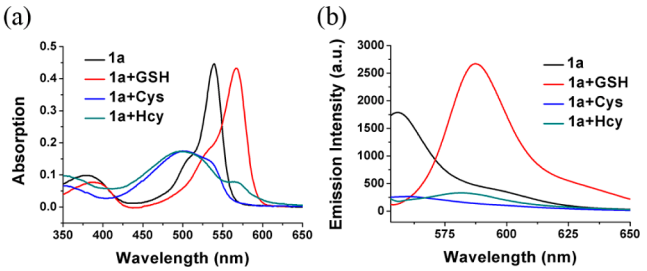

Figure 2. (a) Absorption and (b) fluorescence spectra of **1a** (10 μM) 2 h after addition of 100 equiv of GSH, Cys, or Hcy in acetonitrile/HEPES buffer (5:95 v/v, 20 mM, pH 7.4) at 37 °C with λ_{ex} = 550 nm.

emission of **1c** at 591 nm increased during the initial 5 min, indicating the formation of the thioether by replacement of the chlorine of **1c** with thiolate. However, the emission began to decrease afterward, and an emission band centered at 570 nm formed, suggesting the formation of a new product (**1c-Cys**). We characterized **1b-Cys** instead of **1c-Cys** because it was easier to isolate from the reaction mixture. The mass spectrum manifested a peak at m/z 582.1, which was assigned as [**1b** + Cys − HCl + Na]$^+$, suggesting the attachment of Cys to **1b** (Figure S9). In the ^{1}H NMR spectrum, we assigned a broad signal at ∼6.86 ppm and a triplet at 1.75 ppm to the exchangeable protons of the aromatic amine and SH, respectively, suggesting that Cys is attached to BODIPY through the amino group (Figure S10). This assignment was confirmed by comparison of the spectra of **1b-Cys** and an authentic sample (**1b-N**) obtained by reacting **1b** with butylamine under basic conditions (Figures S11 and S12).

On the basis of these observations, we propose a two-step reaction for **1** with Cys/Hcy. First, the chlorine of BODIPY is rapidly replaced by thiolate. Second, the amino group replaces the thiolate to form amino-substituted BODIPY. This was further confirmed by the control reaction of **1a** with *N*-acetylcysteine, which is structurally similar to Cys but lacks an

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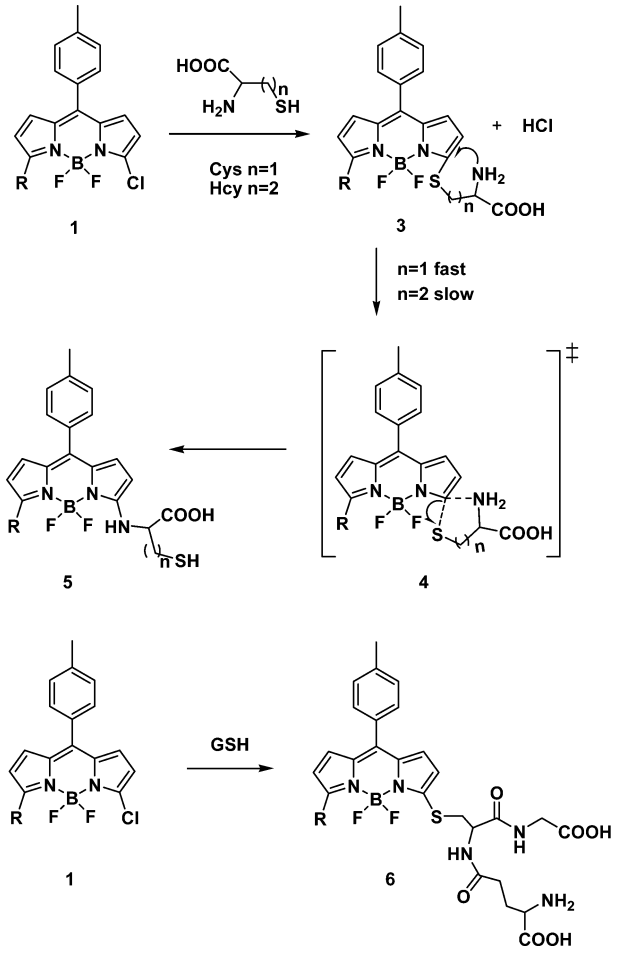
amino group; only the formation of the thioether was observed (Figure S13).

These results are consistent with the reaction mechanism in Scheme 2. Deprotonation of the thiol yields the active

Scheme 2. Proposed Mechanism for the Reactions of 1 with Cys, Hcy, and GSH



nucleophile, thiolate. Nucleophilic aromatic displacement of the chloride generates the kinetic product, thioether **3** or **6**. The primary amine in **3** allows further intramolecular displacement of thiolate to yield the thermodynamic product **5** by five- or six-membered cyclic transition state **4**. A similar reaction in **6** would require a macrocyclic transition state, which may be too unstable to be kinetically significant on the time scale of the experiment.

Accordingly, the discrimination of GSH from Cys and Hcy is due to the propensity of the thioether originally generated with Cys and Hcy to undergo intramolecular displacement to form a secondary amine. Since photophysics of BODIPY derivatives is sensitive to substituents, the thioether product of the reaction between BODIPY and GSH is easily distinguishable spectroscopically from the amine derivative originating from the reaction between BODIPY and Cys or Hcy. Thioether **6** manifests stronger fluorescence than amine **5** with a ~25 nm red-shifted maximum. To the best of our knowledge, **1** is the first ratiometric fluorescent sensor for the selective detection of GSH over Cys/Hcy. The importance of a secondary reaction for discrimination of the SH-containing biomolecules might constitute a general approach for discrimination among other biomolecules containing the same functional groups.

Control experiments were carried out with biologically relevant amino acids and another reactive sulfur species, hydrogen sulfide,[15] under the same physiological conditions (Figure S14). As shown in Figure 3, only GSH induced a high fluorescence intensity ratio under the given conditions.

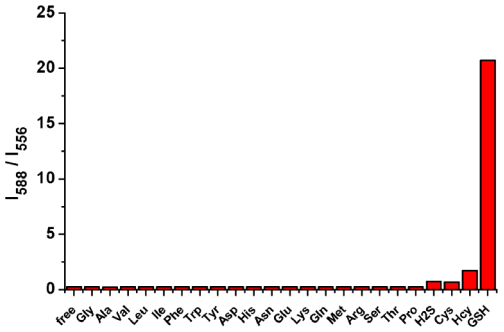


Figure 3. Ratiometric responses of **1a** (10 μM) upon addition of 100 equiv of various analytes. Bars represent the fluorescence intensity ratio I_{588}/I_{556} with λ_{ex} = 550 nm. Each datum was acquired 2 h after the addition of the amino acid in acetonitrile/HEPES buffer (5:95 v/v, 20 mM, pH = 7.4) at 37 °C.

We studied the capacity of **1a** for ratiometric imaging of GSH in living cells. HeLa cells incubated with **1a** (5 μM) for 15 min showed clear fluorescence in two emission channels, green (500−550 nm) and red (570−620 nm) (Figure 4a,b),

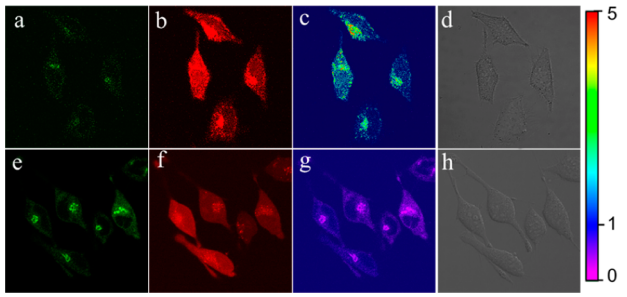


Figure 4. (a−d) Confocal fluorescence and bright-field images of living HeLa cells incubated with probe **1a** (5 μM) for 15 min: (a) green channel at 500−550 nm; (b) red channel at 570−620 nm; (c) ratio image generated from (b) and (a); (d) bright-field transmission image. (e−h) Confocal fluorescence and bright-field images of living HeLa cells incubated with the probe **1a** (5 μM) for 15 min after preincubation with 1 mM diamide for 30 min: (e) green channel at 500−550 nm; (f) red channel at 570−620 nm; (g) ratio image generated from (f) and (e); (h) bright-field transmission image.

confirming that **1a** is cell-permeable. The ratio of the emissions from the red and green channels was ~3 (Figure 4c). Addition of diamide (a GSH-recognized reagent[16,3g]) to the cell culture prior to the addition of **1a** resulted in a ratio of <1 (Figure 4e−g). These results are consistent with the specificity of **1a** for GSH and demonstrate its suitability for ratiometric fluorescent imaging of GSH in living cells.

In conclusion, we have developed a ratiometric fluorescent sensor for discrimination of GSH over Cys and Hcy. The sensor operates by undergoing rapid displacement of chloride with thiolate. The unique example of discrimination of GSH from Cys/Hcy is attributed to subsequent displacement of the

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thiolate by the amino group of Cys/Hcy to form amino-substituted BODIPY, which exhibits dramatically different photophysical properties compared with the sulfur-substituted BODIPY produced by the reaction with GSH. This specific and interesting reaction mechanism may inspire the exploration of new systems for the selective detection of biothiols.

■ ASSOCIATED CONTENT

Supporting Information

Synthesis details and characterization of compounds **1a–f**, **2a**, **1b-Cys**, and **1b-N**; time-dependent fluorescence response of **1a** in the presence of Cys or Hcy; absorption and emission spectra of **1a** with increasing amounts of Cys or GSH; and time-dependent fluorescence responses of **1b** and **1c** in the presence of GSH, Cys, or Hcy. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

qzyang@mail.ipc.ac.cn

Notes

The authors declare no competing financial interest.

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