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A turn-on fluorescent sensor for the discrimination of cystein from homocystein and glutathione†

Cite this: *Chem. Commun.*, 2013, **49**, 1294

Received 23rd November 2012,
Accepted 19th December 2012

DOI: 10.1039/c2cc38429a

www.rsc.org/chemcomm

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We report a turn-on fluorescent sensor based on nitrothiophenolate boron dipyrromethene (BODIPY) derivatives for the discrimination of cystein (Cys) from homocystein (Hcy) and glutathione (GSH). The sensor was applied for detection of Cys in living cells.

Biological thiols, including cystein (Cys), homocystein (Hcy) and glutathione (GSH), play crucial roles in maintaining redox balance of biological systems. Cys deficiency could lead to slow growth, skin lesions, liver damage, *etc.*[1] Hcy is associated with a variety of diseases such as neutral tube defects, cardiovascular and Alzheimer's diseases.[2] GSH is a pivotal indicator for the maintenance of xenobiotic metabolism, intracellular signal transduction and gene regulation.[3] Accordingly, it is of great importance to detect these biological thiols.

A fluorescent sensor is a powerful tool for the detection of mercapto biological molecules in living systems due to its simplicity, high sensitivity and suitability for intracellular detection.[4] Significant effort has been made to develop fluorescent sensors for biothiol detection.[5] However, it still remains a challenge to discriminate between SH-containing molecules because of their similarity in structure and reactivity. Only a few sensors discriminating Cys, Hcy and GSH from one another have been reported so far.[6] Recently we developed the first ratiometric fluorescent sensor for the selective detection of GSH over Cys/Hcy.[7] The chlorine of the monochlorinated boron dipyrromethene (BODIPY) can be rapidly replaced by thiolate. The amino groups of Cys/Hcy, but not of 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/Hcy. However, amino-substituted BODIPY exhibited a limited emission shift relative to monochlorinated BODIPY, which is less suitable for the detection of Cys and Hcy in biological systems. Herein we report a sensor that emits turn-on fluorescence upon addition of thiols. By means of more rapid intramolecular displacement of sulfur with amino group of Cys than Hcy and GSH, the discrimination of Cys from Hcy and GSH is achieved.

BODIPY dyes have many excellent features, such as high molar absorption coefficients and quantum yields, elevated chemical and photostability, which make them good chromophore candidates as fluorescent sensors in biological systems.[8] Furthermore, their spectroscopic and photophysical properties can be well tuned by introducing ancillary residues at the appropriate positions of the dipyrromethene core.[9] By using 3,5-dichlorinated BODIPY as the precursor, we introduced a triethyleneglycol group at the 3-position through Sonogashira coupling followed by click reaction to improve the water-solubility. The chlorine at the 5-position was displaced with nitrothiophenol or nitrophenol to yield compound **1**, which was characterized by ^{1}H NMR, ^{13}C NMR, and high-resolution mass spectrometry (ESI†). A nitrothiophenol or nitrophenol moiety with strong reduction potential attached to a BODIPY fluorophore may incur photo-induced electron transfer (PET) to quench the fluorescence.

In preliminary experiments, we studied the reaction of **1** toward methyl mercaptoacetate as a model thiol (Scheme 1). The resulting products exhibit strong fluorescence, which were identified as compound **2** (Fig. S1 in ESI†). It revealed that methyl mercaptoacetate substituted the nitrothiophenol or nitrophenol group, which blocked the PET process and led to a fluorescence enhancement. The observed rate constant (k_{obs}) for the reaction of **1a** and methyl mercaptoacetate is found to be 2.0×10^{-2} s^{-1} ($t_{1/2}$ = 34.5 s) under a pseudo first order reaction. **1b** showed a slower reactivity ($t_{1/2}$ = 97.4 s), which is probably attributed to the more stable C–O bond compared to the C–S bond of **1a** (Fig. S2 in ESI†). The mild and efficient reaction for the formation of **2** with significant fluorescence enhancement showed that **1** might be candidates for turn-on fluorescent sensors for the detection of thiols.

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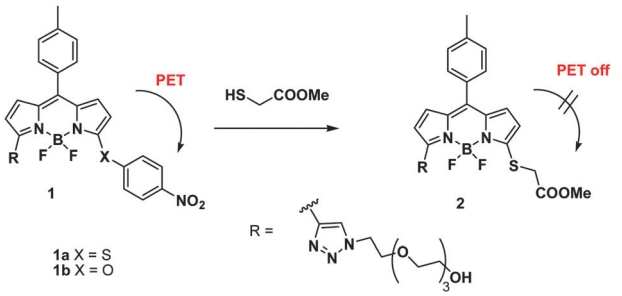
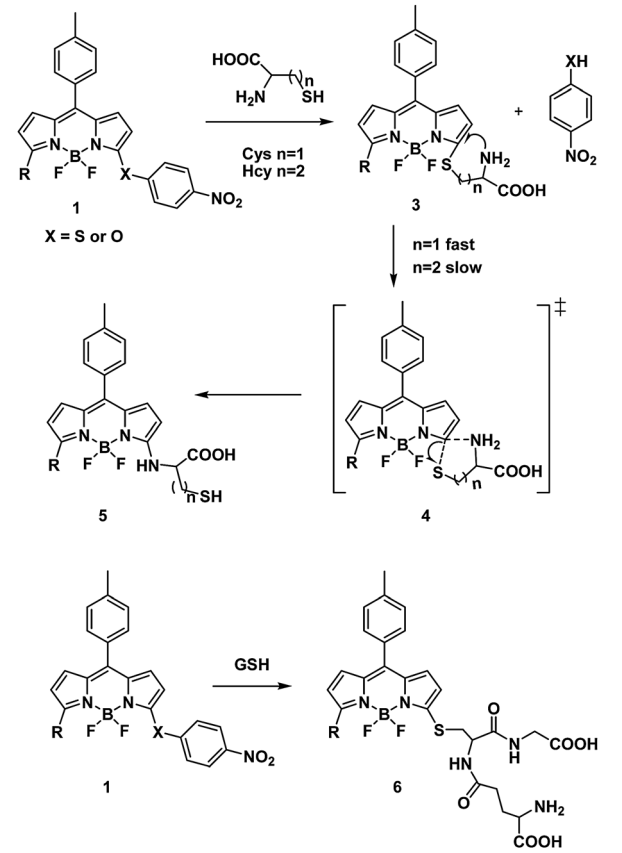
† Electronic supplementary information (ESI) available: Synthetic details and characterizations of compounds **1a** and **1b**; fluorescent response of **1a** in the presence of Hcy or GSH; emission spectra of **1b** in the presence of Cys, Hcy or GSH. See DOI: 10.1039/c2cc38429a



Scheme 1 Reaction of **1** with methyl mercaptoacetate.

Based on our previous work,[7] we hypothesized the reaction mechanism of **1** with Cys, Hcy and GSH as illustrated in Scheme 2. Initially, the thiolate can replace the nitrothiophenyl or nitrophenyl group of **1** to generate compound **3**, which is attributed to a kinetics controlled reaction. Once **3** is formed, the distance between the amine group and the active site of BODIPY is getting close. Driven by thermodynamics, the amino group in compound **3** can further attack the carbon attached to the sulfur by a five- or six-membered cyclic transition state **4**, followed by the formation of the thermodynamically more stable C–N bond and the cleavage of the C–S bond to yield compound **5**. In the case of GSH, after sulfur is substituted for chlorine, intramolecular substitution is difficult to happen since there is no adjacent amine group to form a favored transition state in GSH, and it will result in the stable product **6**.



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

The time-dependent fluorescence response of **1a** in the presence of Cys was investigated at 37 °C in aqueous HEPES buffer (20 mM, pH 7.4) containing 20% acetonitrile (Fig. 1a). Upon addition of Cys, the emission band centered at 588 nm increased in the initial 3 minutes, indicating that the thiolate displaced the nitrothiophenol group and turned off the PET quenching. The emission band then shifted to 564 nm and increased gradually as a result of the formation of amino-substituted product. We studied the fluorescence response of **1a** with Hcy and GSH under otherwise identical conditions (Fig. S3 in ESI†). In the presence of Hcy, the sulfur-substituted product was formed initially as expected. Due to the relatively slow rate of further intramolecular displacement with amino group, the emission of 588 nm increased steadily and maintained its strong fluorescence for a few hours. In the presence of GSH, one can only observe the fluorescence enhancement at 588 nm since intramolecular cyclization cannot happen. Therefore, the discrimination of Cys from Hcy and GSH was achieved (Fig. 1b).

We also measured the fluorescence response of **1b** toward Cys, Hcy and GSH. Similar but relatively slow sensing behavior was observed (Fig. S4 in ESI†).

Fig. 2 showed the absorption and emission changes of **1a** with different concentrations of Cys in acetonitrile/HEPES buffer (2 : 8, v/v, 20 mM, pH 7.4) at 37 °C. With increasing amounts of Cys, the absorption band centered at 571 nm gradually decreased with concomitant growth of a new band at 495 nm. When excited at the isosbestic point of 528 nm,

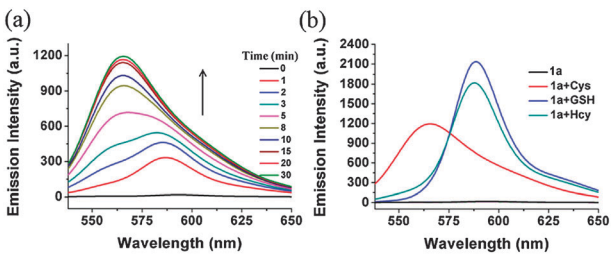


Fig. 1 (a) Time-dependent fluorescence spectra of **1a** (10 μM) with 100 equiv. of Cys. (b) Fluorescence spectra of **1a** (10 μM) after 1 h addition of 100 equiv. of GSH, Cys and Hcy in acetonitrile/HEPES buffer (2 : 8, v/v, 20 mM, pH 7.4) at 37 °C. λ_{ex} = 528 nm.

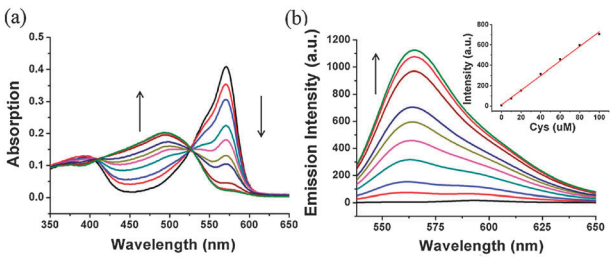


Fig. 2 (a) Absorption and (b) fluorescence spectral changes of **1a** (10 μM) upon addition of Cys (0, 10, 20, 40, 60, 80, 100, 200, 300 and 500 μM). Inset: the fluorescence intensity at 564 nm as a function of the concentrations of Cys. λ_{ex} = 528 nm. Each spectrum was recorded 40 min after Cys addition in acetonitrile/HEPES buffer (2 : 8, v/v, 20 mM, pH 7.4) at 37 °C.

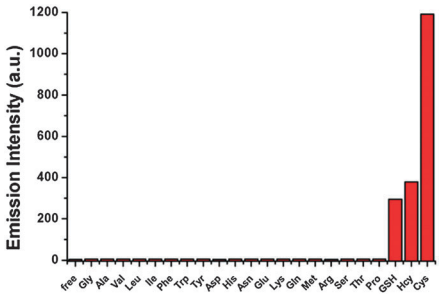


Fig. 3 Emission response at 564 nm of **1a** (10 μM) upon addition of 100 equiv. of various amino acids and GSH. Each data point was acquired 2 h after addition of different amino acids in acetonitrile/HEPES buffer (2 : 8, v/v, 20 mM, pH 7.4) at 37 °C.

the emission at 564 nm increased significantly upon addition of Cys. The emission intensity at 564 nm showed good linear relationship with the Cys concentration (0–100 μM). The detection limit was determined to be 2.12×10^{-7} M (S/N = 3). The recognition of GSH and Hcy were also investigated (Fig. S5 in ESI†).

To examine the selectivity, the fluorescence responses of **1a** to various amino acids were measured under physiological conditions. As shown in Fig. 3, only Cys induced a high fluorescence intensity at 564 nm under the given conditions.

The capability of **1a** for the imaging of Cys was studied in living cells (Fig. 4). When HeLa cells were pretreated with 500 μM Cys for 15 min and then incubated with **1a** (5 μM) for 15 min, strong fluorescence was observed. By contrast, addition of *N*-ethylmaleimides (NEM), a known thiol-blocking agent, to the cell culture prior to the addition of **1a** led to almost no fluorescence. The result exhibits that **1a** is cell-permeable and it can make turn-on detection for Cys in living cells possible.

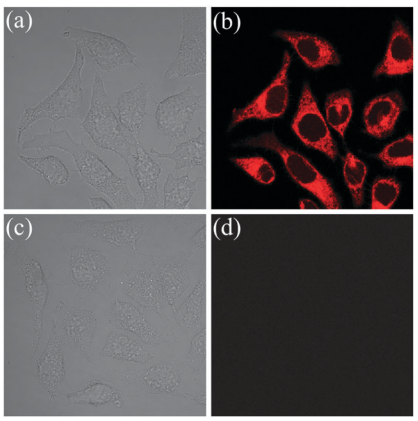


Fig. 4 Confocal fluorescence and bright-field images of living HeLa cells: (a) bright field and (b) fluorescence images of HeLa cells pretreated with 500 μM Cys for 15 min and then incubated with **1a** (5 μM) for 15 min; (c) bright field and (d) fluorescence images of HeLa cells pretreated with 500 μM NEM for 15 min and then incubated with **1a** (5 μM) for 15 min.

In conclusion, we have developed a highly sensitive and selective turn-on fluorescent sensor for the discrimination of Cys from Hcy and GSH. The nitrothiophenol or nitrophenol group can be substituted by thiolate and result in turn-on fluorescence. The discrimination of Cys from Hcy and GSH is achieved through more rapid intramolecular displacement of sulfur with the amino group of Cys rather than that of Hcy and GSH. The interesting reaction mechanism may provide new insight for designing highly selective and sensitive fluorescent sensors for the detection of Cys.

We are grateful for financial support by 973 Program (2013CB933800), the National Natural Science Foundation of China (21222210, 21102155, 21072202), and the Chinese Academy of Sciences (100 Talents Program). We thank Prof. P. Wang (TIPC, CAS) for providing HeLa cells.

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