Communication

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

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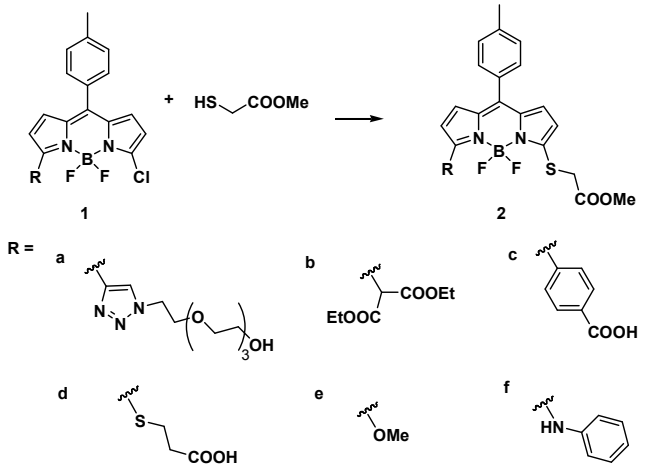
Supporting Information Placeholder

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 thiolate 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. The abnormal level 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]

Fluorescence sensor is well suited for the detection of SH-containing biological molecules in vivo.[4] Most of the existing sensors utilize the strong nucleophilicity of 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. Utilizing the cyclization of Cys/Hcy with aldehydes or acrylates, pioneered by 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 of Cys/Hcy remains a tough task. Recently, based on previous work,[9c] Strongin *et al* reported a sensor for selective detection of GSH in CTAB media.[10] However, the need of a surfactant may limit its application in living system. 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 of GSH, further displace the sulfur to form amino substituted BODIPY which exhibited relatively weak fluorescence as well as blue-shifted emission. Thereby GSH is distinguished from Cys/Hcy.

Scheme 1. Reaction of 1 with methyl mercaptoacetate.



BODIPY dyes have several attributes, such as the relatively high molar absorption coefficients and quantum yields, narrow and high-intensity emission peaks, relative inertness under physiologically relevant conditions and resistance to photobleaching, which make them good candidates as fluorescent sensors in biological systems.[11] Their spectroscopic and photophysical properties can be finely tuned by substitution of the dipyrromethene core. Dehaen *et al* originally performed systematic work on the reactivity of 3,5-dichlorinated BODIPY with carbon, nitrogen, oxygen, sulfur towards nucleophilic aromatic substitution (S_NAr) and palladium-catalyzed cross coupling.[13] Mono- and di-substituted products are prepared selectively by a 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 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 analytes in limited to an increase or a decrease in emission intensity because they measure the intensity ratio of fluorescent at two different wavelengths, which increases the dynamic range and provides built-in correction for environmental effects.[14]

We synthesized a series of monochlorinated BODIPY derivatives **1a-f**. In preliminary experiments we measured time-dependent fluorescent of **1** in the presence of methyl mercaptoacetate as a model thiol in aqueous HEPES buffer (20 mM, pH 7.4, see Figure S1-S3, Supporting Information). Compounds **1a-d** reacted readily with the thiol and the absorbance and the fluorescence of the resulting thioethers (**2a-d**) was red-shifted by ~30 nm. The identity of product **2a** was confirmed by ^{1}H NMR spectroscopy and HRMS (See Supporting Information). In contrast, almost no reaction was observed for **1e** and **1f** with thiols in 3 hours under the identical conditions probably because the electron-rich substituents decrease the reactivity of monochlorinated BODIPY towards nucleophilic aromatic substitution. For **1a** and **1b**, the reaction almost completed within 2 min (the pseudo-1st order reaction rate constants were 4.6×10^{-2} s^{-1} and 3.6×10^{-2} s^{-1}, respectively), indicating that they could be potential candidates 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**). Together with the better water-solubility of **1a** vs. **1b**, **1a** was chosen for further studies as a ratiometric fluorescent sensor for thiol detection.

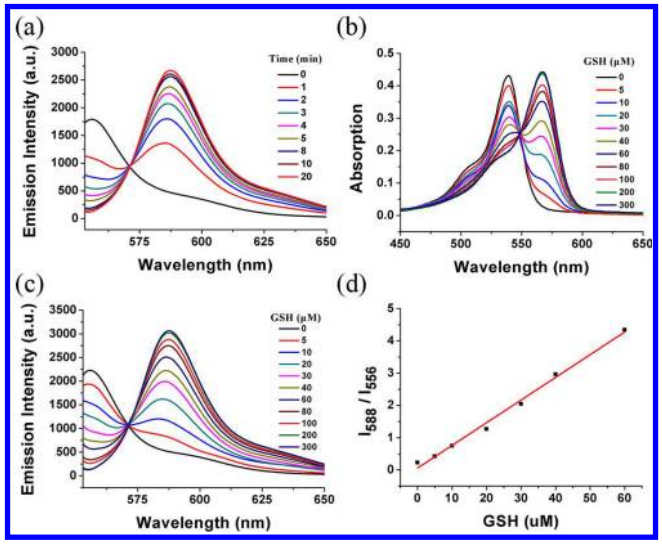


Figure 1. (a) Time-dependent fluorescence spectra of **1a** (10 μM) with 100 equiv. of GSH. (b) Absorption and (c) emission spectra of **1a** (10 μM) after 1h upon addition of increasing concentrations of GSH. (d) Ratio of the fluorescence intensity at 588 nm and 556 nm (I_{588} / I_{556}) as a function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

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 (Figure S4-S5). Upon the addition of Cys, a decrease in the fluorescence intensity at 556 nm was accompanied by the appearance of a new emission band at ~590 nm at the very start, which decreased rapidly. Finally the emission maximum of **1a** 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 of Cys or Hcy, indicating that **1a** can selectively detect GSH over Cys and Hcy under physiological conditions.

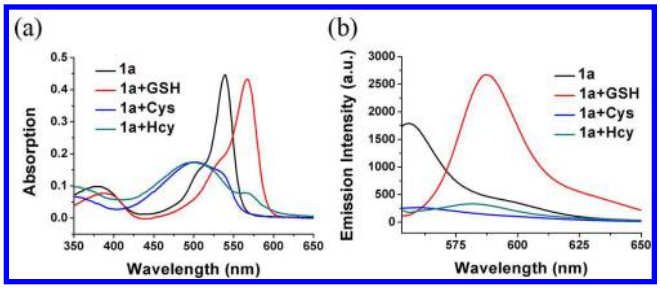


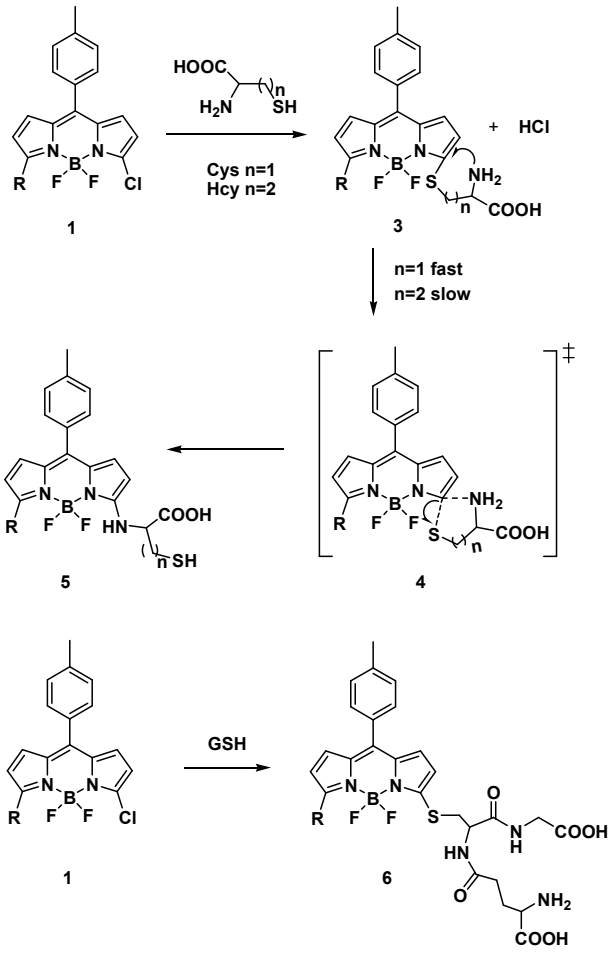
Figure 2. (a) Absorption and (b) fluorescence spectra of **1a** (10 μM) after 2h addition of 100 equiv. of GSH, Cys and Hcy in acetonitrile/HEPES buffer solution (5:95, v/v, 20 mM, pH 7.4) at 37 °C. λ_{ex} = 550 nm.

To better understand 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 (Figure S6-S8). They exhibited similar but delayed reactions because of their lower reactivity towards the thiols. In the presence of Cys, the emission of **1c** at 591 nm increased during the initial 5 min, indicating the formation of thioether by the replacement of the chlorine of **1c** with thiolate. However, it began to decrease afterwards, 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 = 559.6, which was assigned as [**1b** + Cys - Cl], 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 proton of the aromatic amine and of SH, respectively, which suggests that Cys is attached to BODIPY through the amino group (Figure S10). This assignment was confirmed by comparing the spectra of **1b-Cys** with an authentic sample (**1b-N**) obtained by reacting **1b** with butylamine under basic condition (Figure S11-S12).

Besed on 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. It was further confirmed by the control reaction of **1a** with N-acetylcysteine which is structurally similar to Cys but lacking an amino group. Only the formation of thioether was observed (Figure S13).

These results are consistent with the reaction mechanism in Scheme 2. Deprotonation of thiol yields the active nucleophile, thiolate. Nucleophilic aromatic displacement of the chloride generates the kinetic products of thioether **3** or **6**. The primary amine in **3** allows further intramolecular displacement of thiolate to yield the thermodynamic products **5** by a 5- or 6-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 at the timescale of the experiment.

Scheme 2. Proposed reaction mechanism of 1 with Cys, Hcy and GSH



Accordingly, the discrimination of GSH from Cys and Hcy is due to the propensity of the originally generated thioether 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 the ~30 nm red-shifted maximum. To the best of our knowledge, it 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 to the 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 physiology conditions (Figure S14). As shown in Figure 3, only GSH induced a high fluorescence intensity ratio under the given condition.

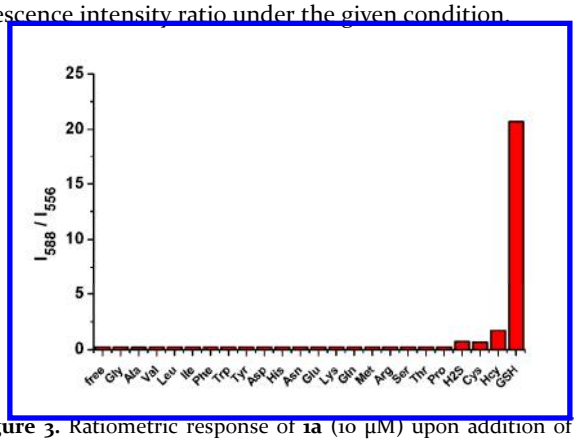


Figure 3. Ratiometric response of **1a** (10 μM) upon addition of 100 equiv. of different analytes. Bars represent fluorescence intensity ratio of I_{588}/I_{556}. λ_{ex} = 550 nm. Each data were acquired 2h after addition of different amino acids in acetonitrile/HEPES buffer solution (5:95, v/v, 20 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 at dual-emission channels of green (500-550 nm) and red (570-620 nm), confirming that **1a** is cell-permeable. The ratio of the emission from the red and green channels was ~ 3. Addition of diamide, a GSH-recognized reagent,[16,3g] to the cell culture prior to the addition of **1a** resulted in the ratio of < 1. The result is consistent with the specificity of **1a** for GSH and demonstrates its suitability for ratiometric fluorescent imaging of GSH in living cells.

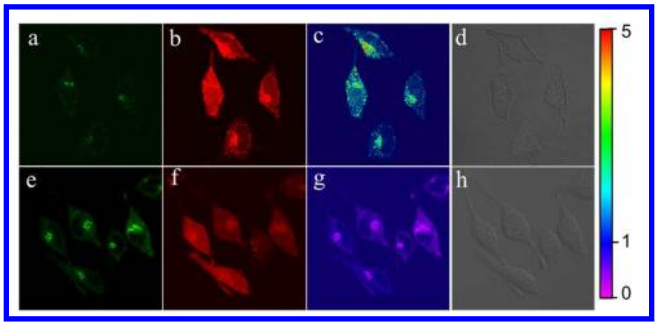


Figure 4. Confocal fluorescence images of living HeLa cells incubated with the probe **1a** (5 μM) for 15 min: (a) green channel at 500-550 nm, (b) red channel at 570-620 nm, (c) ratio image generated from (b) and (a), and (d) bright-field transmission image; Confocal fluorescence images of living HeLa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

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 amino group of Cys/Hcy further replacing the thiolate to form amino substituted BODIPY, which exhibited dramatically different photophysical properties compared to sulfur substituted BODIPY from the reaction of GSH. This specific and interesting reaction mechanism may inspire the exploration of new system 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 and Hcy. Absorption and emission spectra of **1a** with increasing amount of Cys and GSH. Time-dependent fluorescence response of **1b** and **1c** in the presence of GSH, Cys and Hcy. This material is available free of charge via the Internet at http://pubs.acs.org.

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