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COMMUNICATION

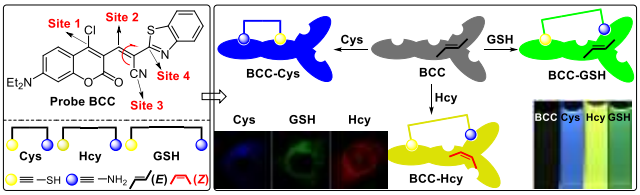
A Multi-signal Fluorescent Probe with Multiple Binding Sites for Simultaneous Sensing of Cys, Hcy and GSH

Guo-xing Yin, Ting-ting Niu, Ya-bing Gan, Ting Yu, Peng Yin, Hai-min Chen,* You-yu Zhang, Hai-tao Li* and Shou-zhuo Yao*

Abstract: A novel fluorescent probe by integrating chlorinated coumarin and benzothiazolylacetonitrile was exploited for simultaneous detection of Cys, Hcy and GSH. Featured with four binding sites and different bonding mechanism between probe and biothiols, this probe exhibited rapid fluorescence turn-on for distinguishing Cys, Hcy and GSH with 108, 128, 30-fold fluorescence increase at 457, 559, 529 nm, respectively, through different excitation wavelengths. Furthermore, the probe was successfully applied to the fluorescence images of cellular Cys, GSH and exogenous Cys, Hcy and GSH in living cells.

Small molecule thiols play vital roles in many physiological processes and are closely related to a lot of diseases.[1] Cysteine (Cys), homocysteine (Hcy) and glutathione (GSH) are commonly abundant and the most important biological thiols. Though they have the similar structures and reactivity, the roles played by Cys, Hcy and GSH are totally different. Cys is an essential amino acid for the synthesis of proteins and abnormal level of Cys is related to edema, slowed growth in children, lethargy, cardiovascular diseases, liver damage, etc.[2] The role of Hcy in diseases still remains a highly contentious topic.[3] And its normal concentration in serum is approximately 5-15 μM and abnormal total homocysteine might cause cognitive impairment in the elderly.[4] GSH is well-known to be associated with a series of disorders including cancer, Alzheimer's and other ailments.[5] Considering the important biological roles of biothiols and the relationship of many diseases with their concentrations' changes (the intracellular concentration for Cys: 30-200 μM;[6] for GSH: 1-10 mM[5]), it is of great value to develop effective methods for real-time simultaneous monitoring of intracellular Cys, Hcy and GSH, respectively, in biological systems.

High-performance liquid chromatography (HPLC), mass spectrometry (MS), capillary electrophoresis and HPLC-MS/MS have been applied to the detection and determination of specific biothiols.[7] However, owing to their non-invasiveness, high selectivity, high sensitivity, relative low cost and operational simplicity, a large number of fluorescent probes have been developed to detect and sense biothiols recently.[8] Despite of similarity among the structures and properties of Cys, Hcy and GSH, several fluorescent probes that allow for selective detection of two species in one time have been reported.[9] But, it is more challenging to discriminate the three thiols from each other simultaneously. Guo and co-workers have developed a chlorinated coumarin-hemicyanine probe with three potential reaction sites for simultaneous detection of Cys and GSH from different emission channels.[10] Though the signaling promoted by Hcy was poor and 60 min was needed for the sensing, their work exhibits an excellent perspective for simultaneous Cys/Hcy/GSH detection. Liang and co-workers reported a sulfonamide-based self-quenched fluorescent probe for simultaneously detecting biothiols.[11] And each product for the probe with Cys/Hcy/GSH exhibited different emission maxima, but the difference between the emissions from probe with Cys and Hcy was very small (5 nm) and it was required 2 h at 37 °C for the detection. In 2016, Sun and partners discovered a commercial fluorescent probe (2,3,5,6-tetrafluoro-terephthalonitrile) for differentiating Cys, Hcy and GSH in living cells.[12] Though the probe has four potential reaction sites for detecting biological thiols, long sensing time (up to 2 h) was needed to reach an optimal signal and the addition of CTAB (cetyl trimethylammonium bromide) was required to discriminate Hcy from GSH, which are significant barriers to the simultaneous monitoring of Cys, Hcy and GSH in living cells. Simultaneous analysis of biothiols (Cys/Hcy/GSH) in vivo/vitro from different emission channels by single molecule fluorescent probe is still quite rare, which is highly valuable but even more challenging. Herein, we presented a novel fluorescent probe with four potential reaction sites for simultaneous and rapid sensing of Cys, Hcy and GSH from three emission channels, which based on different reaction mechanisms between probe and biothiols (Scheme 1). This probe was capable of not only fluorescence imaging of exogenous Cys, Hcy and GSH but also simultaneous detection of endogenous Cys and GSH from different emission channels in living cells. And it has great potential to real-time simultaneous and quantitative monitoring of cellular Cys, Hcy and GSH, which will greatly promote the clarification of their complicated relationship and functions in various physiological processes.



Scheme 1. Simultaneous Sensing of Cys, Hcy and GSH Based on Four-Binding Sites of Probe **BCC**.

Probe **BCC** we have rationally designed has four potential reaction sites, using coumarin as a fluorophore (Scheme 1). The chloro atom in the 4-position of the coumarin moiety (site 1) is

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reactive and facilitates the thiol-halogen S_NAr nucleophilic aromatic substitution between probe **BCC** and thiols. And α, β-unsaturated bond is a well-known Michael acceptor (site 2). The cyano group could be utilized as an electrophile (site 3), and under the effect of the cyano group, the unsaturated bond in the benzothiazolium moiety could be used to another reaction site (site 4) for discriminating biothiols. On the other hand, the steric distance between thiol group and amino group in biothiols should be: Cys < Hcy < GSH (Scheme S1). A probe with four potential reaction sites in different steric distances would be possible to discriminate similar biothiols.

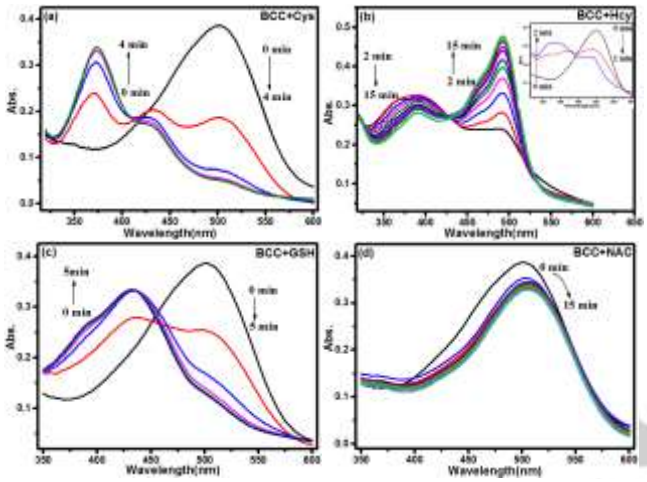

Figure 1. (a−d) Time-dependent absorption spectra of **BCC** (10 μM) in the presence of 10 equiv. of Cys, Hcy, GSH and NAC in DMSO-PBS (pH 7.4, 10 mM, v/v, 6/4) at r.t.

Initially, we synthesized probe **BCC** (Supporting Information) and examined its sensing behavior towards Cys/Hcy/GSH by utilization of time-dependent UV-vis and fluorescence spectra in DMSO-PBS (pH 7.4, 10 mM, v/v, 6/4) at room temperature. As shown in Figure 1, the UV−vis spectra of free **BCC** showed a main absorption at 500 nm (ε = 3.86 × 10^4 L mol^{-1} cm^{-1}, quantum yield (QY) = 0.003, with Rhodamine B as reference). Upon addition of Cys, the initial absorption peak at 500 nm decreased dramatically while a new peak at 374 nm appeared with a 126 nm blue shift (Figure 1a) (details in Figure S1), which indicated that the conjugation between the coumarin and benzothiazolylacetonitrile is broken due to the Michael addition of Cys to site 2 in probe **BCC**. The product BCC-Cys with a seven-membered ring (QY = 0.313, with quinine sulfate as reference) might be yielded shortly (Scheme S3a). Correspondingly, a significant fluorescence turn-on with 108-fold enhancement at 457 nm was observed in time-dependent fluorescence spectra at 360 nm excitation within 15 min, but there was only a slight fluorescence enhancement for Hcy (3.5-fold), GSH (11-fold) and NAC (N-Acetylcysteine) (1.2-fold) under the same condition (Figures 2a and 2b, details in Figures S5-S6).

For Hcy, the time-dependent UV-vis spectrum is similar to Cys in the beginning 2 min, along with the decrease of the initial absorption peak at 500 nm and a simultaneous increase of a new broad absorption peak at 391 nm (insert, Figure 1b). After that, the absorption at 391 nm showed a slight decrease and a new peak at 492 nm increased slowly and reached a plateau after 15 min (details in Figure S2). The absorption at 391 nm would be assigned to the intermediate, which was further transferred to the final product BCC-Hcy (QY = 0.474, with Rhodamine B as reference). The configuration changes (from *E* to *Z*) of the intermediate might happen, which would be favorable for the addition of free amine group to the cyano group (Scheme S3b). And supported by the fluorescence color changes and the time-dependent fluorescence spectra at 360 nm excitation, the slight emission at 444 nm was decreased slowly after 2 min and emission at 559 nm was enhanced (Figure S6). More importantly, a large fluorescence turn-on at 559 nm with 128-fold enhancement was observed (λ_{ex} = 480 nm) (Figures 2c and 2d), and the fluorescence enhancement at 559 nm (λ_{ex} = 480 nm) for Cys (2.4-fold), GSH (13.7-fold) and NAC (0.2-fold) could be neglected (details in Figure S7).

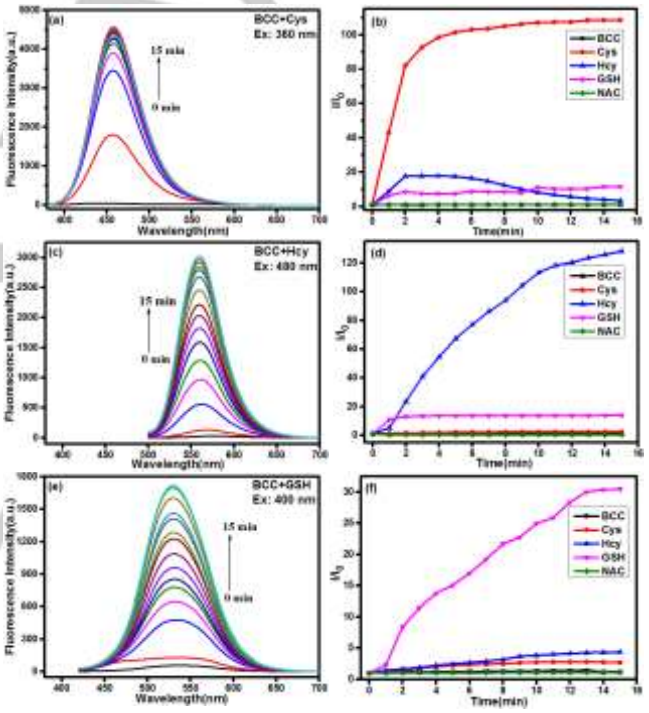

Figure 2. Time-dependent fluorescence spectra of **BCC** (10 μM) in the presence of (a) 10 equiv. of Cys excited at 360 nm, (c) 10 equiv. of Hcy excited at 480 nm, (e) 10 equiv. of GSH excited at 400 nm, respectively. Time-dependent fluorescence intensity changes toward 10 eq. of biothiols excited at (b) 360 nm, (d) 480 nm and (f) 400 nm respectively. Condition: DMSO-PBS (pH 7.4, 10 mM, v/v, 6/4) at room temperature. Slit (nm): 2.5/2.5.

We continued to investigate the performance of GSH. As shown in Figure 1c (details in Figure S3), initial absorption peak at 500 nm decreased dramatically after the addition of GSH, and a new absorption peak was found at 432 nm with a 68 nm blue shift, which indicated that the nucleophilic addition of the amine group in GSH to site 4 in probe **BCC** (Scheme S3c). As a model compound of amino-free thiols, NAC was also tested and the initial absorption decreased slightly probably due to a thioether substitution (Figure 1d, Scheme S3d) (details in Figure S4). The

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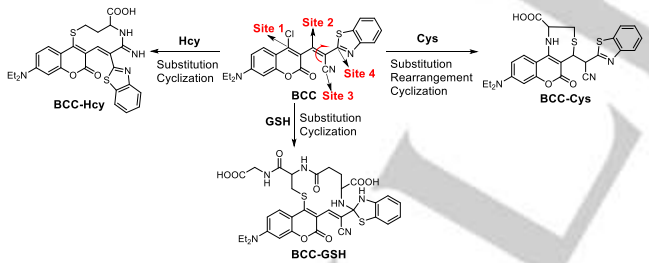
time-dependent fluorescence spectra for probe with GSH (λ_{ex} = 360 and 480 nm) exhibited weak fluorescence (Figures S5 and S7), but a 30-fold fluorescence enhancement at 529 nm was achieved (λ_{ex} = 400 nm) (QY = 0.097, with quinine sulfate as reference). While, Cys, Hcy and NAC displayed less than 5-fold fluorescence enhancement at 529 nm (Figures 2e and 2f, details in Figure S8). Furthermore, probe **BCC** exhibited little fluorescence (λ_{ex} = 360, 400 and 480 nm, respectively) and was stable for a long time (Figure S9).

Taken together, the results from spectral studies demonstrated that probe **BCC** could distinguish Cys, Hcy and GSH by different fluorescence signals, which showed excellent performances for sensing biothiols (Table S1). Encouraging by these results, we further evaluated the selectivity of probe **BCC** over other biologically related species, including various amino acids and representative anions (Figures S10-S12). Only Cys, Hcy and GSH could promote obvious fluorescence intensity enhancement at 457, 559 and 529 nm (λ_{ex} = 360, 480 and 400 nm, respectively) with short time (15 min), confirming excellent selectivity of probe **BCC** for Cys, Hcy and GSH. Furthermore, results from the detection of Cys, Hcy and GSH in the mixture of the three biothiols demonstrated that probe **BCC** could be applied to simultaneously sensing of Cys, Hcy and GSH in complex biological environments (Figures S16-S23).

In addition, the fluorescence titration experiments under the same conditions were conducted. As shown in Figures S13-S15, an excellent linear from 0-3 equiv. of Cys was obtained and the detection limit was calculated to be as low as 0.5 nM based on S/N = 3. The fluorescence intensities were linearly proportional to the amount of Hcy and GSH from 0 to 2 equiv., and the detection limits were calculated to be as 3.6 nM and 6.9 nM, respectively. In view of these results, probe **BCC** could be applied to sense intracellular biothiols.



Scheme 2. Proposed mechanisms of probe **BCC** with Cys, Hcy and GSH.

Next, to further investigate the reaction mechanism between probe **BCC** and Cys/Hcy/GSH, products of BCC-Cys, BCC-Hcy and BCC-GSH were observed in the corresponding HRMS titration experiments (Figures S38-S40). Though the isolation of product BCC-Cys and BCC-GSH was failed, the product BCC-Hcy between probe **BCC** and Hcy was successfully obtained, which was well characterized. So we could propose the reaction mechanism between probe **BCC** with biothiols (Scheme 2, details in Scheme S3), which was based on spectral studies and NMR analyses (Figures S24-S25; S36-S47). As four binding sites in the probe and the differences from the structure of Cys, Hcy and GSH, there compounds with different photophysical properties would be yielded after the reactions between probe **BCC** with Cys, Hcy and GSH respectively. Further mechanism studies are still in progress in our lab.

Subsequently, the capabilities of probe **BCC** to selectively sense exogenous and intercellular Cys, Hcy and GSH were evaluated. Firstly, the results from MTT assays showed that probe **BCC** has good cell permeability and low cytotoxicity to living cells within a short time (Figure S26). For exogenous biothiols, the cells were firstly pretreated with NEM and cellular biothiols and SH-containing proteins were deactivated. After incubation with probe **BCC**, almost no fluorescence could be observed (Figure S27, A1-A3). While, after subsequently treated with Cys, Hcy and GSH respectively, blue, red and green fluorescence were observed from three different emission channels in living cells with high selectivity (Figure 3, C1-C3; Figure S27). Fluorescence images for BEL-7402 cells with different concentrations of exogenous Cys (or GSH) were also conducted, the differences in fluorescence intensities indicated the different concentrations of Cys/GSH in living cells (Figure S28).

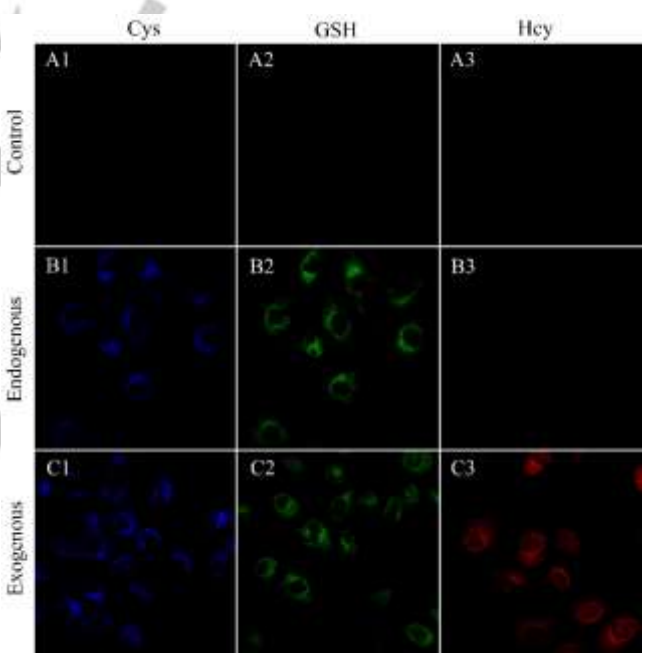


Figure 3. Confocal fluorescence images of Cys, GSH and Hcy in BEL-7402 cells. (A1−A3) Cells were incubated for 30 min, then imaged. (B1−B3) Cells were incubated with probe **BCC** (2.5 μM) for 30 min, then imaged. (C1−C3) Cells were pretreated with NEM (0.5 mM, 30min), subsequently incubated with Cys/GSH/Hcy (500 μM, 30 min) and probe **BCC** (2.5 μM, 30 min), then imaged (λ_{ex} = 405 nm, λ_{em} = 421-475 nm for the blue channel; λ_{ex} = 458 nm, λ_{em} = 500-550 nm for the green channel; and λ_{ex} = 543 nm, λ_{em} = 552-617 nm for the red channel). Scale bar: 20 μm.

For the images of cellular biothiols, cells incubated without probe **BCC** exhibited almost no fluorescence (Figure 3, A1-A3). After incubation with probe **BCC** for 30 min, BEL-7402 cells exhibited different blue fluorescence (from blue channel) for Cys detection and green fluorescence (from green channel) for GSH detection. And almost no fluorescence (from red channel) was

observed for Hcy, which may be due to the concentration of Hcy in living cells is very small (Figure 3, B1-B3). Fluorescence images for cellular Cys and GSH in different kinds of cells were further performed (Figure S29). Thus, this probe could be further applied to the real-time quantitative monitoring of cellular Cys and GSH.

In summary, we have designed and synthesized a novel chlorinated coumarin-benzothiazolium fluorescent probe **BCC** with four binding sites, which could simultaneously and selectively detect Cys, Hcy and GSH from three different emission channels. The fluorescence increments of probe **BCC** for Cys, Hcy and GSH at 457, 559 and 529 nm are 108, 128 and 30-fold increase, respectively (λ_{ex} = 360, 480, and 400 nm) based on different bonding mechanism, with high selectivity and high sensitivity. Furthermore, fluorescence imaging studies in different living cells demonstrated that probe **BCC** is able to simultaneously monitor endogenous Cys and GSH and exogeneous Cys, Hcy and GSH via multicolor imaging. This study would strongly improve the further exploration about biothiols' dynamics and their function in biological systems and promote the research of biothiols in biomedicine and diagnostics.

Acknowledgements

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Keywords: multi-signal • multiple binding sites • fluorescent probe • simultaneous sensing • biothiols

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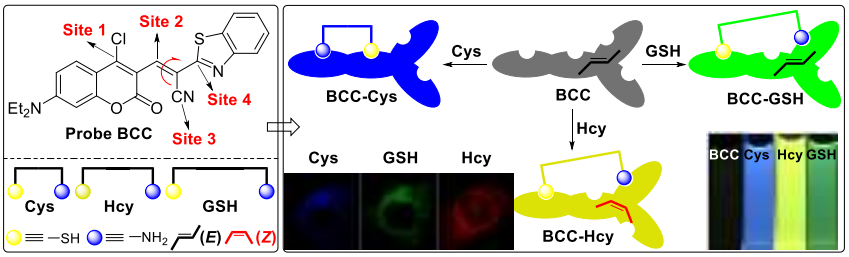

A multi-signal fluorescent probe was exploited for simultaneously distinguishing and sensing of cysteine, homocysteine and glutathione in living cells.

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