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ARTICLE

A dual-response BODIPY-based fluorescent probe for the discrimination of Glutathione from Cystein and Homocystein

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In situ monitoring intracellular thiol activity in cell growth and function is highly desirable. However, the discriminative detection of glutathione (GSH) over cysteine (Cys) and homocystein (Hcy) from common amino acids still remains a challenge due to the similar reactivity of thiol group in these amino acids. Here we report a novel strategy for selectively sensing GSH by dual-response mechanism. Integrating two independent reaction sites with disulfide linker and thioether function into a fluorescent BODIPY-based chemsensor can guarantee the synergetic dual-response for the elegant fashion to address the discrimination of GSH. In the first synergetic reaction process, the thiol group in GSH and Cys/Hcy induces disulfide cleavage and subsequent intramolecular cyclization to release the masked phenol-based BODIPY (*discriminating thiol amino acids over other amino acids*). In the second synergic process, upon the substitution of thioether with the nucleophilic thiolate to form sulfenyl-BODIPY, only the amino groups of Cys/Hcy but not GSH further undergoes intramolecular displacement to yield amino-substituted BODIPY. In this way, we make full use of the kinetically favorable cyclic transition state in the intramolecular rearrangement, and enable the distinct photophysical difference between sulfenyl- and amino-substituted BODIPY for allowing the *discriminative detection of GSH over Cys/Hcy and thiol-lacking amino acids* under physiological conditions. Moreover, this probe exhibits distinguishable ratiometric fluorescence pattern generated from orange imaging channel to red channel, which proves to differentiate GSH from Cys/Hcy in living cells.

Introduction

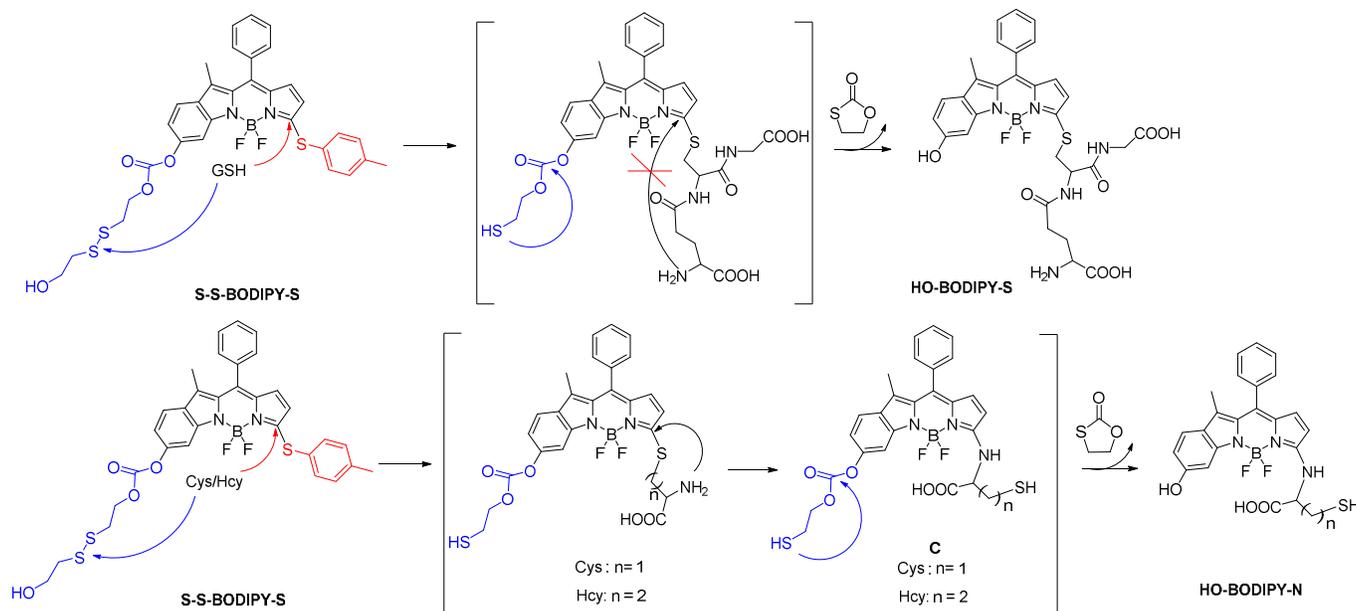
Since intracellular thiols, such as glutathione (GSH), cysteine (Cys) and homocystein (Hcy), play pivotal roles in physiological and pathological events, it is highly desirable to develop sensing probes to *in situ* monitor and quantify these thiols activity in cell growth and function.¹ Specifically, GSH is the most abundant intracellular thiol with the concentration in the millimolar range,² functioning as an essential endogenous antioxidant primarily involved in maintaining biological redox homeostasis.³ Inappropriate level of GSH is directly associated with cancer, Alzheimer's and other ailments. Accordingly, the dynamic and quantification of GSH has become the object of great interest in the sensing communities.

Up to date, significant progresses have been achieved in the development of fluorescent probes⁴ toward detection of thiols by employing the strong nucleophilicity of thiol group.⁵⁻⁹ However, the discriminative detection of GSH over Cys and Hcy still remains a tough task.¹⁰ This challenge arises from the similar reactivity of thiol group in these amino acids. To

achieve the differentiating detection, we focus on a dual-response fluorescent chemsensor, that is, specifically incorporating two independent reaction sites with synergetic response toward these thiol-containing amino acids. Herein we present the synthesis and biological evaluations of a BODIPY-based probe, **S-S-BODIPY-S**, consisting of two key independent reaction sites with disulfide linker and thioether function (Scheme 1). As demonstrated, in the first synergetic reaction process for discriminating thiol amino acids (GSH and Cys/Hcy) over other amino acids, the disulfide (S-S) bond could be cleaved by the thiol group and followed by intramolecular cyclization and cleavage of a neighboring carbonate bond, thus triggering the unmasking of the hydroxyl group to afford the phenol-based BODIPY. In the second synergetic step for discriminating GSH over Cys/Hcy, upon the substitution of thioether with the nucleophilic thiolate to form sulfenyl-BODIPY, the subsequent intramolecular displacement takes place driven only by the amino groups of Cys/Hcy but not GSH, yielding an amino-substituted BODIPY (Scheme 1). As a

consequence, GSH triggers the transformation from **S-S-BODIPY-S** to **HO-BODIPY-S**, while Cys and Hcy produce **HO-BODIPY-N**. It should be noted that, in the second synergetic step, the kinetically favorable cyclic transition state is very critical for discriminating GSH over Cys and Hcy. Exactly, the bulkiness of GSH significantly hinders the intramolecular rearrangement, thus offering only the production of sulfenyl-BODIPY. Given the remarkably different photophysical properties of sulfenyl- and amino-substituted BODIPY, the dual-response probe provides an easily distinguishable fluorescence signal to make a sense of GSH

over Cys and Hcy. To the best of our knowledge, this is the first fluorescent chemosensor that directly explores two synergetic reaction sites to achieve the selective determination of GSH from Cys/Hcy with ratiometric bioimaging mode in living cells.



Scheme 1. Chemical structure of **S-S-BODIPY-S** and its discriminative sensing mechanisms of **S-S-BODIPY-S** toward GSH and Cys/Hcy. Note: the intramolecular rearrangement by five- or six-membered cyclic transition state with Cys/Hcy to form amino-BODIPY is kinetically favored. In contrast, the bulkiness of GSH would significantly hinder the intramolecular rearrangement, thus offering the sulfenyl-BODIPY (**HO-BODIPY-S**).

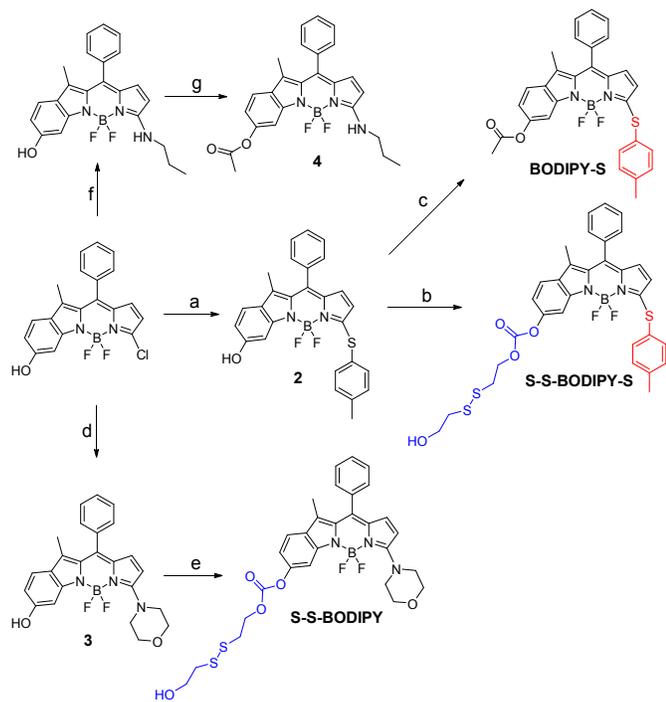
Results and Discussion

As well known, the spectroscopic properties of BODIPY is very sensitive to the substitution on the dipyrromethene core.¹⁰⁻¹² For instance, the sulfenyl- and amino-substituted BODIPY display distinct photophysical properties in absorption and emission spectra. With this in mind, we designed and evaluated a dual-response probe, **S-S-BODIPY-S**. Also its two model compounds (**BODIPY-S** and **S-S-BODIPY**) were synthesized as outlined in **Scheme 2**. **HO-BODIPY-Cl** smoothly reacted with *p*-thiocresol or morpholine through nucleophilic aromatic substitution to offer **2** and **3**. The hydroxy group was then activated by *N,N'*-carbonyldiimidazole (CDI) and coupled with 2,2'-dithiodiethanol to form **S-S-BODIPY-S** in 14% yield.

Initially, we determined the reactivity of the disulfide and thioether with the common thiol-containing and thiol-lacking amino acids using the two model compounds **S-S-BODIPY** and

BODIPY-S. **BODIPY-S** displayed an absorption band at 556 nm and was almost non-fluorescent. Upon addition of GSH and other thiol-lacking amino acids to a solution of **BODIPY-S** in acetonitrile / HEPES buffer (1:1, v/v, 20 mM, pH 7.4, **Fig. S1 in ESI†**), minimal changes in absorption and fluorescence spectra were observed. However, mass study manifested the peak at 718.1 (corresponding to $[\text{Ac-BODIPY-S} + \text{Na}]^+$), showing that the substitution of thioether by GSH proceeded readily (**Fig. S2†** and **Scheme S1†**). In the case of Cys and Hcy, a rapid decrease in the absorption band of free **BODIPY-S** at 556 nm was observed, along with the simultaneous buildup of a new band at 488 nm. Additionally, a remarkable increase by 145 fold in the fluorescence intensity at 552 nm was found (**Fig. S1†**). Mass studies with the peak at 508.1 (identical to $[\text{Ac-BODIPY-N} - \text{H}]$) indicated that the thioether unit in **BODIPY-S** was replaced by Cys (**Fig. S2†**). The observed photophysical properties were matched well with the amino-BODIPY **4** (**Fig. S3†**), indicative of the new product as

amino-BODIPY. Based on the reaction products and previous works,¹⁰ we can reason that the sulfenyl-substituted BODIPY is initially formed through nucleophilic substitution, followed by an intramolecular attack at the thioether carbon by the amino group in Cys/Hcy through a five- or six-membered transition state, yielding amino-substituted BODIPY (Scheme S1†). This cascade substitution-intramolecular displacement mechanism can be further supported by the fact that **BODIPY-S** displayed no response to the thiol-lacking amino acids, suggestive of no direct amino-induced substitution reaction. In the case of GSH, an unfavorable macrocyclic transition state can hinder the formation amino-BODIPY. That is, the bulkiness of GSH significantly hinders the intramolecular rearrangement, thus offering only the sulfenyl-BODIPY. In this regard, the discriminative detection of Cys/Hcy over GSH and thiol-lacking amino acids might be expected by **BODIPY-S**. However, we cannot distinguish GSH and thiol-lacking amino acids with **BODIPY-S** because the substitution of thioether by GSH induce minimal fluorescence change, which is very similar with the thiol-lacking amino acids.



Scheme 2. Synthesis of **S-S-BODIPY-S** and two model compounds **BODIPY-S** and **S-S-BODIPY**. Reaction conditions: (a) *p*-thiocresol/ $\text{Et}_3\text{N}/\text{CH}_3\text{CN}$, rt, 88%; (b) 1,2-dichloroethane/1,1-carbonyldiimidazole/bis(2-hydroxyethyl) disulfide, reflux, 14%; (c) DMAP/DCM/acetic anhydride, rt, 83%; (d) morpholine/ CH_3CN , rt, 90%; (e) 1,2-dichloroethane/1,1-carbonyldiimidazole/bis(2-hydroxyethyl) disulfide, reflux, 20%; (f) propylamine/ CH_3CN , rt, 90%; (g) $\text{Et}_3\text{N}/\text{DCM}/\text{acetic anhydride}$, rt, 85%.

The response behavior of model compound **S-S-BODIPY** toward the common thiol-containing and thiol-lacking amino acids was further determined. It was found that only the three thiol-containing amino acids (GSH, Cys and Hcy) elicited a

time-dependent ratiometric fluorescence change, resulting in a decrease in the emission band at 563 nm and a concomitant increase of a new band at 598 nm (Fig. S4†). All TLC, ^1H NMR and HRMS experiments showed that the disulfide bond in **S-S-BODIPY** was cleaved by the specific nucleophilic thiol group, followed by an intramolecular cyclization to break down the carbonate bonds and yield compound **3** (Scheme S2 and Fig. S5†). Clearly, **S-S-BODIPY** is susceptible to discriminate thiol-containing amino acids over other amino acids since the disulfide bond is inactive to thiol-lacking amino acids. Nevertheless, we cannot expect the disulfide reduction mediated by the thiol group to discriminate these thiol-containing amino acids such as GSH, Cys and Hcy.

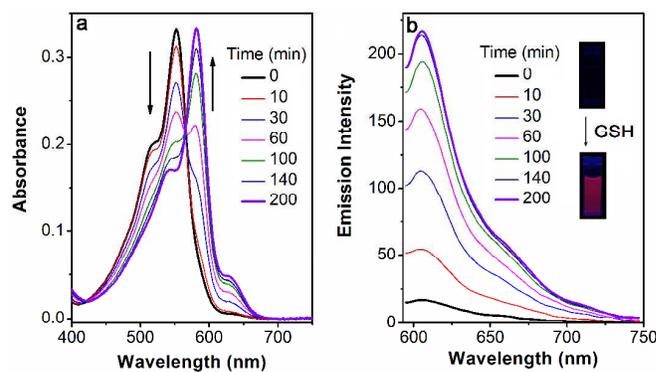


Fig. 1 Time-dependent spectral changes of **S-S-BODIPY-S** (5 μM) in the absence and presence of 5 mM GSH in acetonitrile / HEPES buffer (1:1, v/v, 20 mM, pH 7.4) at 37 $^\circ\text{C}$. (a) Absorption and (b) emission spectra, $\lambda_{\text{ex}} = 565$ nm. Inset: the fluorescence color change of **S-S-BODIPY-S** in the presence of GSH.

Based on the above-mentioned essential weakness of two model systems, the spectral properties of **S-S-BODIPY-S** consisting of the two key independent reaction sites with disulfide linker and thioether function were further investigated. We envisioned that the synergetic dual-responses of the two reaction sites can guarantee the selective detection of GSH. Initially, **S-S-BODIPY-S** exhibited a strong absorption band at 552 nm as well as a weak emission peak at 605 nm with a quantum yield around 0.001. As shown in Fig. 1, addition of 5 mM GSH to a solution of **S-S-BODIPY-S** in acetonitrile / HEPES buffer (1:1, v/v, 20 mM, pH 7.4) triggered a dramatic decrease of the absorption band at 552 nm, along with a simultaneous absorption buildup at 581 nm, displaying a distinct isosbestic point at 565 nm. In fluorescence spectra, addition of GSH elicited a red fluorescence turn-on signal at 605 nm, enabling **S-S-BODIPY-S** a promising probe for GSH. The increment of fluorescence intensity is time-dependent and the observed rate constant was determined to be 0.0175 min^{-1} under the pseudo-first-order conditions (Fig. S6†). It has been demonstrated that GSH can displace thioether in **BODIPY-S** and unmask the protective ester group in **S-S-BODIPY**. Therefore, we reasoned that the incubation of **S-S-BODIPY-S** with GSH triggered both reactions to give **HO-BODIPY-S**

with a quantum yield around 0.06, as illustrated in Scheme 1. HRMS study also revealed the cleavage of the disulfide and thioether linkers to form **HO-BODIPY-S** (Fig. S7†). The reaction product manifests a mass peak at 654.1993 identical to $[\text{HO-BODIPY-S}]^+$.

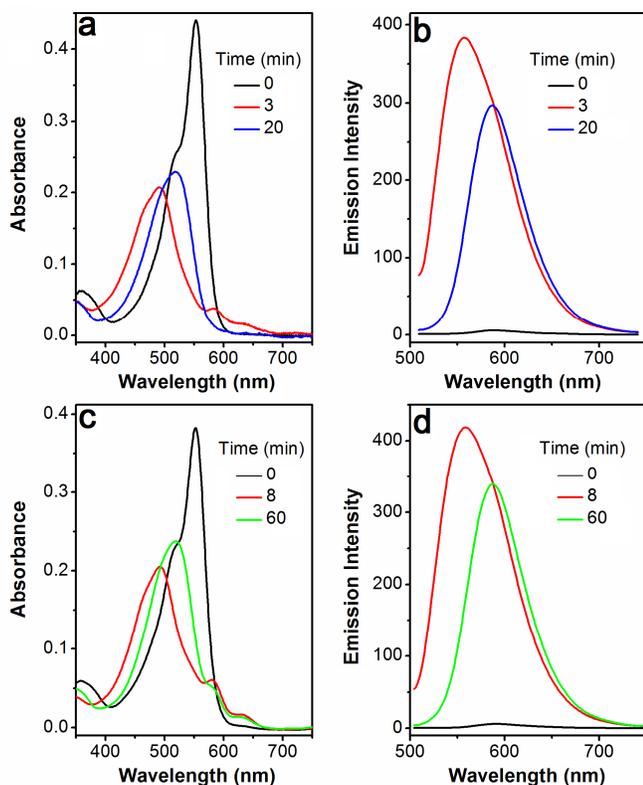


Fig. 2 Time-dependent spectral changes of **S-S-BODIPY-S** ($5 \mu\text{M}$) in the absence and presence of Cys (5 mM) or Hcy (5 mM) in acetonitrile/HEPES buffer ($1:1, v/v$, 20 mM , $\text{pH } 7.4$) at 37°C . Cys: (a) Absorption and (b) emission spectra; Hcy: (c) Absorption and (d) emission spectra. $\lambda_{\text{ex}} = 490 \text{ nm}$.

The spectra profile changes were then investigated in the presence of Cys or Hcy (Fig. 2 and Fig. S8†). A biphasic nature of the spectra changes was observed upon addition of Cys. In the initial 3 min, the original absorption at 552 nm became decreased and a new absorption band at 490 nm increased simultaneously. Subsequently the absorption band at 490 nm was shifted to 520 nm . The optical density value reached a plateau within 20 min. Additionally, a remarkable increment in fluorescence intensity at 556 nm was observed upon excitation at 490 nm during the initial 3 min. Then a fluorescence red-shift took place, and finally switched to 587 nm . The easy-to-monitor fluorescence color change was observed from dark to yellow and eventually to orange (Fig. S9†). Based on the two model compounds, we can infer the formation of **HO-BODIPY-N** with a quantum yield around 0.13. Again, HRMS study also revealed the release of **HO-BODIPY-N** by breaking down the disulfide and thioether bonds (Fig. S7†). Similar spectroscopic changes were also found in the presence

of Hcy with exception of prolonged incubation time. The spectral profile variations were presumably due to the formation of intermediate **C** (Scheme 1) with yellow fluorescence color (556 nm). Since the subsequent intramolecular cyclization and unmasking of the hydroxyl group introduced the removal of electron-withdrawing ester, **HO-BODIPY-N** exhibited red-shifted fluorescence at 587 nm .

Taken together, GSH triggered the transformation from **S-S-BODIPY-S** to **HO-BODIPY-S**, while Cys and Hcy led to produce **HO-BODIPY-N** (Scheme 1). Thus, the photophysical features of **S-S-BODIPY-S** in the presence of GSH are significantly different from those in the presence of Cys/Hcy, enabling **S-S-BODIPY-S** for selective detection of GSH over Cys and Hcy under physiological conditions. As a consequence, the incorporation of two independent disulfide linker and thioether function realizes the synergetic response to GSH, Cys and Hcy. In the first synergetic step, the thiol group in the three thiol amino acids (GSH, Cys and Hcy) induce disulfide cleavage and subsequent intramolecular cyclization to release the masked phenol-based BODIPY (*discriminating thiol amino acids over other amino acids*). In the second synergetic step, after the substitution of thioether with the nucleophilic thiolate to form sulfenyl-BODIPY, only the amino groups of Cys/Hcy but not GSH further undergoes intramolecular displacement to yield amino-substituted BODIPY (*discriminating GSH over Cys and Hcy in thiol amino acids*).

To further confirm the specificity of **S-S-BODIPY-S** toward GSH, the probe was then separately incubated with various biologically relevant amino acids and hydrogen sulfide under the same physiological conditions. As can be seen from Fig. 3, **S-S-BODIPY-S** is a highly selective probe and can monitor GSH with minimum interference from other relevant analytes.

Titration experiments were also performed to evaluate the efficiency of **S-S-BODIPY-S** in the measurement of various concentrations of GSH. It is clear that the enhancement of fluorescence intensity is GSH concentration dependent and a linear relationship can be observed with the concentration of GSH up to $200 \mu\text{M}$ (Fig. 4). The detection limit was then determined as $8.5 \times 10^{-7} \text{ M}$, which indicates that **S-S-BODIPY-S** can readily detect micromolar concentrations of GSH under physiological conditions.

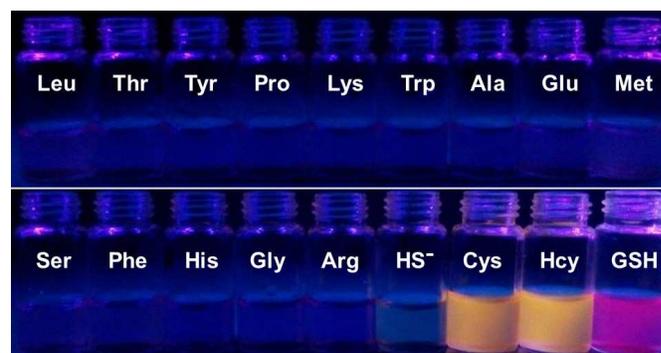


Fig. 3 Fluorescence response of **S-S-BODIPY-S** in the presence of GSH, Cys, Hcy, SH^- and other related amino acids. All spectra were acquired at 2.5 h after addition of analytes.

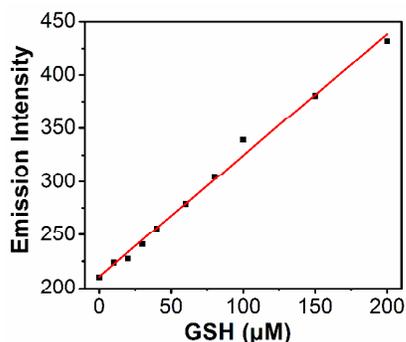


Fig. 4 Plot of fluorescence intensity changes at 610 nm of **S-S-BODIPY-S** as a function of GSH concentrations, with the detection limit 8.5×10^{-7} M for GSH, data acquired at 2.5 h after addition of GSH.

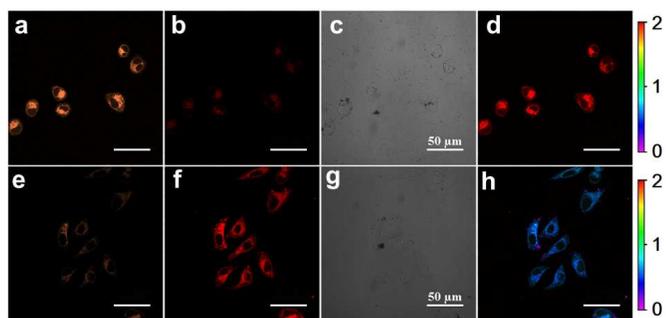


Fig. 5 Confocal fluorescence image of (a-d) MKN-45 cells and (e-h) HeLa cells treated with **S-S-BODIPY-S** (10 μM) for 4 h, the excitation wavelength was 514 nm and the emission was collected at 600-620 nm for red channel and 560-580 nm for orange channel (a and e) fluorescence image at orange channel, (b and f) fluorescence image at red channel, (c and g) bright field, (d and h) ratio images generated from orange channel to red channel. Scale bar is 50 μm.

Inspired by capability of **S-S-BODIPY-S** for detection of GSH and Cys/Hcy in distinct fluorescence patterns, we finally sought to apply this probe for fluorescence imaging applications. Obviously, the reaction of **S-S-BODIPY-S** with GSH features red fluorescence color, whereas that of **S-S-BODIPY-S** with Cys/Hcy offers orange color (Fig. 3). Thus, we investigated the feasibility of the probe to detect these thiols in a dual-color manner to interrogate the discriminative capacity of the probe. Imaging of cellular GSH in living HeLa cells was firstly carried out. Upon loading 10 μM of **S-S-BODIPY-S** at 37 °C, these cells displayed bright red fluorescence image and relatively weak orange fluorescence image (Fig. 5), showing a ratio of 0.5 from orange channel to red channel. In contrast, addition of *N*-methylmaleimide (a trapping reagent of thiols) to the cell culture prior to the incubation with **S-S-BODIPY-S** resulted in minimal fluorescence signals in both channels (Fig. S10†). These results suggest that **S-S-BODIPY-S** is cell membrane permeable and amenable for fluorescent imaging GSH in living cells. For imaging of Cys/Hcy, MKN-45 cells were explored. When these cells loaded with **S-S-BODIPY-S**, bright fluorescence signals

in the orange channel were observed, whereas weak red fluorescence signals were noted. The ratio of the two emissions from orange channel to red channel is about 2, resulted in a remarkable 4-fold enhancement of the emission ratio when compared to the image with GSH. These experiments clearly revealed that **S-S-BODIPY-S** exhibited distinguishable fluorescence images for GSH and Cys/Hcy in living cells.

Conclusions

In conclusion, we have constructed a dual response BODIPY-based fluorescent probe for the discriminative detection of GSH over Cys and Hcy from common amino acids. This unique probe is designed to have two independent reaction sites, a disulfide linker and a thioether function. In the first synergetic process for discriminating thiol amino acids (GSH and Cys/Hcy) over other amino acids, the disulfide bond was cleaved by the thiol group and followed by intramolecular cyclization and cleavage of a neighboring carbonate bond, thus triggering the unmasking of the hydroxyl group to afford the phenol-based BODIPY. In the second synergetic step for discriminating GSH over Cys/Hcy, upon the substitution of thioether with the nucleophilic thiolate to form sulfenyl-BODIPY, the subsequent intramolecular displacement takes place driven only by the amino groups of Cys/Hcy but not GSH, yielding an amino-substituted BODIPY. As a consequence, GSH triggered production of hydroxyl-based sulfenyl-BODIPY, while Cys/Hcy induced the formation of hydroxyl-based amino-BODIPY. Thus, the photophysical features of **S-S-BODIPY-S** in the presence of GSH are significantly different from those in the presence of Cys/Hcy and other thiol-lacking amino acids, enabling **S-S-BODIPY-S** for selective detection of GSH over Cys/Hcy under physiological conditions. Furthermore, **S-S-BODIPY-S** can monitor GSH with minimum interference from other relevant analytes. Importantly, **S-S-BODIPY-S** is cell membrane permeable and amenable for fluorescent imaging these thiols in living cells with distinguishable fluorescence images in a dual-channel manner.

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Notes and references

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† Electronic Supplementary Information (ESI) available: synthesis, spectroscopic properties, NMR and Mass spectra. See DOI: 10.1039/b000000x/

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*Graphics for Contents***A dual-response BODIPY-based fluorescent probe for the discrimination of Glutathione from Cystein and Homocystein**

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With employing a dual response approach, the distinguishable fluorescence signals is initiated by GSH-mediated and Cys/Hcy-induced cascade reactions, thus allowing the selective detection.

