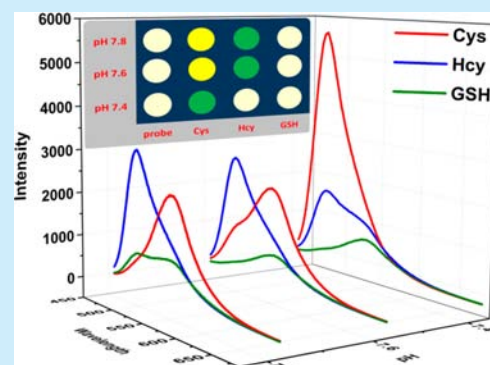


pH-Dependent Fluorescent Probe That Can Be Tuned for Cysteine or Homocysteine

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S Supporting Information

ABSTRACT: The very close structural similarities between cysteine and homocysteine present a great challenge to achieve their selective detection using regular fluorescent probes, limiting the biological and pathological studies of these two amino thiols. A coumarin-based fluorescent probe was designed featuring pH-promoted distinct turn-on followed by ratiometric fluorescence responses for Cys and turn-on fluorescence response for Hcy through two different reaction paths. These specific responses demonstrate the activity differences between Cys and Hcy qualitatively for the first time. The probe could also be used for Cys and Hcy imaging in living cells.



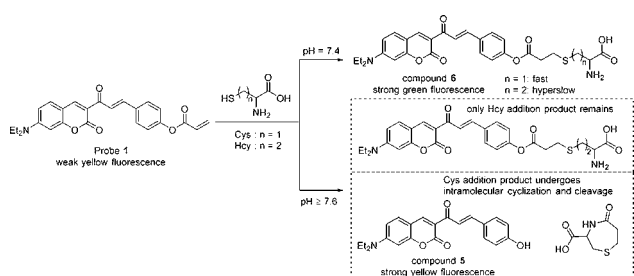
Cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) are closely related to the pathology of many diseases. The deficiency of Cys was reported to cause slowed growth, edema, liver damage, skin lesions, and weakness.¹ The normal intracellular concentration of Cys is 30–200 μM .² For Hcy, the normal concentration in serum is approximately 5–12 μM . Excess Hcy is associated with diseases including Alzheimer's, neural tube defects, and mental disorders.³ GSH intracellular concentrations are 1–10 mM.⁴ Because of the similar structures and chemical properties among Cys, Hcy, and GSH, most commercially available probes for thiols do not enable their discrimination, thus impeding further studies in their roles on pathogenesis and physiological activities.⁵ There have been few probes reported to date with distinct detection characteristics for these three biothiols.⁶ In 2011, the Strongin group utilized the acrylate moiety to synthesize a benzothiazole derivative for discriminative detection of exogenous Cys and Hcy in human plasma through a nucleophilic addition-cyclization process.^{6a} In a later work, a micelle-catalyzed detection procedure specific for GSH was developed on the basis of the same strategy.^{6b} Subsequently, the Yang group reported a BODIPY-based fluorescent probe for specific detection of GSH over Cys and Hcy through a nucleophilic chloride displacement by the thiol group.^{6c} For Cys and Hcy, the thiol-substituted products further undergo an intramolecular substitution of the thiol group by the amino group.

For GSH, its tripeptide structure hinders the attack by the amino group. Other probes have been developed in recent years that were based on this design strategy and have made additional progress in biothiol detection.^{6d,7} Thus, the focus should be to further develop fluorescent probes that address discriminative detection between Cys and Hcy.^{8,7d} Concentrated on this point, the distinction of the pK_a values ($\text{pK}_{a\text{Cys}} = 8.0$; $\text{pK}_{a\text{Hcy}} = 8.87$; $\text{pK}_{a\text{GSH}} = 9.20$), which may reflect the nucleophilic addition activity of thiol moieties depend on the pH environment of the three biothiols and afford the opportunity to detect them separately.⁹ Additionally, the different steric-hindrance effects of the three biothiols can also be used for discriminant detection.¹⁰ For the reaction site, the acryloyl group displayed excellent sensitivity and selectivity for Cys,¹¹ especially the work for discriminant detection of Cys and Hcy as reported by the Strongin group.^{6a} Based on this schematic, we modified the coumarin moiety to synthesize probe 1 for fluorescence detection of thiols (Scheme 1). The detection mechanisms of probe 1 toward Cys and Hcy were the thiol-induced nucleophilic addition–cyclization process and sensitive pH-promoted nucleophilic addition in aqueous solution, respectively. ¹H NMR titration experiments and mass (MS) data confirmed these processes.

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Scheme 1. Design of Probe 1 and the Divisional Detection of Cys and Hcy



The spectroscopic properties of probe 1 toward biothiols were measured through time-dependent UV-vis and fluorescence spectrometry in Hepes buffer/DMSO (1:1, v/v, pH 7.4) solution. As shown in Figure 1a, probe 1 itself displayed

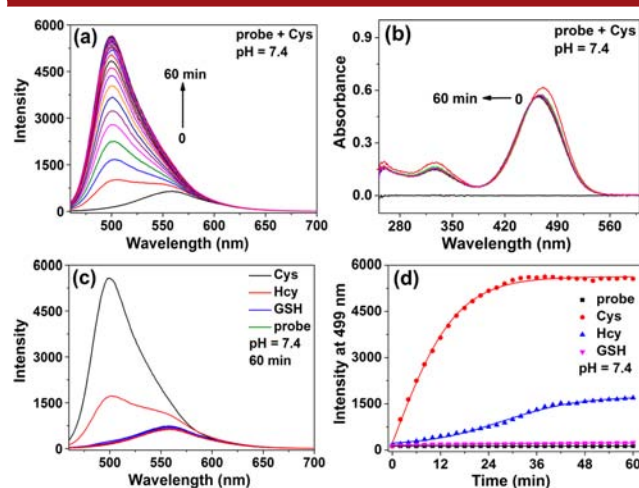


Figure 1. (a) Time-dependent fluorescence emission spectra of 1 (30 μM) in the presence of 10 equiv of Cys in Hepes buffer/DMSO (1:1, v/v, pH 7.4) at 25 °C. (b) Corresponding time-dependent UV-vis spectra of 1 (30 μM) in the presence of 10 equiv of Cys in Hepes buffer/DMSO (1:1, v/v, pH 7.4) at 25 °C. (c) Fluorescence emission spectra of 1 (30 μM) upon addition of 10 equiv of Cys, Hcy, GSH, Ala, Asn, Arg, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val in Hepes buffer/DMSO (1:1, v/v, pH 7.4) at 25 °C. (d) Time-dependent fluorescence emission intensity changes of 1 toward 10 equiv of biothiols at 499 nm (λ_{ex} = 447 nm, slit: 5 nm/5 nm).

dim fluorescence emission at 559 nm. However, the addition of Cys into the probe 1 containing buffer induced a significant blueshifted fluorescence emission at 499 nm with the excitation at 447 nm and plateaued with 44-fold enhancement in 30 min. The corresponding UV-vis spectrum displayed 7 nm blue shift within 60 min (Figure 1b). For Hcy addition, a quarter-fold fluorescence emission enhancement at 499 nm was observed compared with the Cys-probe system, and the UV-vis spectrum was almost invariable (Figure S4). At the same time, the responses of probe 1 toward Hcy in this system were hysteretic (Figure 1d). However, GSH was virtually inert to probe 1 in the detection environment (Figure S4). These optical responses of probe 1 toward the three thiol-containing amino acids might be caused by the nucleophilic addition process displayed in Scheme 1, which resulted in the specific detection of Cys in Hepes buffer/DMSO (1:1, v/v, pH 7.4).

Encouraged by our previous work¹² and the emergence of the weak responses of probe 1 toward Hcy in the above system, we further measured the time-dependent UV-vis and fluorescence spectra in Hepes buffer/DMSO (1:1, v/v) with a slightly higher pH value (pH = 7.8). As expected, the responses of probe 1 toward both Cys and Hcy were accelerated (Figure 2). As a result, the turn-on fluorescence

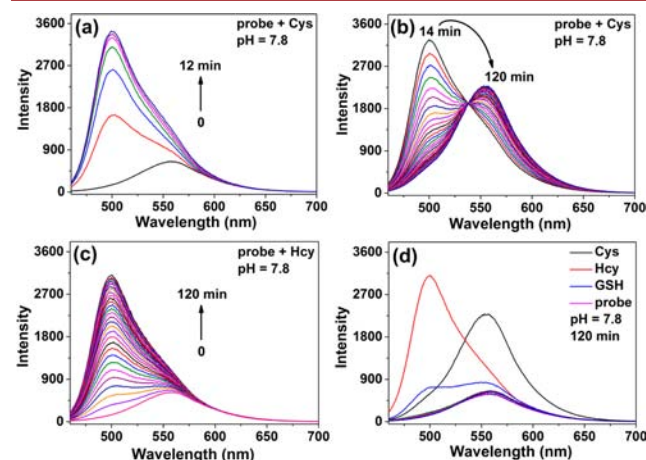


Figure 2. (a, b) Time-dependent fluorescence emission spectra of 1 (30 μM) in the presence of 10 equiv of Cys in Hepes buffer/DMSO (1:1, v/v, pH 7.8) at 25 °C. (c) Time-dependent fluorescence emission spectra of 1 (30 μM) in the presence of 10 equiv of Hcy in Hepes buffer/DMSO (1:1, v/v, pH 7.8) at 25 °C. (d) Fluorescence spectra of 1 (30 μM) upon addition of 10 equiv of Cys, Hcy, GSH, Ala, Asn, Arg, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val in Hepes buffer/DMSO (1:1, v/v, pH 7.8) at 25 °C. (λ_{ex} = 447 nm, slit: 5 nm/5 nm).

responses of probe 1 to Cys at 499 nm peaked within 12 min, then decreased, accompanied by the increase of a new signal centered at 554 nm over extended periods of time (Figure 2a,b). Corresponding to the fluorescence changes, the UV-vis responses of probe 1 to Cys displayed an apparent blue shift within the first 30 min and then reversed with red shift changes in the subsequent 90 min (Figure S6). Neither Hcy nor GSH induced the second fluorescence signal in the present detection system. Interestingly for GSH, pH 7.8 induced a slight responses compared to pH 7.4, which further proved the pH-regulated nucleophilic addition activities of the three thiols. The specific pH-promoted changes of probe 1 toward Cys may be caused by the stepwise detection processes presented in Scheme 1. The pH modulated turn-on and subsequent ratiometric fluorescence responses of probe 1 with Cys, Hcy, and GSH provided chances for distinct detection of the three similar biothiols. To verify the threshold of the pH-regulated two-stage responses, we further studied the detection systems at pH 7.6 and 8.0. The result showed that the differences in the fluorescence sensing of probe 1 toward Cys, Hcy, and GSH occur when the pH value of the system exceeds 7.6 and that there is a proportional relation between fluorescence change and increasing pH value (Figures S5 and S7). These specific fluorescence responses induced by minor pH changes reflected the activity differences among Cys, Hcy, and GSH qualitatively.

The probe not only showed a pH-promoted sensitive response to the biothiols but also had an excellent selectivity toward Cys, Hcy, and GSH over various canonical amino acids. As shown in Figure 1c, only Cys and Hcy could induce the

turn-on fluorescence responses at 499 nm within 60 min, and other amino acids (which included GSH) did not interfere with the detection process. Furthermore, in the Hepes buffer/DMSO (1:1, v/v, pH 7.8) system, probe 1 displayed distinct fluorescence responses toward Cys and Hcy without interference of other amino acids (Figure 2d). Similarly, the detection processes in the pH 7.6 and 8.0 systems maintained the same results as above (Figure S8). These data showed that probe 1 could selectively detect Cys and Hcy through two emission channels.

To further confirm the detection mechanisms of probe 1 toward Cys and Hcy, we carried out time-dependent ^1H NMR experiments upon addition of equivalent amounts of Cys and Hcy to probe 1 in DMSO- d_6 (Figure S1). As shown in Figure 3,

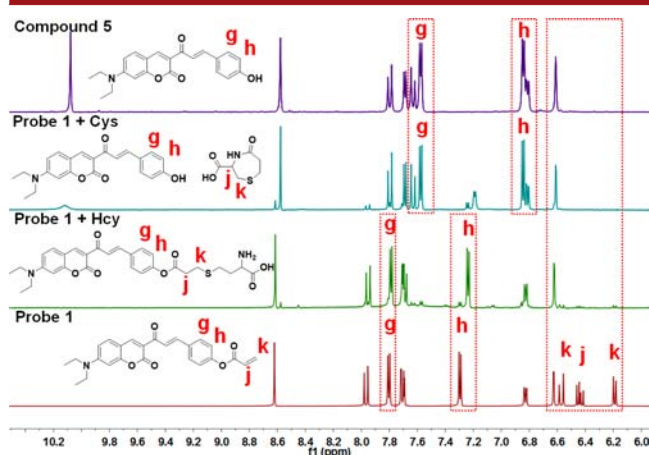


Figure 3. Time-dependent ^1H NMR experiments of probe 1 toward Cys and Hcy in DMSO- d_6 . Spectra for probe 1 + Cys, and probe 1 + Hcy were obtained 30 min after addition.

the addition of Cys reduced the signals at 6.19, 6.44, and 6.57 ppm (j, k) that belong to the acryloyl group and disappeared completely after 30 min. At the same time, the signals at 7.29 (h) and 7.80 ppm (g) reduced gradually and two new signals at 6.84 and 7.57 emerged which are consistent with the ^1H NMR data of compound 5. These signal changes demonstrated how the nucleophilic addition–cyclization process occurred during the detection process of probe 1 toward Cys selectivity. Furthermore, the HR-MS data of the Cys-probe 1 system in Figure S2 also supported the same aforementioned mechanism. For Hcy, however, the original ^1H NMR signals at 6.19, 6.44, and 6.57 ppm (j, k) decreased while other signals downfield remained the same (Figure 3), which indicated that the olefinic bond of the acryloyl group in probe 1 was broken but the ester group still remained intact. Moreover, in the ESI-MS data in Figure S3 we found $m/z = 574.75$ for compound 6 ($n = 2$) $[\text{M} + \text{Na}]^+$. These changes in the reaction processes may be caused by the more stable 7-membered ring in the case of Cys compared to the 8-membered ring for Hcy.

To evaluate the applicability of probe 1 in biological systems, we measured the MTT assay with HepG2 cells, and the results showed minimal cytotoxicity of probe 1 at a concentration of 50 μM (87.6% viability) (Figure S9). The cell-imaging experiments were measured with HepG2 cells in the pH 7.4 system. As shown in Figure 4, cells preincubated with 1 mM NEM and then 5 μM probe 1 in HBSS buffer (containing 10 μM nigericin, an H^+/K^+ ionophore to homogenize the intra- and extracellular pH)¹³ displayed nearly nonfluorescence

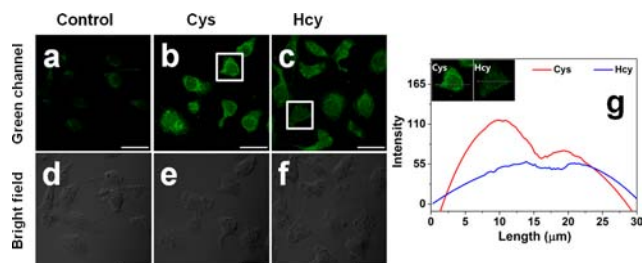


Figure 4. (a, d) Confocal fluorescence image of HepG2 cells preincubated with 1 mM NEM for 30 min and further incubated with HBSS (Hanks' Balanced Salt Solution (with Ca^{2+} , Mg^{2+})) of pH 7.4 in the presence of 10 μM nigericin and 5 μM probe 1 for 30 min. (b, e) Using the control procedures, the cells were further incubated with 100 μM Cys in HBSS of pH 7.4 in the presence of 10 μM nigericin for 60 min. (c, f) Using the control procedures, the cells were further incubated with 100 μM Hcy in HBSS of pH 7.4 in the presence of 10 μM nigericin for 60 min. (g) Cross-sectional analysis along the white line in the insets (single cell in white squares in (b) and (c), respectively). $\lambda_{\text{ex}} = 458$ nm; scale bar = 30 μm . Green channel: 500 ± 20 nm.

emission (a). However, the further exogenous Cys and Hcy induced noticeable fluorescence emission (b, c), and interestingly, the cross-sectional analysis of a single cell for Cys and Hcy respectively displayed distinct intensity differences (g). At the same time, the exogenous GSH displayed nonvariance with the controlled trial (Figure S10). These results demonstrated that probe 1 could detect Cys and Hcy specifically under physiological conditions.

To further value the discriminative detection of Cys and Hcy in living cells, we measured the fluorescence imaging experiments with HepG2 cells in pH 7.8. As shown in Figure 5, cells precultured with 1 mM NEM and then 5 μM probe 1 in HBSS buffer (containing 10 μM nigericin) displayed nearly nonfluorescence emission in both the green and red channels (a, d). Further incubation with Cys induced distinct

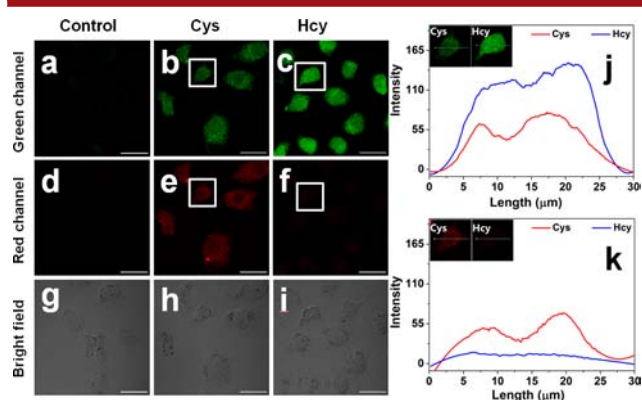


Figure 5. (a, d, g) Confocal fluorescence image of HepG2 cells preincubated with 1 mM NEM for 30 min and further incubated with HBSS (Hanks' Balanced Salt Solution (with Ca^{2+} , Mg^{2+})) of pH 7.8 in the presence of 10 μM nigericin and 5 μM probe 1 for 30 min. (b, e, h) Using the control procedures, the cells were further incubated with 100 μM Cys in HBSS of pH 7.8 in the presence of 10 μM nigericin for 60 min. (c, f, i) Using the control procedures, the cells were further incubated with 100 μM Hcy in HBSS of pH 7.8 in the presence of 10 μM nigericin for 60 min. (j, k) Cross-sectional analysis along the white line in the insets (single cell in white squares in (b) and (c), (e) and (f) respectively). $\lambda_{\text{ex}} = 458$ nm; scale bar = 30 μm . Green channel: 500 ± 20 nm; Red channel: 600 ± 25 nm.

fluorescence emission in the two emission channels (b, e). As for Hcy, these cells displayed strong fluorescence emission in the green channel but very low emission in the red channel (c, f). The cross-sectional analysis of a single cell in the green and red channels for Cys and Hcy, respectively, displayed a large signal ratio that demonstrated the utility of probe **1** for discrimination detection of Cys and Hcy in living cells (j, k) (Figure S11). Consistently, the exogenous GSH could not induce the fluorescence responses in the pH 7.8 system (Figure S12). These minor pH changes induced fluorescence responses of probe **1** toward Cys and Hcy in living cells promoted a deeper insight into the activities of the biothiols in biological systems.

In conclusion, we designed a coumarin-based fluorescent probe for discriminative detection of Cys and Hcy through two emission channels. The reaction mechanism involves a nucleophilic addition step followed by intramolecular cyclization and cleavage. Probe **1** was able to detect Cys through a turn-on-ratiometric fluorescence response when the pH of the reaction was set at 7.6, while Hcy would only undergo the nucleophilic addition step with a turn-on fluorescence signal. Increasing the reaction pH up to 8.0 modulated the turn-on fluorescence at 499 nm of probe **1** toward Cys, Hcy, and GSH with high selectivity. Further, imaging studies with HepG2 cells showed that probe **1** can detect Cys and Hcy in live cells using two emission channels. These results have promoted ongoing related studies of fluorescent probes for thiols in subcellular structures at either pH 8.0 in mitochondria or at pH 4.5 in lysosomes. The pH-promoted detection mechanism provides a new pathway for the design of thiol probes and may bring a deeper insight into the biological activities of these amino thiols.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.orglett.6b03357.

Synthesis of the probe, structure characterizations, additional UV-vis and fluorescence spectra, and additional fluorescence images (PDF)

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Notes

The authors declare no competing financial interest.

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