

Simultaneous Fluorescence Sensing of Cys and GSH from Different Emission Channels

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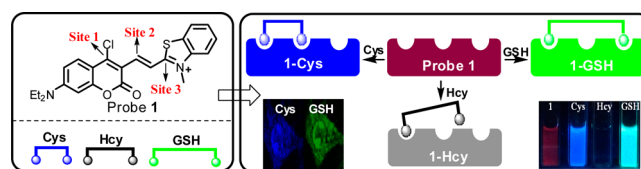
S Supporting Information

ABSTRACT: A chlorinated coumarin-hemicyanine dye with three potential reaction sites was exploited as fluorescent probe for biothiols. The Cys-induced substitution–rearrangement–cyclization, Hcy-induced substitution–rearrangement, and GSH-induced substitution–cyclization cascades lead to the corresponding amino-coumarin, amino-coumarin-hemicyanine, thiol-coumarin with distinct photophysical properties, enabling Cys and GSH to be selectively detected from different emission channels at two different excitation wavelengths.

Since the fluorescent indicators for calcium ion were reported by Tsien in the early 1980s,¹ fluorescent probes have been recognized as the efficient molecular tools that can help monitor and visualize trace amounts of samples in live cells or tissues because of its high sensitivity and high spatiotemporal resolution.² Given that biothiols, including cysteine (Cys), homocysteine (Hcy), and glutathione (GSH), play crucial roles in many physiological processes and are closely related to many diseases,³ a large number of fluorescent probes have been developed in recent years to detect and sense these biologically important species.⁴ However, although these probes can highly selectively distinguish these biothiols from other amino acids, most of them can not distinguish Cys/Hcy/GSH from each other due to the similar structures and reactivity of these biothiols. In fact, the discrimination between them has been a focal point and also a tough challenge for researchers, albeit some advances have been obtained.

Pioneered by Strongin's group, the selective detection of Cys/Hcy over GSH could be realized based on the cyclization of Cys/Hcy with aldehydes^{5a} or acrylates.^{5b} From then on, some more specific probes for Cys or Hcy were developed based on the extended version of the two strategies.^{6,7} Very recently, the discrimination of Cys from Hcy/GSH was also achieved by taking advantage of either the Cys-induced S_NAr substitution–rearrangement reaction⁸ or Michael addition combined with steric and electrostatic interactions.⁹ By comparison, the discrimination of GSH from Cys/Hcy was addressed in relatively less extent, and as far as we know, only several examples were reported.¹⁰ One of attractive strategy, reported very recently, is based on the thiol-halogen S_NAr substitution reaction between GSH and a chlorinated Bodipy,^{10c} which is distinct from Cys/Hcy-induced S_NAr substitution–rearrangement cascade reaction, thereby enabling GSH to be highly selectively discriminated from Cys/Hcy.

Noteworthy is that while the above results represent the important advances in this field, almost all of them can selectively sense only one of these biothiols in one time except that reported by Strongin.^{5b} In fact, to clarify the complicated relationship between these biothiols in various physiological processes, the development of small molecule fluorescent probe that can selectively sense two or all three of them simultaneously from different emission channels is highly valuable but even more challenging. Herein, we present a novel discrimination strategy for biothiols based on three potential reaction sites of a chlorinated coumarin-hemicyanine¹¹ fluorescent probe **1** (Scheme 1). The probe could not only

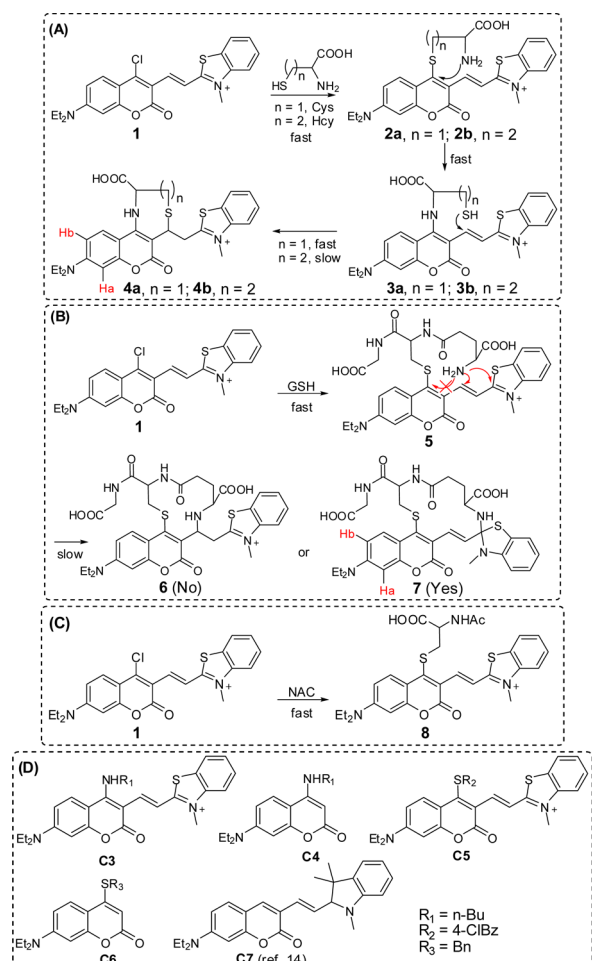
Scheme 1. Simultaneous Sensing of Cys and GSH Based on Three Potential Reaction Sites of **1**

discriminate Cys (or GSH) from Hcy/GSH (or Cys/Hcy) but also simultaneously detect Cys and GSH from different emission channels and thus holds great potential in biological applications.

The design rationale is depicted in Scheme 2 and illustrated as follows. Site 1 lies in 4-position of coumarin moiety of **1**, which is doubly activated and thus is reactive.¹² Moreover, a chloro leaving group in the site would enable the thiol-halogen S_NAr nucleophilic substitution between **1** and thiols. Thus, it was expected that the chloro group of **1** could initially be replaced by thiol group of Cys (or Hcy) to produce thio-coumarin-hemicyanine **2a** (or **2b**), and the following rearrangement would lead to amino-coumarin-hemicyanine **3** (Scheme 2A).^{10c} However, if the reaction only stops at this stage, it appears to be difficult to discriminate between Cys and Hcy because products **3a** and **3b** were expected to have similar photophysical properties, thereby necessitating an additional discriminating factor in the molecule, here that is site 2 (a Michael receptor). We speculated that an intramolecular cyclization between the thiol group and the adjacent site 2 in **3** would lead to amino-coumarin **4**. If so, it would be possible to

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Scheme 2. (A–C) Proposed Reaction Mechanisms of Probe 1 with Cys, Hcy, GSH, and NAC (N-Acetylcysteine) and (D) Control Compounds C3–C7 of 3–7



distinguish Cys and Hcy because the reaction to form a seven-membered ring (for **4a**) should be kinetically favored relative to the formation of eight-membered ring (for **4b**).^{5b} In other words, the reaction of **1** with Cys would ultimately produce **4a** and with Hcy would mainly stay at the stage of **3b**. Now, let us turn our attention to the reaction of **1** with GSH. Similarly, the initial thiol-halogen S_NAr nucleophilic substitution would lead to thio-coumarin-hemicyanine **5** (Scheme 2B). However, due to the unstable 10-membered macrocyclic transition state, it is difficult for **5** to undergo the aforementioned intramolecular rearrangement to produce the corresponding amino-coumarin-hemicyanine.^{10c} Alternatively, it is possible that the free amino group in **5** would attack sites 2 or 3 to produce the 12- or 14-membered ring products thio-coumarin **6** or **7**. In fact, the speculation could be partially supported by a recent publication on a large ring product that resulted from the reaction of GSH with acrylate.^{10b} Overall, if our working hypothesis is rational, it would be promising to realize the selective discrimination of Cys, Hcy, and GSH in terms of the different chemical structures and thus the distinct photophysical properties of the corresponding amino-coumarin **4a**, amino-coumarin-hemicyanine **3b**, and thiol-coumarin **6** or **7**.

To test the above-mentioned possibility, we synthesized probe **1** (Supporting Information) and examined its reactivity toward Cys/Hcy/GSH through time-dependent UV–vis

spectra in PB buffer (10 mM, pH 7.4, containing 1 mM CTAB)¹³ at 25 °C. As shown in Figure 1A (details in Figure

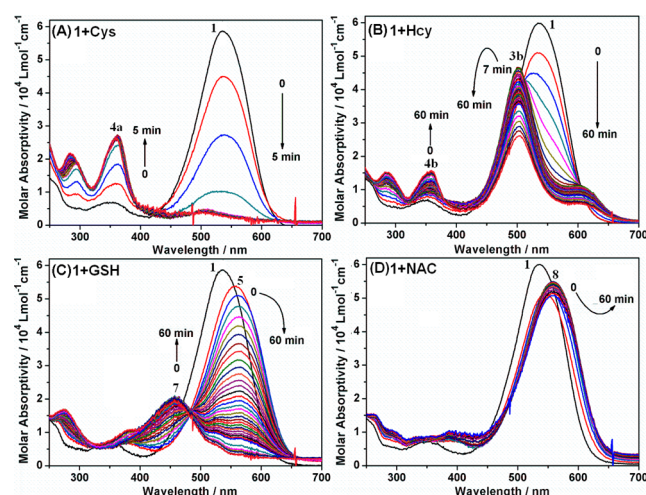


Figure 1. (A–D) Time-dependent absorption spectra of **1 (2 μM) in the presence of 10 equiv of Cys, Hcy, GSH, and NAC in PB buffer (10 mM, pH 7.4, 1 mM CTAB) at 25 °C.**

S1), the UV–vis spectra of free **1** showed a main absorption at 535 nm ($\epsilon = 5.87 \times 10^4 \text{ L mol}^{-1} \text{ cm}^{-1}$; $\Phi_f = 0.012$ with rhodamine B as reference), characteristic for coumarin–hemicyanine dyes. Upon addition of Cys, the initial absorption peak decreased gradually, along with the simultaneous emergence of the blue-shifted new peak at 360 nm. Such a big blue shift in absorption wavelength indicates that the above reaction breaks the π -conjugation of **1**. According to the aforementioned speculation, the absorption at 360 nm should be assigned to **4a**, which was supported by control compound **C4**, whose absorption maximum was found at around 360 nm (Figure S5A). However, in the titration process we did not observe the corresponding absorption peaks of intermediates **3a** and **2a**, presumably due to the fast reaction kinetics. However, their traces could be indicated in the subsequent time-dependent UV–vis spectra of **1** upon addition of Hcy, GSH and NAC, respectively.

As shown in Figure 1B (details in Figure S2), addition of Hcy to the solution of **1** led to the decrease of the initial absorption peak at 535 nm, followed by a simultaneous increase of a new absorption at 500 nm; after that, the absorption at 500 nm decreased very slowly, along with a slight increase of the absorption at 360 nm. As the case of Cys, the poor absorption at 360 nm could be assigned to **4b**. However, the blue-shifted absorption at 500 nm should be assigned to amino-coumarin-hemicyanine **3b**, which was then transformed to **4b** considerably slowly due to kinetically unstable 8-membered ring transition state. The assign could be supported by control compound **C3** (Abs_{max} : ca. 500 nm, Figure S5B).

Next, let us see what is about GSH. As shown in Figure 1C (details in Figure S3), addition of GSH to the solution of **1** initially led to a red-shifted absorption at 558 nm, which then decreased gradually along with the simultaneous emergence of the blue-shifted absorption at 450 nm. The absorption at 558 nm could be assigned to thio-coumarin-hemicyanine **5**, which was supported by either the control compound **C5** (Abs_{max} : ~558 nm) or the reaction of **1** with NAC (Figure S5D). Note that the absorption at 450 nm, which is longer than that of amino-coumarin **4** (360 nm) but shorter than that of amino-

coumarin-hemicyanine **3** (500 nm) was initially assigned to thio-coumarin **6** by us. However, the assignment did not match the absorption spectrum of the corresponding control compound **C6** (Abs_{max} : 385 nm, Figure S5C). Thus, we speculated that thio-coumarin **7**, which has a more extended π conjugation than **6**, may be the desired product, although its absorption maximum was ~ 40 nm longer than that of control compound **C7**¹⁴ likely due to a thioether substitution-induced red shift.¹⁵

Taken together, the above experimental results are in good agreement with our proposed reaction mechanisms. In addition, adducts **4a**, **3b**, and **7** were also observable in the corresponding HRMS titration experiments (Figure S6). We also performed ¹H NMR titration studies. It was found that upon addition of Cys or GSH to **1**, the coumarin protons H_a and H_b displayed the obvious upfield shifts when compared with those of coumarin-hemicyanine dyes **1**, **C3**, and **C5** (Figure S7). This is in fact consistent with our proposed cyclization reactions (Scheme 2A,B), which break the conjugation between 7-diethylamino-coumarin moiety and electron-withdrawing benzothiazolium cation, resulting in the increased electron density around H_a and H_b in **4a** or **7** and thus the upfield shifts. Given the distinct chemical structures and absorption wavelengths of **4a**, **3b**, and **7**, it is very promising to sense Cys, Hcy, and GSH simultaneously and selectively by use of **1**. Subsequently, we examined that emission behaviors of **1** upon addition of Cys, Hcy, and GSH, respectively, through time-dependent fluorescence spectra in the same conditions (Figure 2). As a model compound of amino-free thiols, NAC was also tested in the study.

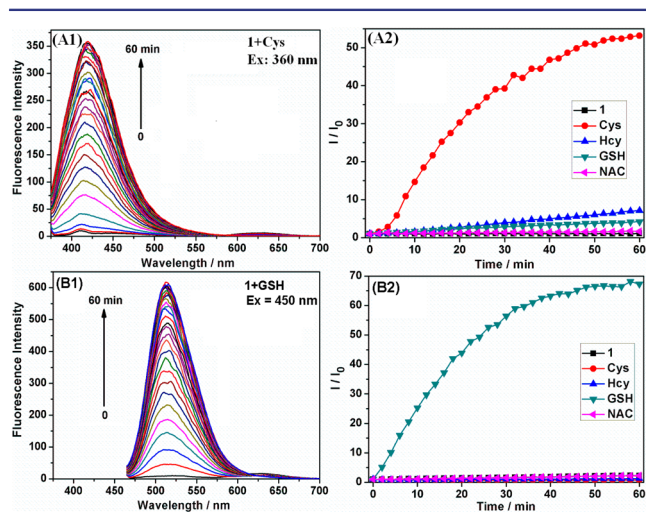


Figure 2. Time-dependent fluorescence response of **1** ($1\ \mu\text{M}$) toward 10 equiv of Cys excited at 360 nm (A1) or 10 equiv of GSH excited at 450 nm (B1). Time-dependent fluorescence intensity changes toward 10 equiv of biothiols excited at 360 nm (A2) or 450 nm (B2). Condition: slits, 10/10 nm; PB buffer (10 mM, pH 7.4, 1 mM CTAB) at 25 °C.

First, we selected the absorption maximum of **4a** at 360 nm as excitation wavelength to probe Cys (Figure 2A1,A2, details in Figure S8). With the excitation, probe **1** is almost nonemissive. Upon addition of Cys, a new emission peak appeared at 420 nm and reached equilibrium within 60 min. In this case, an approximate 53-fold increase in fluorescence signal could be observed. By comparison, Hcy and GSH only elicited a 7- and 4-fold emission enhancement when monitoring at 420

nm, and NAC hardly contributes to any interference, indicative of the ability of probe **1** to discriminate Cys from Hcy/GSH/NAC. Second, we selected the absorption maximum of **3b** at 500 nm as excitation wavelength to probe Hcy (details in Figure S9). With the excitation, probe **1** shows a poor emission at 625 nm, characteristic for the coumarin-hemicyanine dyes. The addition of Hcy led to the decrease of the emission at 625 nm along with a very poor new emission at 545 nm that could be assigned to **3b**. Thus, probe **1** is not proper to sense Hcy due to the lack of an efficient fluorescence turn-on. Third, we selected the absorption maximum of **7** at 450 nm as excitation wavelength to probe GSH (Figure 2B1,B2, details in Figure S10). With the excitation, probe **1** is also nonemissive. However, upon addition of GSH, a new emission peak appeared at 512 nm and reached equilibrium within 60 min. In this case, an approximate 67-fold increase in fluorescence signal was observed. Importantly, Cys/Hcy/NAC hardly elicited any significant fluorescence changes of **1**, indicative of the strong ability of probe **1** to discriminate GSH from Hcy/Cys/NAC. Overall, these results confirm that probe **1** possesses the capability of highly selective detection of Cys and GSH from the different emission channels. In addition, probe **1** is also stable at the time range of 0–60 min in the condition, as indicated by its time-dependent absorption and fluorescence spectra (Figure S11).

Further, we evaluated the selectivity of **1** toward other biologically related species, including various amino acids and representative anions and cations (Figures S12 and S13). In fact, only Cys and GSH promoted significant fluorescence intensity changes at 420 and 512 nm, respectively, confirming the high selectivity of probe **1** for Cys or GSH.

Next, we performed the fluorescence titration experiments at 60 min in the same condition. As shown in Figures S14A and S15A, it was found that approximate 1 equiv of GSH (or Cys) could complete the reactions. For GSH, the fluorescent intensity is linearly proportional to the amount of GSH from 0 to 0.9 equiv (Figure S14B). The detection limits for GSH were determined as $0.05\ \mu\text{M}$ based on $S/N = 3$. However, for Cys, the obvious fluorescence enhancement occurred only when more than 0.4 equiv of Cys was added (Figure S15A), and the linearity range was from 0.4 to 0.8 equiv (Figure S15B). We speculated that the initial fluorescence silence is probably due to an intermolecular thiol-halogen nucleophilic substitution between intermediate **3a** and unreacted **1** that results in a nonemissive 2:1 1-Cys adduct **9** (Figure S16A), which was supported by the respective absorption bands of amino- and thio-coumarin-hemicyanine moieties of **9** when 0.1–0.4 equiv Cys was added to **1** (Figure S16B) as well as the HRMS assay, where the 2:1 1-Cys adduct **9** could be clearly observed (Figure S17). In view of these results, probe **1** could at least detect $>0.4\ \mu\text{M}$ Cys in the condition (the intracellular concentration for Cys: $30\text{--}200\ \mu\text{M}$,^{16a,b} for GSH: $1\text{--}10\ \text{mM}$ ^{16c}).

Subsequently, we evaluated the capability of **1** to selectively sense Cys and GSH in biological system. COS-7 cells were found to have almost no fluorescence in both blue and green channels (Figure 3A1,B1). When COS-7 cells are incubated with **1** ($10\ \mu\text{M}$), they gave fluorescence in both blue (Figure 3A2) and green (Figure 3B2) channels, indicating that **1** is responsive to intracellular Cys and GSH. When COS-7 cells were pretreated with 0.5 mM Cys and then incubated with **1**, a marked increase in blue emission (Figure 3A3) and a slight decrease in green emission (Figure 3B3) were observed. When COS-7 cells were pretreated with 0.5 mM GSH and then

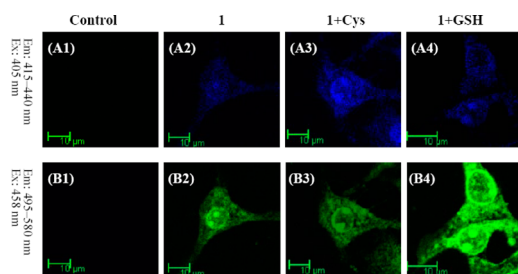


Figure 3. (A1–A4) Confocal fluorescence images of Cys in COS-7 cells: emission was collected at 415–440 nm for blue channel (excited at 405 nm). (B1–B4) GSH images: emission was collected at 495–580 nm for green channel (excited at 458 nm). (A1, B1) Control (no probe). (A2, B2) COS-7 cells incubated with 10 μ M of **1** and CTAB (1 mM). (A3, B3) COS-7 cells preincubated with 0.5 mM Cys and then treated with 10 μ M of **1** and CTAB (1 mM). (A4, B4) COS-7 cells preincubated with 0.5 mM GSH and then treated with 10 μ M of **1** and CTAB (1 mM).

incubated with **1**, a marked increase in green emission (Figure 3B4) and a slight decrease in blue emission (Figure 3A4) were observed. These results are in agreement with the specificity of probe **1** for Cys or GSH and also demonstrate the potential of **1** to sense intracellular Cys and GSH simultaneously from different emission channels. In addition, the MTT assay for **1** was also conducted, and the results showed that the probe at a concentration of 10 μ M has only minimal cytotoxicity (Figure S18).

Finally, we also evaluated how the mixture of Cys/Hcy/GSH affects the emission of **1** (10 μ M) *in vitro*. It was found that addition of the high concentration of Cys/Hcy/GSH (100 μ M/100 μ M/1 mM) to **1** could elicit the obvious fluorescence turn-on at 420 nm (for Cys) excited at 360 nm (Figure S19A1), but almost no fluorescence changes at 512 nm (for GSH) excited at 450 nm. This could be attributed to the higher reactivity of Cys than GSH. However, in the presence of the low concentration of bi thiol mixture (Cys/Hcy/GSH: 1/1/10 μ M), GSH could elicit the obvious fluorescence turn-on at 512 nm excited at 450 nm (Figure S19B1). These results indicated that probe **1** could selectively detect Cys or GSH in the presence of other bi thiols.

In summary, we have presented a chlorinated coumarin-hemicyanine fluorescence probe **1** that can selectively sense Cys and GSH from different emission channels. We also demonstrated that probe **1** is able to simultaneously monitoring Cys and GSH in COS-7 cells in multicolor imaging. All these results are based on three reaction sites of probe **1**, which elicit three different chemical reactions toward Cys, Hcy, and GSH, respectively. We hope that the novel strategy could inspire the exploration of new systems to sense bi thiols with improved sensitivity, greater fluorescence turn-on or ratiometric response for probing bi thiols function in biological systems.

■ ASSOCIATED CONTENT

● Supporting Information

Experimental procedures and data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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