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A multi-emissive fluorescent probe for the discrimination of glutathione and cysteine

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ABSTRACT

Glutathione (GSH) and cysteine (Cys) play different roles in biological systems, thus the discrimination between them is of great importance. Herein we report a multi-emissive fluorescent probe for the selective detection of GSH and Cys. The probe was composed of covalently linked BODIPY and coumarin fluorophores. The BODIPY fluorophore was designed to react with GSH and Cys and generate different products with distinct photophysical properties, and the coumarin fluorophore acted as an internal standard. The probe exhibited green emission in aqueous solution. Upon addition of Cys, it yielded nitrogen-substituted BODIPY with weak fluorescence and free coumarin with blue emission. In the presence of glutathione, it generated mono- and di-sulfur substituted BODIPY and coumarin, resulting in various emission colors at different concentrations of GSH. Interestingly, the solution exhibited white fluorescence at GSH concentration of 0.4 mM. The probe was capable of detecting and imaging GSH and Cys in living HeLa cells, indicating its significant potential in biological applications.

1. Introduction

Biothiols play crucial roles in maintaining biological redox homeostasis in biological systems (Peng et al., 2012). Especially glutathione (GSH) and cysteine (Cys) are two most abundant low-molecular-weight thiols in living cells. It has been demonstrated that abnormal levels of biothiols are associated with certain diseases. The deficiency of Cys can cause slowed growth, edema, lethargy, liver damage, muscle and fat loss, skin lesions, and weakness, etc (Lipton et al., 2002). The ratio of free GSH to its oxidized state glutathione disulfide is an indicator for the corresponding enzyme activity and the redox state of the cell, and the alteration of the ratios can lead to human pathologies such as heart disease, cancer, strokes, and many neurological disorders (Monostori et al., 2009; Forman et al., 2009). Consequently, the assessment of abnormal levels of thiol-containing substances in biological system may provide valuable information for early diagnosis of some diseases.

Fluorescent probes are considered to be an effective molecular tool that monitor and visualize intracellular analytes (Zhou et al., 2015). Great efforts have been devoted to develop reaction based fluorescent sensors for the detection of thiols in living systems (Niu et al., 2015a; Jung et al., 2013; Wang et al., 2014b). Most of the sensors were able to distinguish biothiols from other amino acids by taking advantage of the strong nucleophilic ability of thiols (Chen et al., 2010). However, the discriminations between biothiols are hampered on account of their similar structures and reactivity. Given that the levels of Cys, Hcy and GSH are related to different physiological processes and diseases, fluorescent sensors that can discriminate between them are of great importance and have attracted particular interest of researchers.

Lately, considerable efforts have been made to develop fluorescent sensors with selective response to Cys, Hcy, or GSH (Niu et al., 2015a). For instance, the selective detections of Cys/Hcy were usually performed based on the strategy of cyclization with aldehydes (Rusin et al., 2004; Wang et al., 2012; Barve et al., 2014) and conjugate addition–cyclization with acrylates (Yang et al., 2011; Niu et al., 2016; Han et al., 2015; Guo et al., 2012). On the other hand, the discriminations of GSH from Cys were achieved using aromatic substitution-rearrangement reactions, etc (Niu et al., 2012; Wang et al., 2014c; Lim et al., 2014; Niu et al., 2015b; Işık et al., 2014). To clarify the relationship between these biothiols in complicated physiological processes, the development of a single fluorescent probe that can simultaneously selectively sense two or three biothiols is highly attractive (Liu et al., 2014; Jia et al., 2015; Niu et al., 2015a).

Herein, we reported a fluorescent probe **BC** for the simultaneous detection of Cys and GSH based on the combination of aromatic

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substitution-rearrangement and dual-fluorophore fragmentation strategy. Coumarin, served as an internal standard fluorophore, was covalently linked to BODIPY core. After the reaction with Cys, **BC** yielded free coumarin and nitrogen-substituted BODIPY with low fluorescence; in the presence of GSH, **BC** generated coumarin and highly fluorescent disulfur-substituted BODIPY, thus distinct fluorescence spectra were obtained. In particular, white fluorescence was observed at the GSH concentration of 0.4 mM. **BC** was successfully used for the selective detection of GSH and Cys in living cells.

2. Experimental section

2.1. Materials and instruments

All the reagents and solvents are of commercial quality and without further purification. ^{1}H and ^{13}C NMR spectra were recorded on an Advance Bruker 400 M spectrometer and referenced to solvent signals. Mass spectra were obtained on a Bruker Apex IV Fourier Transform Mass Spectrometer. Fluorescence spectra were determined on a Hitachi 4500 spectrophotometer. Absorption spectra were determined on a Shimadzu UV-1601PC UV-Visible spectrophotometer. HPLC analysis was performed on a Hitachi ELITE LaChrom system with Silica gel column (YMC-Pack SIL) in normal phase (eluent: CH_2Cl_2/MeOH =90/10). The absorbance at 500 nm was monitored for detection.

2.2. Cell culture and fluorescence imaging

HeLa cells were cultured in culture media (DMEM/F12 supplemented with 10% FBS, 50 unit/mL penicillin, and 50 μg/mL of streptomycin) at 37 °C under a humidified atmosphere containing 5% CO_2 for 24 h. The cells were seeded in a 6-well plate at a density of 104 cells per well in culture media. The cells were treated with 1 mM GSH or 500 μM Cys in culture media for 15 min at 37 °C in a humidified incubator. After washing with PBS, the cells were further incubated with 5 μM of **BC** in culture media for 15 min. For the control experiment, the cells were treated with 1 mM N-ethylmaleimide (NEM) in culture media for 30 min at 37 °C in a humidified incubator. After washing with PBS, the cells were further incubated with 5 μM of **BC** in culture media for 15 min. Confocal fluorescence imaging was performed with Nikon A1R MP multiphoton microscopy with a 100× oil-immersion objective lens. Blue fluorescence was excited at 405 nm and emission was collected by a 425–475 nm band pass filter. Red fluorescence was excited at 561 nm and emission was collected by a 570–620 nm band pass filter.

2.3. Synthesis

BODIPY-Cl$_2$ (350 mg, 1 mmol) was dissolved in acetonitrile (50 mL), and followed by the addition of 7-hydroxycoumarin (162 mg, 1 mmol) and one drop of trimethylamine. The resulting solution was stirred at room temperature for 30 min, and then the solvent was evaporated under reduced pressure. The residue was purified by chromatography on silica gel (dichloromethane / petroleum ether =1/1 as a eluent) to give **BC** as an orange solid (300 mg, 63%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.72 (d, 2 h, *J*=9.6 Hz), 7.55 (d, 2 h, *J*=8.4 Hz), 7.40 (d, 2 h, *J*=7.6 Hz), 7.32 (d, 2 h, *J*=7.6 Hz), 7.27 (s, 1 h), 7.23 (d, 2 h, *J*=8.8 Hz), 6.92 (d, 1 h, *J*=4.4 Hz), 6.76 (d, 1 h, *J*=4.0 Hz), 6.43 (d, 1 h, *J*=9.6 Hz), 6.37 (d, 1 h, *J*=4.0 Hz), 5.91 (d, 1 h, *J*=4.4 Hz), 2.46 (s, 3 h). ^{13}C NMR (100 MHz, $CDCl_3$): 164.33, 160.00, 156.71, 155.11, 143.23, 142.66, 141.10, 140.31, 133.69, 132.28, 130.46, 129.80, 129.60, 129.50, 129.27, 128.76, 116.85, 116.68, 116.24, 108.53, 105.84, 21.45. ESI-HRMS: calculated for $[M+H]^+$ 477.09877, found 477.09925. HPLC (eluent CH_2Cl_2/MeOH =90/10): 3.17 min

3. Results and discussion

3.1. Design rationale

We developed the BODIPY-based fluorescent probe to selectively detect GSH over Cys/Hcy based on an aromatic substitution-rearrangement reaction (Niu et al., 2012), which have become a widely used strategy for the discrimination between GSH and Cys/Hcy (Wang et al., 2014c; Wang et al., 2014a; Liu et al., 2016; Zhang et al., 2016; Liu et al., 2015; Pan et al., 2015; Kim et al., 2015; Gao et al., 2015; Wang et al., 2015). However, in the previous work, in the presence of Cys, the resulting nitrogen-substituted product shows relatively weak fluorescence, which made the probe not suitable for the simultaneous detection of Cys and GSH in living system. Therefore, we introduced a covalently linked coumarin as the internal standard fluorophore (Hammers et al., 2014; Chen et al., 2016; Hu et al., 2016). We anticipated that, upon addition of Cys, free coumarin would be released, and the internal standard signal from coumarin would be observed other than the weakly fluorescent nitrogen-BODIPY. On the other hand, the probe would generate mono- or di-sulfur substituted BODIPY in the presence of different concentrations of GSH, and various emissions would be achieved for the quantitative detection of GSH.

Probe **BC** was conveniently synthesized by treatment of 3,5-dichlorinated BODIPY with hydroxycoumarin as nucleophile in acetonitrile at room temperature with yield of 63%. Compound **BC** was well characterized by ^{1}H NMR and ^{13}C NMR spectroscopy and HRMS. Control compounds **BC-N**, **BC-S** and **BC-2S** were also synthesized for the mechanism study (Scheme 1).

3.2. Optical properties of *BC*

To evaluate the suitability of our design strategy of **BC** as a fluorescent probe for biothiols, we first tested the time-dependent absorption and emission response of **BC** in the presence of 5 mM Cys, Hcy and GSH. As shown in Fig. 1 and Figure S1, probe **BC** itself displayed an orange color with an absorption peak at 508 nm. Upon addition of Cys, a broad blue-shifted absorption peak at 474 nm and an absorption peak at 327 nm emerged, and the solution color changed to yellow. By contrast, in the presence of GSH and Hcy, the original absorption red shifted to 536 nm and 570 nm with different intensities, with the emergent of a new absorption peak at 327 nm. It should be noticed that a weak absorption at around 470 nm was also observed 1 h after upon addition of Hcy (Figure S1e). In the fluorescence spectra, the free **BC** showed an emission peak at 528 nm with green fluorescence. It displayed a strong emission at 450 nm and a weak fluorescence at 534 nm in the presence of Cys, and the emission color turned to blue. However, upon addition of GSH, three emission peaks at 450 nm, 558 nm and 588 nm was observed. Probe **BC** manifested a much stronger fluorescence at 588 nm in the presence of Hcy than GSH, thus their solutions exhibited different emission colors. The solution of probe **BC** exhibited distinct absorption and emission spectra 1 h after the addition of Cys, Hcy and GSH, thus enabling their discrimination even by naked eyes (Fig. 1).

3.3. Reaction mechanism of *BC* towards thiols

In order to explore the reaction mechanism, control reactions of **BC** with *n*-butylamine or methyl mercaptoacetate as model compounds were performed. Probe **BC** was mixed with *n*-butylamine to give products of amino BODIPY (**BC-N**) and coumarin, while the reaction of **BC** with methyl mercaptoacetate generated mono- and di-sulfur BODIPY (**BC-S** and **BC-2 S**) and coumarin. The products were characterized by ^{1}H NMR spectroscopy (See Supporting Information). Their absorption spectra matched well with the products of **BC** with Cys, Hcy or GSH (Fig. S2). Based on above observations and