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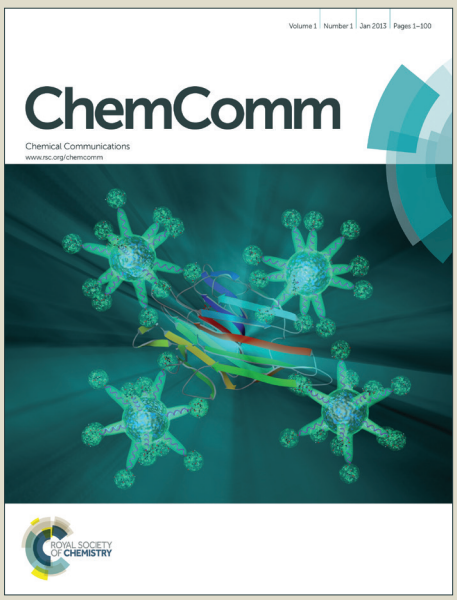

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Dual Emission Channels for Sensitive Discrimination of Cys/Hcy and GSH in Plasma and Cells

Cite this: DOI: 10.1039/x0xx00000x

Yuanlin Zhang,[a] Xiangmin Shao,[a] Yue Wang,[a] Fuchao Pan,[a] Ruixue Kang,[a] Fangfang Peng,[a] Zhentao Huang,[a] Weijuan Zhang*[a] and Weili Zhao*[ab]

Received 00th January 2012,
Accepted 00th January 2012

DOI: 10.1039/x0xx00000x

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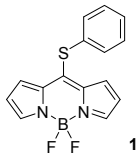
A new selective fluorescent and colorimetric chemosensor for the detection of GSH was developed. The discrimination of GSH from Cys and Hcy is achieved through two emission channel detection. The detection limit of probe 1 for GSH reached 10 nM (3 ppb). The excellent sensitivity and selectivity of probe 1 allow selective detection of GSH over Cys and Hcy, which can be visualized colormetrically and/or fluorescently. The sensitive detection of GSH allowed convenient measurement of GSH content in human plasma. The presence of GSH in cells was demonstrated through cell imaging.

Pioneered by Strongin's group, fluorescent probes for biothiols including cysteine (Cys), homocysteine (Hcy), as well as reduced glutathione (GSH) have been the focused area due to their significance in biological processes.[1,2] GSH as a tripeptide of glutamic acid, cysteine, and glycine, is the most abundant intra-cellular nonprotein thiol and a biomarker of oxidative stress.[3] Aberrant levels of GSH have been correlated with various diseases, such as cancer, AIDS, liver damage and neurodegenerative diseases.[4] Modern intelligent drug delivery systems have been developed by taking advantages of the differences between intracellular and extracellular thiol concentrations in cancer cells.[5] Some Antibody-drug-conjugates (ADCs) were also believed to release the cytotoxic payloads by reduction of disulfide linker with high content of intraceller GSH.[6] Abnormal levels of Cys have been associated with many kinds of diseases such as slow growth, hair depigmentation, liver damage, loss of muscle and fat, skin lesion, and cancer.[7] As a risk factor for disorders including cardiovascular diseases and Alzheimer's disease,[8] Hcy has been implicated in various types of vascular and renal diseases.[9] Thus, the detection of thiol is highly important for early diagnosis of diseases, evaluation of disease progression and therapy efficiency of disulfide linked ADC-drugs.

Although discrimination of biothiols from other amino acids by taking advantage of the unique nucleophilicity of the thiol groups was fulfilled, distinguishing Cys/Hcy/GSH from each other turned out to be more difficult due to the similar structures and reactivity of these biothiols. While selective detection of Cys/Hcy over GSH could be realized in some recent reports.[10] discrimination of GSH from Cys/Hcy was much more difficult and was addressed in much less extent.[11] A noteworthy advance reported recently is S_NAr mechanism-based substitution reaction to allow differentiation of GSH, as well as detect Cys/Hcy via different emission channels to be possible,[11f-k] However, the interference of the probe itself and the significant overlap of sensing products on absorption and emission spectra limited the potential applications, especially in the presence of large excess of interfering biothiol species. It is known that intracellular concentration of GSH is much higher than Cys (Cys: 30−200 μM; GSH: 1−10 mM),[11e] On the contrary in healthy human plasma Cys concentration is typically 10 times that of GSH, 20–30 times that of Hcy which normally presents below 12− 15 μM,[12] So far, detection of GSH in the presence of large excess of interference is still illusive. There is a pressing need for the identification of newer more effective probe for fast and selective GSH sensing. In this communication we report a highly selective BODIPY-based fluorescence chemosensor for fast discrimination of GSH over Cys/Hcy, and selective detection of GSH in plasma and in living cells.

The dipyrrometheneboron difluoride (difluoroboradiaza-s-indacene, BODIPY) fluorescent dyes have found widespread applications because of many advantages, such as high molar absorption coefficients and quantum yields, elevated chemical and photo stability.[13] It was demonstrated that *meso* position of BODIPY is the most sensitive position to the electronic nature of the substituent on emission maxima and/or fluorescent quantum yield.[13] We have rich experience on BODIPY dyes in recent years.[14] We speculated that once a good leaving group was connected to the *meso* position, an unprecedented faster

and sensitive sensing through replacement by a nucleophile may be realized. Literature search suggested that only a few *meso*-heteroatom substituted BODIPYs were documented and only one 8-heteroatom substituted BODIPY was reported to act as fluorescent sensor for Hg and very recently for Cys/Hcy.[15,16] A thorough screening of various 8-heteroatom substitution patterns resulted in a discovery of a known compound 8-phenylmecapto BODIPY **1**[15d] as fast responsive probe for Cys/Hcy, as well as GSH.



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Probe **1** is essentially almost non-fluorescent (Φ_f = 0.00045) in acetonitrile/HEPES buffer (2 : 8, v/v, 10 mM, pH 7.4), which suggested that a photo-induced electron transfer (PET) process took place from the electron-donating phenylmarcapto group to the electron-deficient BODIPY core. When probe **1** was subjected to various nucleophiles, we were pleased that very fast responses to biothiols were observed as shown in Fig. 1.

Drastic changes in absorption spectra of probe **1** against Cys/Hcy were noticed in Fig. 1a. From Fig. 1b, it could be observed that the nearly non-fluorescent probe **1** generated fluorescent species which emit around 470 nm for Cys and Hcy. In the case of GSH, stronger fluorescent product (Φ_f = 0.17) emits at 535 nm was noticed. Importantly, the different emission regions for Cys/Hcy from GSH constituted the basis for the discriminations of GSH from Cys/Hcy and vice visa. Favorably, the two series of fluorescent spectra generated do not interfere with each other and may allow clear differentiation. The longer wavelength and strong fluorescence generated from GSH facilitate fast and sensitive sensing of GSH. The dramatic fluorescence off-on of probe **1** in response to GSH (Φ_f 0.00045→0.17) promoted us for further investigation of GSH detection.

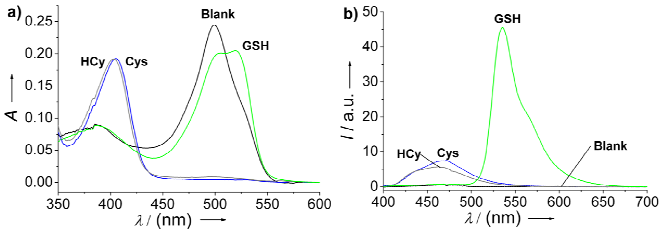

Figure 1. Absorption and emission spectra of probe **1** (10 μM) prior to and after addition of Cys, Hcy, GSH (200 μM each) in acetonitrile/HEPES buffer (2 : 8, v/v, 10 mM, pH 7.4) at 37 °C for 20 min. λ_{ex} = 390 nm..

To evaluate the selectivity of the developed probe **1** for GSH, a series of biologically relevant amino acids and another reactive sulfur species, H_2S, were examined in acetonitrile/HEPES buffer (2 : 8, v/v, 10 mM, pH 7.4) at 37 °C (Fig. 2). The fluorescence intensity at 535 nm (excited with 480 nm light) after addition of various analytes was measured, only GSH was observed to induce high fluorescence enhancement and more than 500 fold increase in the emission intensity was noticed under the given conditions. In a stark contrast, only HS^- resulted in fairly slight interference (merely about 5-fold enhancement). More importantly, under the given conditions, neither Cys nor Hcy displays fluorescence interference at 535 nm. These experimental results demonstrated the excellent selectivity of probe **1** for GSH.

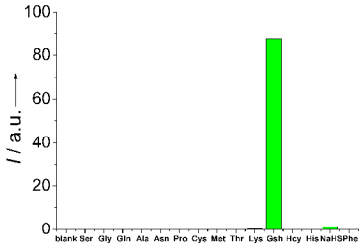

Figure 2. Fluorescent intensity at 535 nm (excited at 480 nm) of probe **1** (10 μM) upon addition of 100 equiv of various analytes. Each data point was acquired 20 min after addition in acetonitrile/HEPES buffer (2:8, v/v, 10 mM, pH 7.4) at 37 °C.

The time-dependent fluorescence response of probe **1** (10 μM) in the presence of GSH (200 μM) was measured at 37 °C in acetonitrile/HEPES buffer (2 : 8, v/v, 10 mM, pH 7.4) (Fig. 3a and Fig. 3b). As the reaction progressed, the emission band centered at 535 nm quickly increased. A complete response was finished within 20 min. Concentration-dependant response profiles are shown in Fig. 3c and Fig. 3d. The excellent sensitivity and selectivity of probe **1** allow selective detection of GSH over Cys and Hcy with a detection limit of 10 nM (3 ppb).

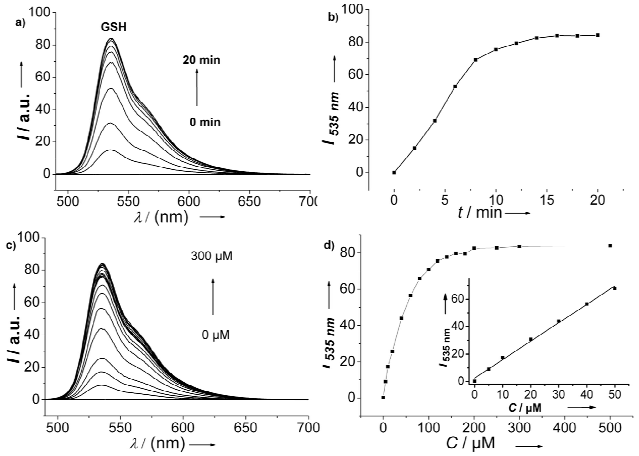

Figure 3. a) Fluorescence responses of probe **1** (10 μM) to GSH (20 equiv) recorded in 2 min interval; b) Time course fluorescence responses recorded at 535 nm; c) Emission spectra of probe **1** (10 μM) upon addition of increasing concentrations of GSH; d) Fluorescence intensity as a function of GSH concentration at 535 nm. Inset: Fluorescent intensity as linear response of concentration of the GSH at 535 nm. All spectra were acquired in acetonitrile/HEPES buffer (2 : 8, v/v, 10 mM, pH 7.4) at 37 °C. λ_{ex} = 480 nm..

One unique feature of probe **1** was that it also provided an opportunity to differentiate Cys/Hcy from GSH when monitored in blue channel (e.g 460–470 nm) as already shown in Fig. 1. The probe showed fast response towards Cys/Hcy. The concentration-dependent responses of probe **1** to Cys/Hcy were summarized in Figures S1 and S2 respectively. Linear response to Cys was in the range of 1–20 μM, and to Hcy was 1–40 μM. The advantages of probe **1** as smart probe to be capable of differentiation of GSH from Cys/Hcy and Cys/Hcy from GSH were shown in Fig. 4. When monitored at 535 nm, highly sensitive and selective discrimination for GSH over Cys/Hcy was fulfilled within 30 min (Fig. 4a). Surprisingly, when monitored at 462 nm, differentiation of Cys/Hcy

from GSH was reached within 10 min and Cys reacted with probe **1** even faster than Hcy and went to completion within 6 min (Fig 4b). Importantly, under such conditions, GSH does not interfere with the detection of Cys/Hcy.

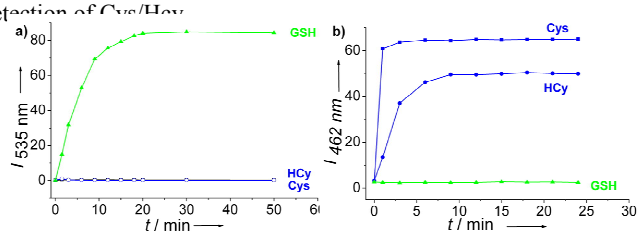

Figure 4. Time course fluorescence response of probe **1** (10 μM) towards Cys, Hcy, and GSH (200 μM each) in acetonitrile/HEPES buffer (2 : 8, v/v, 10 mM, pH 7.4) at 37 °C. a) recorded at 535 nm; b) recorded at 462 nm.

The smartness of probe **1** for sensing GSH, Cys/Hcy can be visualized colormetrically and fluorescently as shown in Fig. 5. The presence of Cys/Hcy can be recognized by naked eyes and/or by the blue emission generated UV irradiation. However, GSH can only be differentiated by the yellow-green fluorescence generated under UV irradiation.

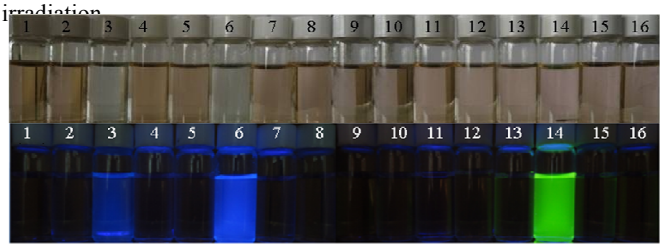

Figure 5. Photo images of probe **1** prior to after the addition of 100 equiv of various amino acids (from left to right: Blank, Ser, Hcy, Gly, Ala, Cys, Asn, Pro, Met, Thr, Lys, His, Gln, GSH, NaHS, Phe) by naked eyes (upper row) compared with UV irradiation from a hand-held UV lamp (bottom row) in acetonitrile/HEPES buffer (2 : 8, v/v, 10 mM, pH 7.4) at 37 °C.

The reaction-based probe **1** for discrimination GSH over Cys/Hcy was believed to follow the similar mechanism as reported by Yang et al.[10i] Such mechanism was proposed and studied in several recent publications including the fairly closely-related 8-Methylmercapto BODIPY,[16b] coumarine dye,[11g] heptacyanine,[11j] as well as Rhodol derivative.[11k]

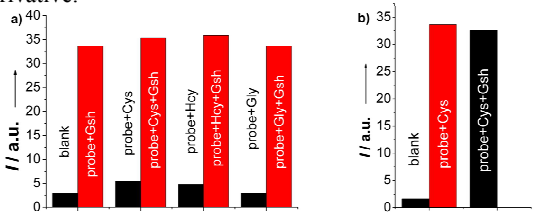

Figure 6. a) Fluorescent Intensity at 535 nm (excited at 480 nm) of probe **1** (10 μM) toward GSH (0.2 μM) in the absence/presence of Cys (6 μM)/Hcy (1 μM)/ Gly (6 μM). b) Fluorescence intensity at 462 nm (excited at 390 nm) of probe **1** (20 μM) toward Cys (5 μM) in the absence/presence of GSH (500 μM). All data were acquired 40 min after addition in acetonitrile/HEPES buffer (2 : 8, v/v, 10 mM, pH 7.4) at 37 °C.

The clearly differentiated detection channels for GSH and Cys/Hcy are advantages to discriminate the desired analyte even in the presence of excess interference (see Fig. 6). In the presence of large excess analytes over GSH (30 equiv of Cys, 5 equiv of Hcy, 30 equiv of Gly), when monitored at 535 nm, significant fluorescence enhancement was observed only when GSH existed (Fig. 6a). On the contrary, when monitored at 462 nm, even in the presence of 100 equiv of GSH, Cys was effectively detected as shown in Fig. 6b. From Fig. 6, it is clearly shown that probe **1** is very powerful for the detection of GSH and Cys from two different emission channels even in the presence of large amount of interference.

We next turned our attentions for the detection of GSH and measurement of GSH concentration in plasma. The detection of GSH was performed in 10% deproteinized human plasma. The emission spectra of the plasma in the absence and presence of probe **1** are shown in Fig. 7a. Characteristic emission from the product of GSH with probe **1** was observed and when compared with the linear response of GSH concentration to probe **1** (Fig. 7b), the GSH content of the plasma sample measured was calculated to be 4.5 μM.

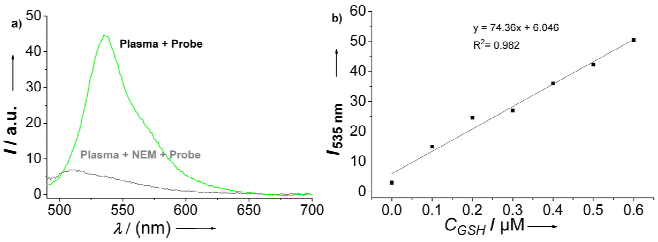

Figure 7. a) Fluorescence intensity (excited at 480 nm) of probe **1** (10 μM) in plasma. b) The fluorescence linear response of probe **1** (10 μM) toward GSH in 0–0.6 μM. All data were recorded at 20 min after addition of GSH.

To evaluate the function of the probe for biological applications, we performed an assay to detect intracellular GSH in live cells. Human Hepatoma SMMC-7721 cells were used in our study. An MTT experiment was performed to assess the cytotoxicity of the probe. The MTT assay results in SMMC-7721 cells with probe concentrations from 2.5 to 80 μM were collected in Fig. S3. The cell growth was not affected by the probe, which suggested that probe **1** was not toxic to culture cells under the experimental conditions.

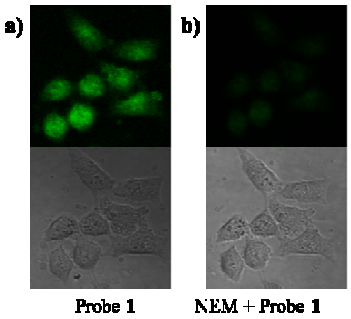

Figure 8. Confocal laser scanning microscopic images of probe **1** (λ_{ex} = 488 nm) to GSH in SMMC-7721 cells. (a) Fluorescence image of SMMC-7721 cells incubated with probe **1** (10 μM) for 60 min. (b) Fluorescence image of SMMC-7721 cells pretreated with NEM (5 mM, 60 min) and then incubated with probe **1** for 60 min.

Confocol laser scanning microscopic fluorescence imaging was used for GSH detection. As shown in Fig. 8, green fluorescence could be visualized in SMMC-7721 cells upon addition of the probe

1 (Fig. 8a). When SMMC-7721 cells were pretreated with 5 mM of N-ethylmaleimide (NEM) and subsequently incubated with the probe **1** (Fig. 8b), weak green fluorescence was noticed. These results clearly reflected the change of GSH level in cells.

In conclusion, we have described a new selective fluorescence and colorimetric chemosensor for the detection of GSH. The discrimination of GSH from Cys and Hcy is achieved through two emission channel detection. Highly sensitive and selective detection of GSH is feasible with probe **1** with the detection limit of 3 ppb. The current studies provide potential applications for quick GSH detection in plasma and in living cells.

This research was supported by NSFC (21372063) and Program for Changjiang Scholars and Innovative Research Team in University, No. PCS IRT1126. We thank Mr. Jian Zhang for performing MTT assay and cell imaging experiments.

Notes and references

[a] Key Laboratory for Special Functional Materials of the Ministry of Education, Henan University, Kaifeng, 475004, P. R. China, E-mail: zhaow@henu.edu.cn. Tel./fax: +86-371-23881358.

[b] School of Pharmacy, Fudan University, Shanghai, 201203, P. R. China. Email: zhaoweili@fudan.edu.cn. Tel/Fax: +86-21-51980111.

Electronic Supplementary Information (ESI) available: The synthesis, preocedures for GSH detection in plasma and cells, Absorption and fluorescence responses to Cys, Hcy, copies of NMR spectra. See DOI: 10.1039/c000000x/

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