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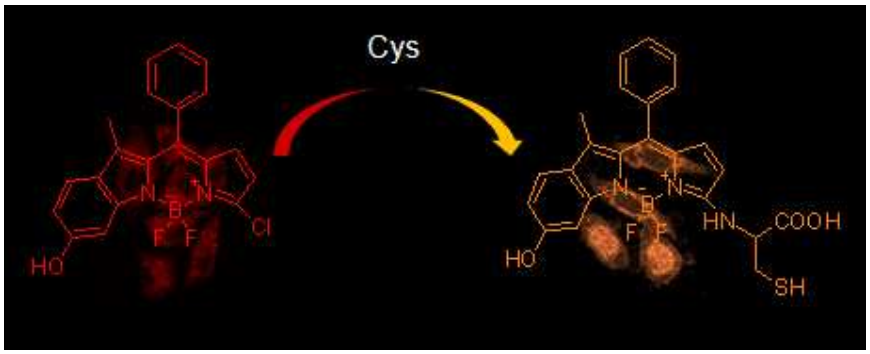
We developed a cystein specific probe by utilizing the remarkable difference in reactivity toward discriminating cystein from homocystein and glutathione. This probe was also successfully used for detection of Cys in living cells and monitoring Cystathionine γ-lyase activity in vitro.



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ARTICLE

Construction of a fluorescence turn-on probe for highly discriminating detection of cysteine

Cite this: DOI: 10.1039/x0xx00000x

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Received ooth January 2012,
Accepted ooth January 2012

DOI: 10.1039/x0xx00000x

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It is still a challenge to construction of probes for discriminating thiols due to the similar structure and reactivities of thiol-containing molecules. We here developed a Cys specific probe by utilizing the remarkable difference in reactivity toward Cys, Hcy and GSH. The reaction between designed probe and Cys produces an amino-substituted BODIPY, giving a yellow fluorescence turn-on response. The response to Hcy or GSH shows a red fluorescence turn-on signal, due to the formation of sulfenyl-substituted BODIPY. These distinct fluorescence turn-on responses allow Cys to be distinguished from Hcy and GSH. This probe was also utilized for detection of Cys in living cells and monitoring Cystathionine γ-lyase activity in vitro.

Itroduction

Biothiols have caused continuing concern due to their critical roles in biological systems.[1] Exploitation of probes for selective and sensitive detection of these species by fluorescence techniques has therefore emerged as a focal point in the sensing communities.[2] The prime strategy pursued for development such probes involves the use of reaction-based indicator systems, generally employing the strong nucleophilicity of the thiols group to trigger a specific reaction between probes and thiols, such as Michael additions,[3] cyclization reactions with aldehyde,[4] cleavage of disulfide and sulfonamide,[5] and others.[6] Although significant progresses have been made toward thiols detection, it is still a challenge to construction of probes for discriminating them due to the similar structure and reactivities of thiol-containing molecules. It is established that reaction-based probes need to fulfill several crucial design requirements, such as operating with high selectivity, and generating product that can be used as a marker for the analyte of interest.[7] Therefore, rationally designed probe bearing the aforementioned key requirements may pave to differentiate thiols.

We and others have recently demonstrated that change in electron-donating nature of the substituent on monochlorinated boron dipyrromethene (BODIPY) can result in remarkable difference in reactivity toward nucleophilic reagents.[8] By employing this promising feature, the selective detection of glutathione (GSH) over cysteine (Cys) and homocysteine (Hcy) has been obtained. However, few monochlorinated boron dipyrromethene-based probes have fulfilled the discrimination of Cys over Hcy and GSH. In the present study, we developed a 6-hydroxylindole-based monochlorinated BODIPY for highly discriminating detection of cysteine over Hcy and GSH based on the distinct electrophilic reactivity of the probe toward them. 6-hydroxylindole was introduced to a monochlorinated BODIPY core, rendering the BODIPY core with electron-rich nature and enabling the difference in reactivity toward Cys, Hcy, and GSH, thus leading to the high selectivity of the probe toward nucleophilic analytes. Detailed studies demonstrated that replacement of chlorine with thiolate in Hcy and GSH through nucleophilic aromatic substitution (S_NAr) reaction yields sulfenyl-substituted BODIPY with red-shifted absorption and emission features. The sulfenyl-substituted BODIPY derived from Cys is unstable and undetectable, which immediately undergoes intramolecular displacement with the amino group displacing of the sulfur function to form amino-substituted BODIPY, thus resulting in a significant hypsochromic shift in the optical spectra. These distinct fluorescence turn-on responses allow Cys to be distinguished

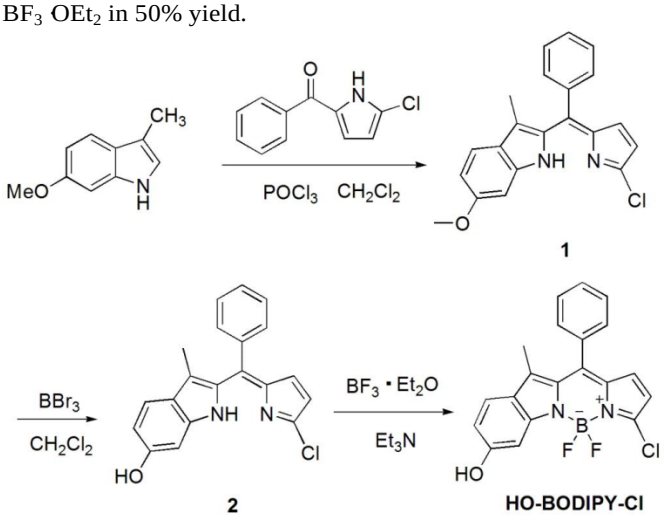
from Hcy and GSH. We further testified the potential application of this probe for detection of Cys in living cells.

Cystathionine γ-lyase (CGL) is a pyridoxal 5'-phosphate (PLP)-dependent enzyme, which catalyzes the decomposition of cystathionine to generate cysteine and other transsulfurations.[9] CGL plays a central role in amino acid catabolism and deregulation level of CGL is linked to cystathioninuria and cystinosis.[10] Therefore, monitoring CGL activity could provide valuable insight into early detection of the related metabolic disorders. However, few fluorescent probes have been developed for evaluating CGL activity. Significantly, the fluorescent turn-on probe reported here is capable of assay of cystathionine γ-lyase under physiological conditions.

Results and discussion

Synthesis

HO-BODIPY-Cl was prepared in a three-step reaction starting from 3-methyl-6-methoxyindole and 2-benzoyl-5-chloropyrrole, obtained by following reported methods. The condensation of 3-methyl-6-methoxyindole with 2-benzoyl-5-chloropyrrole in the presence of $POCl_3$ to furnish intermediate **1**, which was subsequently treated with BBr_3 in CH_2Cl_2 following a standard deprotection protocol to afford intermediate **2**. Finally, **HO-BODIPY-Cl** was obtained through boron insertion with BF_3 OEt_2 in 50% yield.



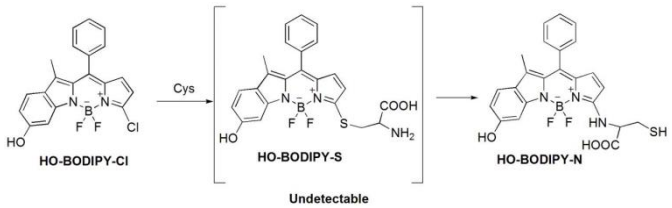
Scheme 1. Synthesis of **HO-BODIPY-Cl**.

Spectroscopic properties

In buffer solutions (acetonitrile / HEPES buffer, 1:1, v/v, 20 mM, pH 7.4), **HO-BODIPY-Cl** displays an intense absorption at 557 nm with a shoulder located at 600 nm, which is assigned to the absorption band of phenolate anion **$^-$O-BODIPY-Cl**, consistent with the observation of other phenol-containing BODIPYs.[11] Moreover, negligible emission of neutral phenol form **HO-BODIPY-Cl** could be observed from the fluorescence spectra, whereas only weak emission by **$^-$O-BODIPY-Cl** centered at 645 nm was noted upon photo-excitation. This low background fluorescence feature enables **HO-BODIPY-Cl** a promising platform to construct a fluorescence turn-on probe for monitoring analytes of interest.

We assessed the reactivity of **HO-BODIPY-Cl** with Cys in buffer solutions (acetonitrile / HEPES buffer, 1:1, v/v, 20 mM, pH 7.4) at 37 ℃ by monitoring the changes in UV-vis and fluorescence spectra. The reaction triggered a ratiometric absorption change over time and time-dependent fluorescence increase (Fig. 1). As can be seen in Fig. 1a, the absorption peaked at 557 nm and 600 nm gradually decreased, while a new absorption band at 520 nm was noted to increase simultaneously. This process was accompanied with an isosbestic point at 538 nm, suggesting the potential application of **HO-BODIPY-Cl** as a ratiometric and colorimetric probe for evaluation Cys under physiological condition. In the absence of Cys, **HO-BODIPY-Cl** displayed a weak emission centered at 645 nm. The addition of Cys (5 mM) to a solution of **HO-BODIPY-Cl** in buffer introduced a remarkable fluorescence increment, which was completed within 45 min. Accordingly, the fluorescence was changed from weak pink color to bight orange (Fig. S1). The observed rate constant was then determined to be 0.085 min^{-1} by fitting the data with a pseudo-first-order model (Fig. S2). The enhancement of fluorescence intensity after the reaction with Cys was as high as 10 fold, accompanied by a blue shift in the emission maxima from 645 nm to 586 nm, which enable **HO-BODIPY-Cl** to serve as a suitable fluorescence turn-on probe for Cys in aqueous solution. The reaction between **HO-BODIPY-Cl** and Cys yields amino-substituted BODIPY (**HO-BODIPY-N**), which shows blue-shift in the maximum wavelengths of both the absorption and the fluorescence spectra compared to that of the original probe. The production of **HO-BODIPY-N** after the reaction was confirmed by HRMS analysis as well as high performance liquid chromatography (HPLC) (Figure S3). HPLC analysis of **HO-BODIPY-Cl** + Cys revealed that **HO-BODIPY-Cl** (retention time at 9.306 min) was converted efficiently into **HO-BODIPY-N** (retention time at 6.010 min). In HRMS, the mass peak at 466.1206 corresponding to [**HO-BODIPY-N** - H]$^-$ was noted. Though the photophysical properties of **HO-BODIPY-N** are stable in buffer, it was likely to undergo oxidation upon drying and purifying. Thus, we could not obtain pure product for NMR characterization and observe a triplet signal from the proton of SH. It is worth mentioning that signals of sulfenyl-substituted BODIPY (**HO-BODIPY-S**) in the optical response spectral were not observed on the experimental time scale, indicating that **HO-BODIPY-S** is unstable and undetectable, which immediately undergo intramolecular displacement to form amino-substituted BODIPY (Scheme 2). The formation of amino-substituted BODIPY was also demonstrated by the reaction between **HO-BODIPY-Cl** and N-acetyl-cysteine. N-acetyl-cysteine is structurally similar to Cys but the amino is inactive. This structure feature makes only the product of sulfenyl-substituted

BODIPY possible, thus resulting in the absorption and emission shifted to 581 nm and 608 nm (Fig. S4).



Scheme 2. The reaction of **HO-BODIPY-Cl** with Cys.

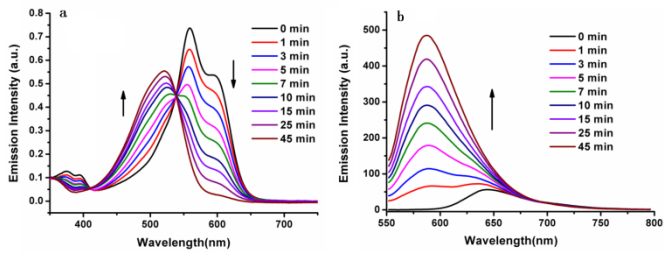


Fig. 1 Time-dependent spectra changes of **HO-BODIPY-Cl** (5μM) in the presence of 5 mM Cys in acetonitrile / HEPES buffer (1:1, v/v , 20 mM, pH 7.4) at 37 ℃. (a) Absorption and (b) emission spectra, λ_{ex} = 538 nm.

The fluorescence change of **HO-BODIPY-Cl** treated with different concentrations of Cys was also recorded in Figure 2. Upon addition of gradually increasing concentration of Cys, a progressive fluorescence intensity increase at 586 nm was observed, which is linearly dependent (R = 0.98) on the Cys concentration over a range of 0−100 μM, essentially falling within the physiological levels of Cys between 240 – 360 μM. Therefore, probe **HO-BODIPY-Cl** can be employed as an ideal candidate for quantitative detection of Cys. The detection limit was then calculated to be 4.1×10^{-7} M, which is comparable to previously reported probes.[12]

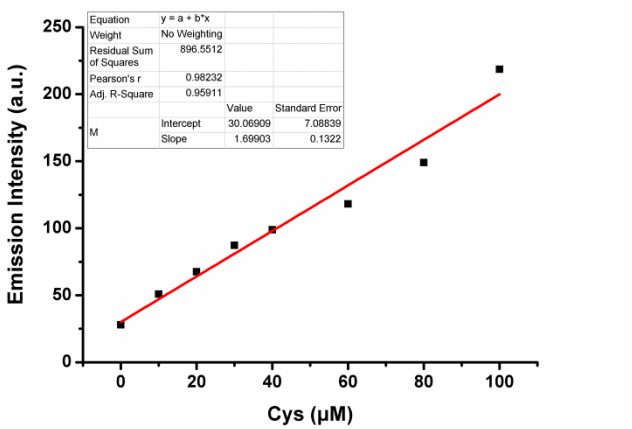


Fig. 2. Plots of fluorescence intensity as a function of Cys concnetrations. The standard deviation obtained by fluorescence responses was determined to be σ = 0.2325 for Cys, therefore, the detection limit was calculated by the formula (3σ/k) and given a result of 4.1×10^{-7}M for Cys.

We next evaluated the inference from Hcy and GSH (Fig. 3). As compared to Cys, both Hcy and GSH elicited inverse changes in the absorption spectra. A dramatic red-shift of the S_0-S_1 transition from 557 nm to 581 nm was introduced. Although Hcy and GSH also caused the blue-shift in the maximum wavelengths of fluorescence spectra, together with the fluoresce intensity increment after the reaction with **HO-BODIPY-Cl**, the fluorescence maximum located at 608 nm with red color, showing 22 nm red-shift relative to that of **HO-BODIPY-Cl** + Cys system. Both the UV-vis and emission spectra demonstrate the formation of single sulfenyl-substituted BODIPYs, **HO-BODIPY-S-Hcy** and **HO-BODIPY-S-GSH** after the reaction with Hcy and GSH, respectively. HPLC analysis and HRMS also confirmed the formation of **HO-BODIPY-S-Hcy** (retention time at 3.859 min, mass peak at 482.1516) and **HO-BODIPY-S-GSH** (retention time at 3.656 min, mass peak at 654.2011). As can be seen from Fig. 3f, the sulfenyl-substituted BODIPYs, **HO-BODIPY-S-Hcy** and **HO-BODIPY-S-GSH**, have minimal absorption at 500 nm compared to **HO-BODIPY-N** formed through the reaction of **HO-BODIPY-Cl** with Cys, therefore the selective excitation of **HO-BODIPY-N** with 500 nm can be performed. In this case, **HO-BODIPY-Cl** showed a very strong orange fluorescence in response to Cys upon excitation at 500 nm, while minimal fluorescence was observed with Hcy or GSH. Thus **HO-BODIPY-Cl** has a high selectivity for Cys, no disturbance by Hcy and GSH on the detection could be observed.

Based on the aforementioned experimental data and the reaction mechanism of two related thiol-response probes,[8] we believe that the spectra change of **BODIPY-Cl** induced by Cys was initiated by a two-step reaction: Displacement of chlorine with thiolate to generate sulfenyl-substituted BODIPY and then intramolecular cyclization to yield amino-substituted BODIPY through a five-membered cyclic transition state. The amino-substituted BODIPY was not observed for Hcy and GSH. This difference between Cys and Hcy/GSH can be attributed to the kinetic rate of the intramolecular cyclization reactions. The intramolecular cyclization reaction to form the six-membered ring with Hcy or a macrocyclic ring with GSH may be kinetically unfavorable.

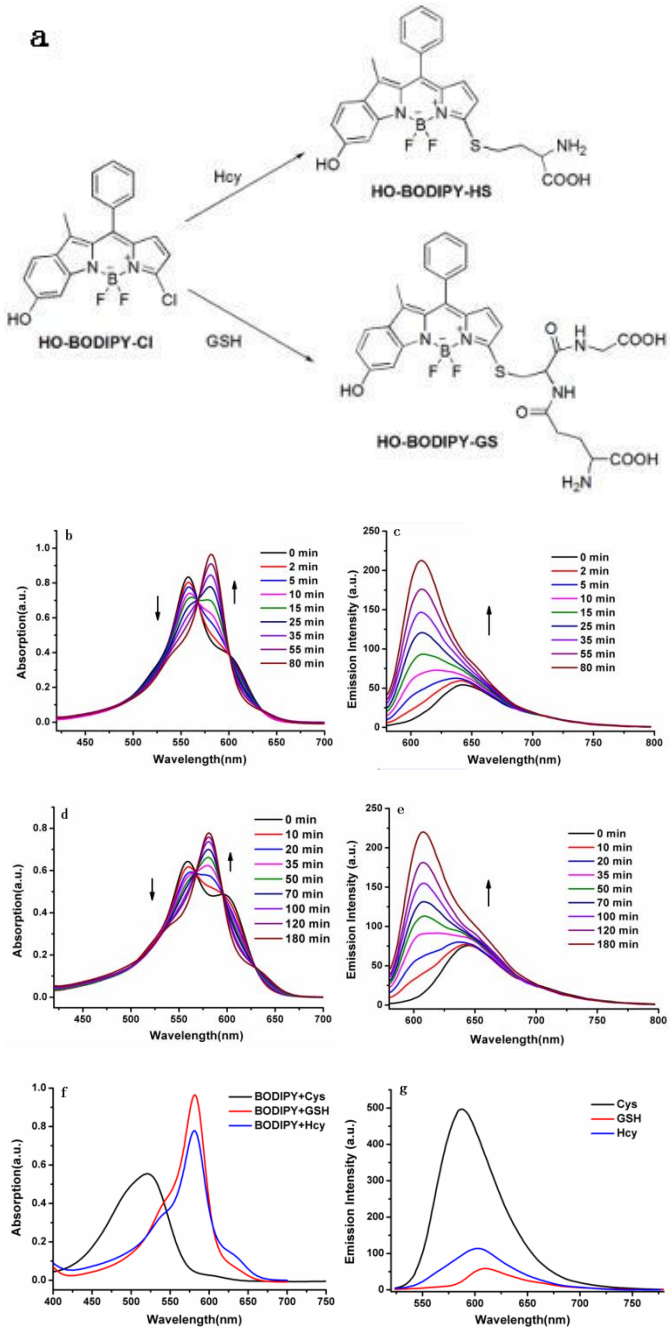


Fig. 3 (a) The reaction of **HO-BODIPY-Cl** with Hcy and GSH. Time-dependent (b) absorption and (c) emission (λ_{ex} = 533 nm) spectra changes of **HO-BODIPY-Cl** (5μM) in the presence of 5 mM GSH in acetonitrile / HEPES buffer (1:1, v/v , 20 mM, pH 7.4) at 37 ℃. Time-dependent (d) absorption and (e) emission (λ_{ex} = 533 nm) spectra changes of **HO-BODIPY-Cl** (5μM) in the presence of 5 mM HCY in acetonitrile / HEPES buffer (1:1, v/v , 20 mM, pH 7.4) at 37 ℃. (f) The absorption and (g) emission of **HO-BODIPY-Cl** (5μM) after incubation with 5 mM of Cys, Hcy and GSH for 20 min, respectively. λ_{ex} = 500 nm.

We then examined the reactivity of **HO-BODIPY-Cl** toward other related thiols and amino acids by means of fluorescence spectrometry (Fig. 4). **HO-BODIPY-Cl** showed negligible fluorescence ratio change (I_{586}/I_{645}) in response to these related species. Taken together, **HO-BODIPY-Cl** offers a good selectivity toward theses competing analytes, indicative of its capability for practical usefulness.

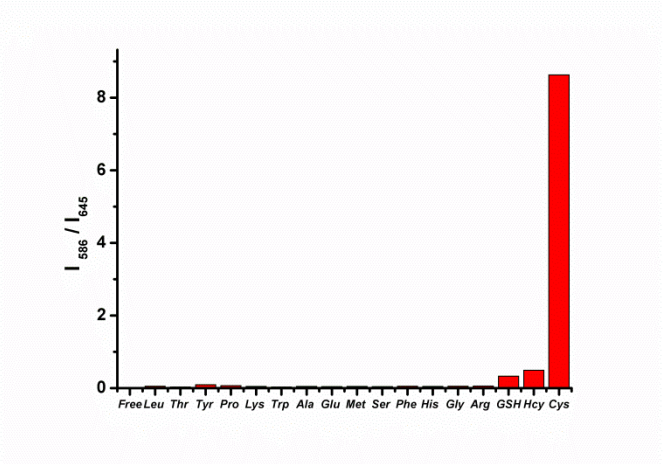


Fig. 4 Ratiometric responses of **HO-BODIPY-Cl** (5μM) toward analytes. λ_{ex} = 538 nm. Each data was acquired 2 h after the addition of the amino acid in acetonitrile/HEPES buffer (1:1 v/v, 20 mM, pH = 7.4) at 37 ℃.

With these characterization studies in hand, the efficacy of **HO-BODIPY-Cl** for live-cell imaging of Cys was explored. As shown in Figure 5, when cells successively pretreated with N-ethylmaleimide (a trapping reagent of thiols) were treated with **HO-BODIPY-Cl** for 20 min in the culture medium, only faint red fluorescence (606 nm -636 nm) signals was observed, which was ascribed to the unreacted probe. In contrast, the yellow channel (572-588 nm) is almost nonfluorescent. The ratio of the two emissions from yellow channel to red channel is about 0.1. More importantly, bright fluorescence in the yellow channel between 572-588 nm was observed in cells upon incubation with 100 μM Cys for 20 min and further incubation with **HO-BODIPY-Cl** for another 20 min in the same culture medium. The ratio was improved to be around 1.6, showing a ca. 16-fold enhancement. These results suggest that **HO-BODIPY-Cl** is a promising probe for living cells imaging of Cys.

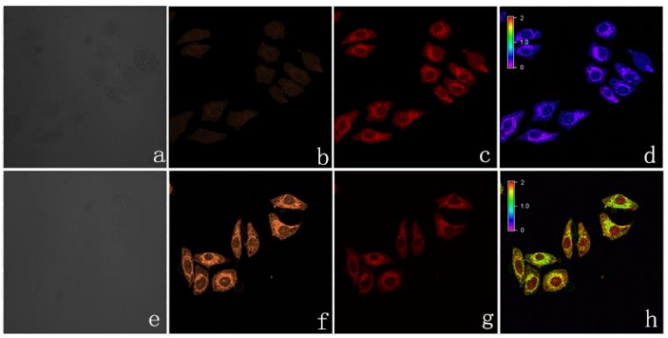


Figure 5. Fluorescent confocal images of HeLa cells: (a-d) cells pretreated with 500 μM N-methylmaleimide for 20 min, further incubated with **HO-BODIPY-Cl** (10μM) for 20 min, the excitation wavelength was 561 nm: (a) bright-field images, (b) yellow channel at 572−588 nm, (c) red channel at 606−636 nm, (d) ratio image generated from (b) and (c); (e-h) cells pretreated with 500 μM N-methylmaleimide for 20 min, further incubated with 100 μM Cys for 20 min, then loaded with **HO-BODIPY-Cl** (10 μM) for 20 min, the excitation wavelength was 561 nm: (e) bright-field images, (f) yellow channel at 572−588 nm, (g) red channel at 606−636 nm, (h) ratio image generate from (f) and (g).

This unique probe was also used to monitor the activities of Cystathionine γ-lyase (CGL) in vitro. It is known that CGL catalyzes the decomposition of cystathionine to generate cysteine. Therefore, probe **HO-BODIPY-Cl** should give rise to a turn-on emission signal in the presence CGL and its substrate cystathionine. This was accomplished by mixing **HO-BODIPY-Cl**, CEL and cystathionine in buffer at 37 ℃. The time dependent fluorescence changes were recorded in Fig. 6 and Fig. S5, indicating a remarkable fluorescence increment accompanying by the emission maximum shifted from 645 nm to 586 nm. More importantly, minimal fluorescence enhancement was observed upon pretreatment with CGL inhibitor, Propargylglycine(PAG), indicative of the blocking effect in formation of Cys. These results verify the capacity of **HO-BODIPY-Cl** for monitoring CGL activity.

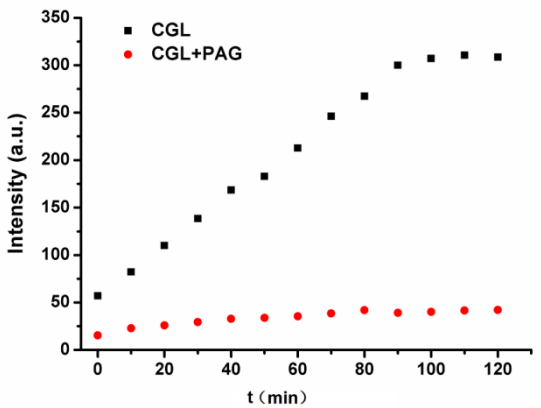


Fig. 6. Kinetics of fluorescence enhancement profile of **HO-BODIPY-Cl** (10 μM) at 586 nm upon incubation with CGL and its substrate cystathionine (3 mM) in the absence and presence of PAG (CGL inhibitor).

Conclusions

In this paper, a 6-hydroxylindole-based monochlorinated BODIPY was designed and synthesized for highly discriminating detection of cysteine over Hcy and GSH. Cys triggered an amino-substituted BODIPY formation with a distinct yellow fluorescence turn-on at 586 nm. In contrast, Hcy and GSH introduced the sulfenyl-substituted BODIPY products with red-shifted absorption and emission features. These distinct fluorescence turn-on responses allow Cys to be distinguished from Hcy and GSH. We further demonstrated the potential application of this probe for detection of Cys in living cells and the capacity for monitoring CGL activity.

Acknowledgements

We gratefully acknowledge the financial support by the National Science Foundation of China (grant no.: 21372083, 21172071, 21190033).

Notes and references

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† Electronic Supplementary Information (ESI) available: UV-vis absorption and emission spectra, Pseudo first-order kinetic profile, ^{1}H NMR, ^{13}C NMR spectra of the new compounds. See DOI: 10.1039/b000000x/.

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