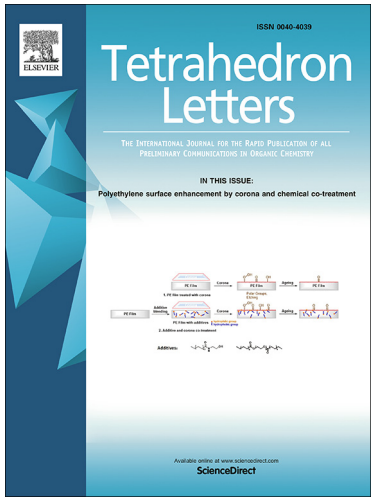

Design of fluorescent probes with optimized responsiveness and selectivity to GSH

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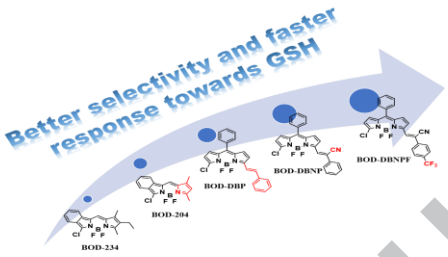
Ji-An Chen, [$a] Zhen-Yu Zhang, [$a] Jie Gao, [a] Jia-Hui Tan [a] and Xian-Feng Gu [*a]



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Design of fluorescent probes with optimized responsiveness and selectivity to GSH

Ji-An Chen, [$a] Zhen-Yu Zhang, [$a] Jie Gao, [a] Jia-Hui Tan [a] and Xian-Feng Gu [a*]

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ABSTRACT

We designed and synthesized a series of BODIPY based probes with fast and distinct ratiometric responsiveness for discriminative detection of GSH from Cys and Hcy. The discriminative detection is based on the different products obtained by the S_NAr between probes and thiol-containing amino acids. The amino group of the obtained thioether from the reaction with Cys or Hcy but not GSH would trigger an intramolecular nucleophilic substitution through five- or six-membered cyclic transition state, finally yielding an amino substituted derivative. To achieve highly discriminative detection and fast response, a series of structure modifications and improvements have been made by elongating the π-conjugation and introducing electron withdrawing groups, finally affording probe **BOD-DBNPF** with optimized responsiveness and selectivity. Importantly, **BOD-DBNPF** was successfully used for the selective detection of GSH from Cys with distinct fluorescent ratiometric responses in living HeLa cells.

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Introduction

Glutathione (GSH) is the most abundant intracellular biothiol in biological systems [1]. As an important antioxidant, Glutathione is capable of protecting cells and organs against the damages caused by ROS such as free radicals, peroxides, lipid peroxides, and heavy metals [2]. It also has other biological functions including maintenance of intracellular redox activity, xenobiotic metabolism, intracellular signal transduction, and gene regulation, which enable it to play crucial roles in many physiological processes [3]. Therefore, it is of great significance to develop highly selective methods to monitor and quantitate GSH under physiological conditions.

In spite of the remarkable advance in the development of fluorescent probes toward biothiols including Cys [4] and Hcy [5] over the past decade, the discriminative detection of GSH from Cys and Hcy still remains a tough task due to their similar structures and reactivity [6]. To achieve accurate and selective detection GSH from other biothiols, the ratiometric fluorescent chemosensor for responsive differences between GSH and other biothiols should be developed, that could exhibit a spectral fluorescent signal shift upon reacting with GSH and thus eliminate most or all ambiguities by self-calibration of two emission bands [7].

Herein, we designed (Figure 1) and synthesized two BODIPY based probes, **BOD-204** and **BOD-234**, consisting of a chlorine as the reaction site which is expected to react with thiols via nucleophilic aromatic substitution (S_NAr) to produce thioether. The discriminative detection of GSH from other biothiols, especially from Cys and Hcy, is based on the different products obtained by the S_NAr between probes and thiol-containing amino acids. The amino group of the obtained thioether from the reaction with Cys or Hcy but not GSH would trigger an intramolecular nucleophilic substitution through five- or six-membered cyclic transition state, finally yielding an amino substituted derivative. As sulfenyl-substituted (thioether) BODIPYs showed markedly different optical properties from amino-substituted BODIPYs [8], thus selective detection of GSH could be furnished by monitoring fluorescence changes. However, **BOD-204** and **BOD-234** still suffered from the drawbacks of relatively low selectivity and slow response in aqueous system. In view of these problems, we further made a series of structure modifications and improvements to the fluorescent dyes. More significant fluorescent ratio changes were attained by elongating the π-conjugation, and faster response towards GSH was achieved by introduction of electron withdrawing groups. Importantly, the optimized probe **BOD-DBNPF** was successfully used for the selective detection of GSH from Cys with unique fluorescent ratiometric responses in living HeLa cells.

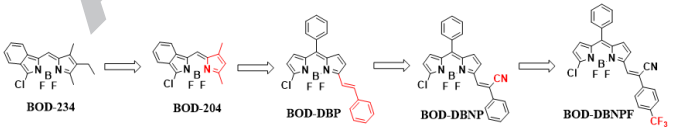
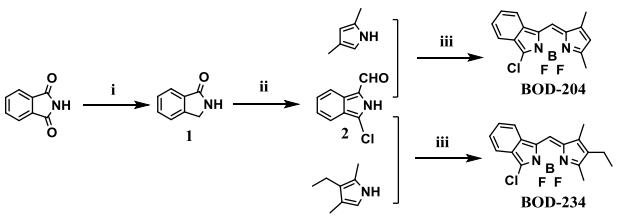


Figure 1. The design of the probes for detection of GSH.



Scheme 1. The syntheses of **BOD-204** and **BOD-234**. Reagents and conditions: (i) AcOH, HCl, Tin, 2.5 h; (ii) $POCl_3$, DMF, DCM, 5 h; (iii) $POCl_3$, DCM, Et_3N, BF_3, 3 h.

Results and discussion

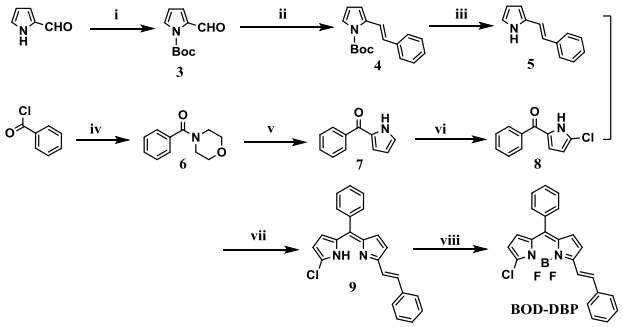
In order to achieve the highly selective detection of GSH over other biothiols especially for Cys and Hcy, two probes **BOD-234** and **BOD-204** were first designed by introducing a chlorine at the 3-position of BODIPY dyes as a leaving group (Figure 1), which is expected to react with thiols via nucleophilic aromatic substitution (S_NAr) to produce thioethers. Once the thioether forming, the amino group of Cys or Hcy but not GSH would trigger an intramolecular nucleophilic substitution as they could go through five- or six-membered cyclic transition state, finally to yield an amino substituted derivative. The distinct photophysical properties between thio- and amino-substituted BODIPYs enabled the selective detection of GSH over Cys and Hcy. **BOD-204** and **BOD-234** were prepared via the condensation of 1,3-dimethyl-pyrrole or 1,3-dimethyl-2-ethyl-pyrrole with compound **2**, followed by boron insertion with BF_3-Et_2O (Scheme 1).

Initially, the spectroscopic responses of **BOD-234** to GSH, Cys and Hcy were investigated (Figure S14). We evaluated the time-dependent absorption and fluorescence response of **BOD-234** to these three analytes. Upon addition of GSH, the absorption at 572 nm decreased gradually, and an increase of a new band at 588 nm were observed with a distinct isosbestic point at 579 nm. Concomitantly, a remarkable fluorescence change was also observed upon addition of GSH with the excitation wavelength at 450 nm. The emission maximum at 581 nm continuously red-shifted to 598 nm with time. The whole process of spectroscopic changes lasted for 270 minutes.

In the case of Cys and Hcy, the absorption at 572 nm decreased gradually upon addition of analytes. Meanwhile, the fluorescence was increased gradually around 570 nm for Cys and Hcy, reaching a plateau in 75 min for Cys and 360 min for Hcy, respectively.

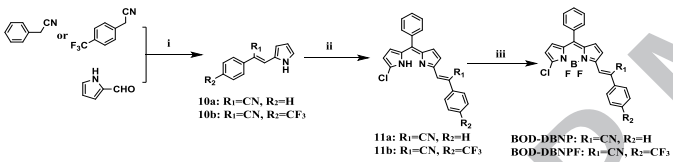
BOD-204, no ethyl substitution at 2-position when compared to **BOD-234**, exhibited very similar photophysical properties as those of **BOD-234** (Figure S15). However, more discriminative fluorescent ratio change caused by GSH (λ_{em} with and without analyte is from 565 nm shifted to 584 mn) and faster fluorescent response to thiols (GSH: 85 min; Cys: 40 min and Hcy: 240 min) was observed. It was deducted that the activation of **BOD-204** towards thiols might because it was more apt to be attacked by nucleophile as there is no ethyl substitution at 2-position that led to less electron cloud density at 3-position of **BOD-204**.

To achieve an optimized probe for GSH, we further incorporated an ethylene phenyl group into BODIPY dye to afford the probe **BOD-DBP** via Witting reaction (Scheme 2). We expected to achieve more significant fluorescent ratio changes by elongating the π-conjugation. During the primary detection, a colorimetric response of **BOD-DBP** to GSH was observed. The absorption maximum at 550 nm continuously red-shifted to 610 nm with time (Figure S16). However, the vital drawbacks includ-



Scheme 2. The synthesis of **BOD-DBP**. Reagents and conditions: (i) NaH, $(Boc)_2O$, THF, 2 h; (ii) NaH, THF, Benzyltriphenylphosphonium bromide, 14 h; (iii) THF, CH_3OH, EtONa, 12 h; (iv) morpholine, Et_3N, DCM, 4 h; (v) pyrrole, $POCl_3$, DCM, overnight; (vi) $CuCl_2$, CH_3CN, 48 h; (vii) $POCl_3$, DCE, overnight; (viii) BF_3, Et_3N, DCM, 12 h.

ing long response time (48 hour) and poor solubility in aqueous system limited its further applications. The further optimization was inspired by **BOD-204**, a probe with more sensitivity to GSH after removing the electron donating ethyl group of **BOD-234**. So, we tried to introduce some electron withdrawing groups, such as cyano or/and trifluoromethyl groups in the structure of **BOD-DBP** and to afford the probes **BOD-DBPN** and **BOD-DBPNF** (Scheme 3). As expected, the fastest response towards GSH and the most significant fluorescent ratio change were attained by using **BOD-DBNPF**, a probe with large conjugating group and two electron withdrawing groups.



Scheme 3. The synthesis of **BOD-DBNP** and **BOD-DBNPF**. Reagents and conditions: (i) NaOH, EtOH, 4 h; (ii) compound **8**, $POCl_3$, DCE, overnight; (iii) BF_3, Et_3N, DCM, 12 h.

BOD-DBPNF exhibit extremely fast response to GSH, Cys and Hcy, as evidenced by the time dependent changes of UV absorption and fluorescence (within 15 min for GSH and Hcy, and 5 min for Cys, Figure S18). Upon addition of GSH, the absorption of at 574 nm decreased gradually with time, and an increase of a new band at 609 nm were observed with a distinct isosbestic point at 550 nm. Concomitantly, a remarkable fluorescence change was also observed upon addition of GSH with the excitation wavelength at 550 nm. The emission maximum at 590 nm continuously red-shifted to 621 nm with time. The whole process of spectroscopic changes lasted for 15 minutes. In the case of Cys and Hcy, upon addition of Cys or Hcy, the absorption at 574 nm decreased gradually with time, and subsequently blue shifted. Meanwhile, the fluorescence turns off gradually at 590 nm and the whole process was all over in 5 min for Cys and 15 min for Hcy. For comparison, the response time of all above probes to three analytes (GSH, Cys and Hcy) was outlined in Figure 2.

In view of the distinct ratiometric response behaviors of above probes to GSH, we evaluated the selectivity of all the probes (5 μM) toward GSH (1 mM) over various amino acids (1 mM) by monitoring the fluorescent ratio change at λ_{em} with and without analyte, except for **BOD-DBP** as its slow response. As shown in Figure 3, only GSH was able to trigger obvious changes of the fluorescent ratio at λ_{em} with and without analyte for all the probes. The most significant fluorescent ratio change (about ten times) was observed when **BOD-DBNPF** as the GSH probe. Otherwise, the response of **BOD-DBNPF** to competing anions was also investigated (Figure S22). The results confirmed the high selectivity of BODIPY-Cl type of probe to GSH by monitoring the change of fluorescent ratio.

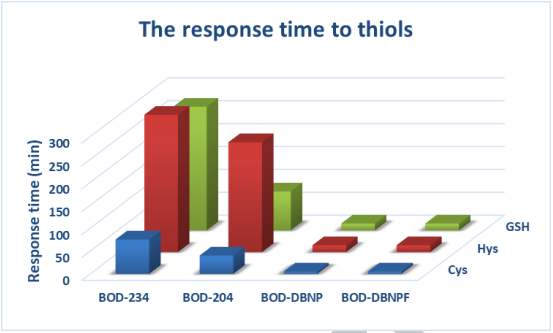


Figure 2. The response time of the probes to GSH, Cys and Hcy.

The spectroscopic responses of above probes toward thiols further verified our proposed the reaction mechanism as illustrated in Scheme 4. Once added Cys or Hcy, the chloro group in the probe is replaced by the thiols, and subsequently the rearrangement reaction takes place by a five- or six-membered cyclic transition state to yield amino substituted products which resulted in a blue-shift in the UV absorption of the probes. In case of GSH, only thiol substituted products produced and the electron-donating effect of the thiols lead to a red-shift of UV absorption for all the probes. The production of BOD-DBNPF-GSH was also identified by the HRMS analysis (Figure S20).

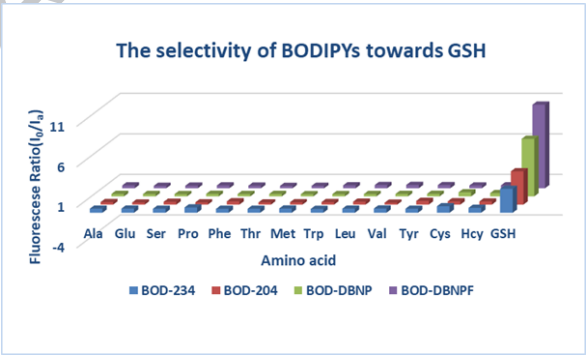
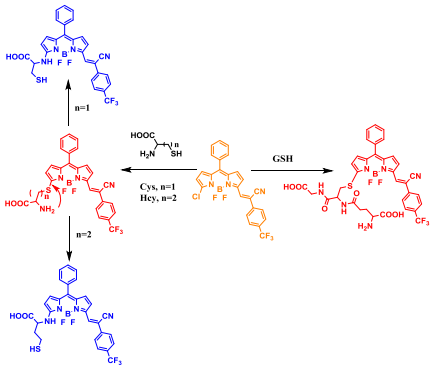


Figure 3. The selectivity of the probes by monitoring the fluorescent intensity ratio. The amino acids concentrations were kept as 1 mM.

The selective detections of GSH and Cys in living HeLa cells were carried out by using confocal fluorescence imaging. Before bioimaging by probe **BOD-DBNPF**, the pH interference and photostability of probe **BOD-DBNPF** were also tested (Figure S23 and S24) which indicated it is suitable for imaging in living cells. Since **BOD-DBNPF** is able to serve as a ratiometric fluorescence probe for monitoring GSH, we investigated the feasibility of this probe to detect HeLa cells in a dual-color manner. Although both green emission (500-530 nm) and red emission (600-640 nm) was observed in all groups (blank, Cys group and GSH group), the stronger fluorescence signals in red channel and relatively low fluorescence signals in green channel were collected in GSH group. In contrast, relatively strong green emission and weak red emission were observed in blank and Cys group. Therefore, the fluorescence ratios of red to green channels were utilized for the discrimination between GSH and Cys in living HeLa cells. In the GSH group, the fluorescence ratio (I_{red}/ I_{green}) was calculated to be around 1.8, In comparison, the fluorescence ratio reduced to about 0.4 in blank and around 0.6 in Cys group. The results demonstrated that **BOD-DBNPF** could be applied for imaging of GSH in living cells (Figure 4). In addition, in vitro cytotoxicity of the probe was valuated with HeLa cells

using CCK8 assay. No obvious adverse effect of **BOD-DBNPF** was found on HeLa cells viability (Figure S21).



Scheme 4. The proposed reaction mechanism of BODIPY-Cl type of probes towards thiols.

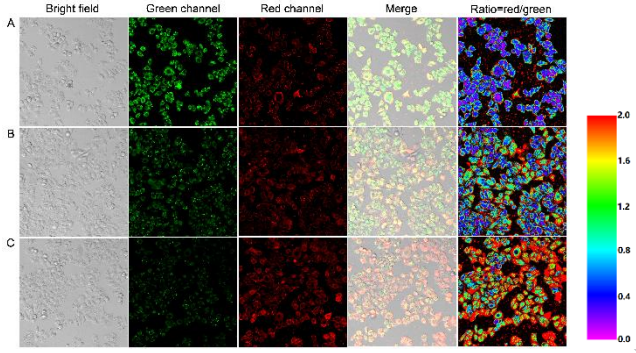


Figure 4. Confocal fluorescence images of living HeLa cells. (A) cells were treated with N-methylmaleimide (1 mM) for 20 min, then incubated with **BOD-DBNPF** (10 μM) for 15 min; (B) cells were treated with N-methylmaleimide (1 mM) for 20 min, then incubated with Cys (1 mM) for 30 min, further incubated with **BOD-DBNPF** (10 μM) for 15 min; (C) cells were treated with N-methylmaleimide (1 mM) for 20 min, then incubated with GSH (1 mM) for 30 min, further incubated with **BOD-DBNPF** (10 μM) for 15 min. Green channel at 500-530 nm; red channel at 600-640 nm; ratio images generated from green channel to red channel.

Conclusions

In summary, we have designed and synthesized several fluorescent probes for discriminative detection of GSH from Cys and Hcy. The discriminative detection is based on the different products obtained by the reaction between probes and GSH, Cys/Hcy. GSH induced the formation of sulfenyl-substituted BODIPYs while Cys/Hcy afforded amino-substituted BODIPYs. The two initial probes **BOD-204** and **BOD-234** showed relatively low selectivity and slow response in aqueous system. To achieve optimized fluorescent probes, structure modification was explored by elongation of π-conjugation and introduction of electron-withdrawing units. As expected, the obtained probe **BOD-DBNPF** showed highly discriminative detection and fast response. Furthermore, **BOD-DBNPF** was capable of selective detection of GSH from Cys with distinct fluorescent ratiometric responses in living HeLa cells.

Acknowledgments

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at

Highlights

1. The fluorescent probe for selective detection of GSH with ratiometric response.
2. The optimized probe with highly selective detection and fast response to GSH.
3. Selective bioimaging GSH in Living cells.