Fluorescence turn-on probe for homocysteine and cysteine in water†

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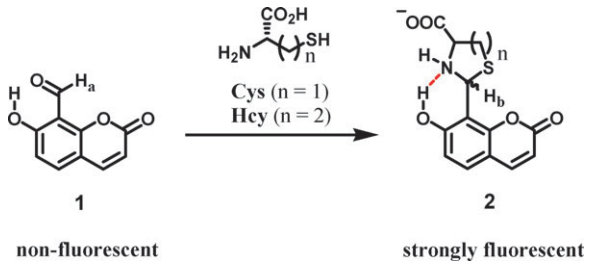
A simple fluorescent probe based on an ortho-hydroxy aldehyde-functionalized coumarin showed selective responses to homocysteine and cysteine by fluorescence turn-on.

Biothiols such as cysteine (Cys), homocysteine (Hcy) and glutathione (GSH) play several important roles in maintaining biological systems.[1–3] For example, Cys and Hcy are essential biological molecules required for the growth of cells and tissues in living systems.[4] The deficiency of Cys is associated with retarded growth, hair depigmentation, edema, lethargy, liver damage, muscle and fat loss, skin lesions, and weakness.[5] An elevated level of Hcy in human plasma is a risk factor for Alzheimer's,[2] cardiovascular diseases,[6] neural tube defect, inflammatory bowel disease, and osteoporosis.[7] Therefore, the selective detection of Hcy and Cys in human blood plasma is very important as a disease-associated biomarker.

Among the available techniques to detect and quantify the presence of biothiols,[8] luminescence methods have been widely employed due to their simplicity and versatility.[9–12] A number of colorimetric and fluorimetric detection methods have been reported for free amino acids,[10] amines,[11] and thiols.[12] Few fluorescent probes for an aminothiol compound, however, are capable of displaying high selectivity over other amino acids and thiols by turning on the fluorescence at neutral pH in pure water.[13]

It is well known that the reaction of aldehydes with the *N*-terminal group of Cys form thiazolidines.[14] The selective reaction has been used to label and immobilize peptides and proteins.[14] Strongin and coworkers made use of a xanthene dye containing a formyl group for the efficient detection of Cys and Hcy by a color change. The experiment was carried out under alkaline condition (pH 9.5).[10d] Cys and Hcy are, however, usually available under physiological conditions; therefore, the development of spectroscopic detection for Cys and Hcy is desirable in a neutral pH environment. For this reason, Huang's group has reported an azo derived choromogenic sensor possesing aldehyde funtionality for Hcy and Cys under neutral pH.[15] Our group has also developed chromogenic[16] and fluorescent[17] sensors for anions with an ortho-hydroxy aldehyde functionalized chromophore. The key substituent, ortho-hydroxy group, was assumed to block the PET quenching due to hydrogen bonding to the nitrogen's lone pair electrons of the ring thiazolidine.[18] Therefore, a coumarinaldehyde (**1**) with an ortho-hydroxy group was prepared according to the previously reported procedure.[17,19]



Scheme 1 Structure of probe **1** and its reaction with Hcy or Cys.

Herein, we report a coumarin-based fluorescent probe **1**, which exhibits a high selectivity toward Hcy and Cys by utilizing a fluorescence turn-on mechanism (Scheme 1). The probe has a coumarin group as a fluorescent signaling unit and a salicylaldehyde functionality as a reaction unit. Both Cys and Hcy react with the carbonyl group of probe **1** to afford a five or six-membered ring. The stable ring formation is attributable to the nucleophilicity of both the nitrogen and sulfur atoms of Cys and Hcy, whereas other amino acids, without the nucleophilic sulfur, can not form the ring compounds.

To confirm that thiazolidine was formed through the reaction of Cys with the aldehyde group of probe **1**, we investigated the ^{1}H NMR spectrum of **1** upon addition of Cys and compared it with that of the probe itself. Complete conversion of **1** to **2** was accomplished by the reaction of Cys and probe **1** in HEPES buffer at room temperature (Fig. 1). As the aldehyde proton (H_a) at around δ 10.2 ppm disappeared, the thiazolidine methine proton (H_b) at around δ 6.35 ppm appeared. Under the same conditions, Hcy also reacted similarly with probe **1** and formed the six-membered ring of thiazinane (Fig. S2).†

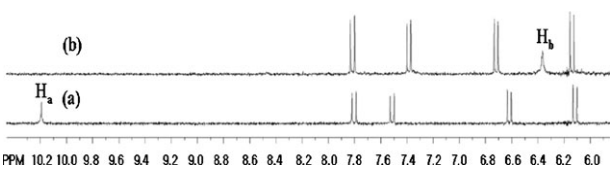


Fig. 1 ^{1}H NMR spectral change of **1** upon addition of L-Cys. (a) **1** only, (b) **1** and 2 equiv. L-Cys. [**1**] = 10 mM in D_2O with HEPES buffer (pH = 7.4) at 25 °C.

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† Electronic supplementary information (ESI) available: ^{1}H NMR spectra of free **1** and its Hcy adduct. Fluorescence titration plot of **1** upon addition of Cys. Selectivity of **1** for Hcy and Cys in the presence of other amino acids and GSH. DFT calculation of **1** and its Hcy adduct. See DOI: 10.1039/b814581d

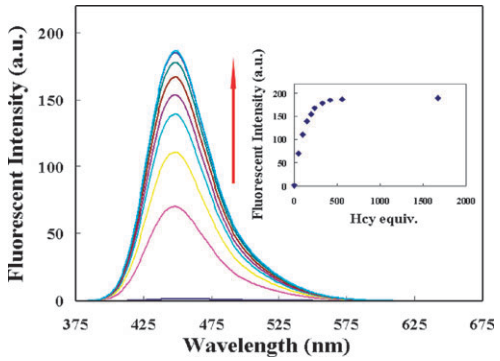

Fig. 2 Fluorescence titration spectra of **1** upon addition of Hcy. Inset: fluorescence change of probe **1** against [Hcy]/[**1**]. ([**1**] = 10 μM, λ_{ex} = 365 nm, λ_{em} = 450 nm).

To measure the fluorescence sensitivity of **1** for Hcy and Cys, fluorescence titration was carried out in aqueous solution (HEPES buffer, pH 7.4). Fluorescence changes were monitored by using a 10 μM solution of **1** in water under biological pH at room temperature. Upon addition of Hcy, the fluorescence emission intensity of **1** at λ_{em} 450 nm was increased more than 100-fold and saturated at 500 equiv. of Hcy (Fig. 2). A similar fluorescence increase was observed for Cys with a 49-fold enhancement at 450 nm. A nonlinear curve fit on the basis of 1 : 1 host–guest binding has shown that the complexation was very strong even in the buffered water solvent, yielding a submillimolar affinity of K_d = 6.05 (±1.16) × 10^{-4} M and 1.20 (±0.11) × 10^{-3} M for Hcy and Cys, respectively, in water at pH 7.4 (Fig. S4 and S6).† Probe **1** showed a slight preference for Hcy over Cys.

To evaluate the selectivity of **1** for Hcy and Cys, we measured the fluorescence intensity changes for various analytes upon addition of excess guests in HEPES buffer at pH 7.4. The fluorescence intensity of **1** was highly enhanced only by the addition of Hcy and Cys. The other amino acids such as N-Boc-Cys, Met, Ser, Thr, Lys, Trp, Asp, Pro, Glu, Leu, Ile, Gly, Ala and GSH did not cause any significant changes in the fluorescence emission intensity (Fig. 3a). A competitive binding assay was performed by adding Hcy to 10 μM of **1** in the presence of 500 equiv. of other analytes, which had also shown the higher selectivity of **1** for Hcy (Fig. S7).† The relative fluorescence intensities at 450 nm of **1** also show a remarkably higher selectivity for Hcy and Cys over the other amino acids and glutathione in water at neutral conditions (Fig. 3b).

A large increase in the fluorescence intensity of Hcy and Cys can be perceived by the naked eye. When probe **1** was excited at 365 nm in the presence 500 equiv. of other amino acids and GSH in HEPES buffer at pH 7.4, the bright blue fluorescence responses were observed only with Hcy and Cys in the solution of 10 μM of probe **1** (Fig. 4).

To see the possible utility of **1** as a sensor for Hcy in human blood, we added Hcy to the samples of deproteinized human blood plasma containing **1** (50 μM) and excess GSH (10 mM). The fluorescence intensities of the serum increased with an increasing amount of Hcy. This demonstrates the potential utility of **1** towards determining a submillimolar concentration of Hcy in blood samples in the presence of other biological ions and GSH in human serum (Fig. 5).

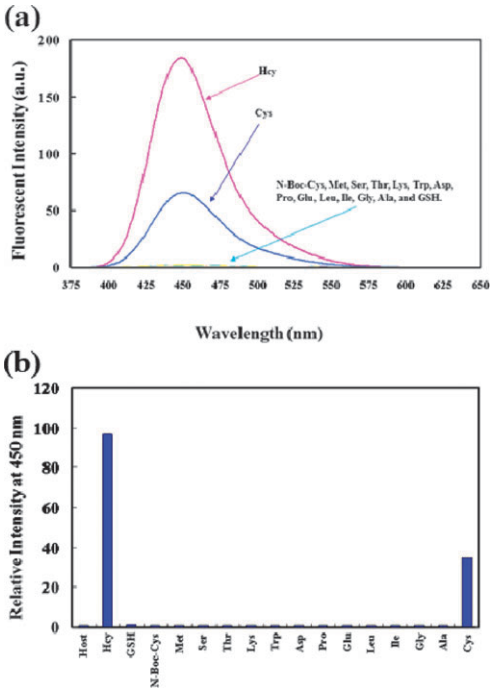

Fig. 3 (a) Fluorescence spectra of **1** (10 μM) upon addition of 500 equiv. of various amino acids or GSH in HEPES buffer. (b) Its relative fluorescence intensities.

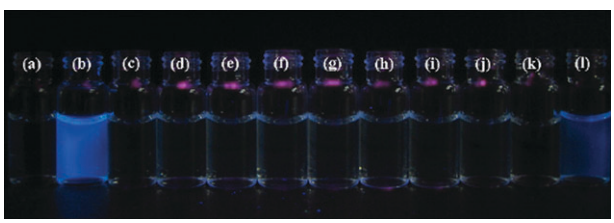

Fig. 4 Fluorescence response of **1** upon addition of 500 equiv. of various amino acids. From left to right: (a) only **1**, (b) Hcy, (c) GSH, (d) N-Boc-Cys, (e) Met, (f) Ser, (g) Thr, (h) Lys, (i) Trp, (j) Asp, (k) Pro, (l) Cys. [**1**] = 10 μM in HEPES (pH 7.4).

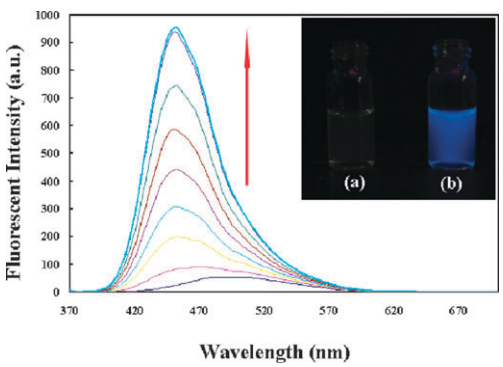

Fig. 5 Fluorescence emission spectral changes of **1** (50 μM) upon addition of Hcy (1.2 × 10^{-4}–1.0 × 10^{-3} M) in human blood plasma in the presence of 10 mM glutathione (pH 7.4, λ_{ex} = 365 nm). (Inset) Fluorescence response of **1** (50 μM) in the blood plasma: (a) **1** + GSH (b) **1** + GSH + Hcy.

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We propose two factors for the fluorescence enhancement upon addition of Hcy or Cys to probe **1**. First, a hydrogen bond between the phenol proton and thiazinane nitrogen atom of **1**-Hcy is assumed to inhibit the possible PET quenching observed in the previous thiazinane coumarin complex.[18] Computational structure of **1**–Hcy explicitly shows the plausible hydrogen bonding between the phenol proton and nitrogen atom (Fig. S8).† Secondly, the fluorescence quenching due to a possible charge transfer from the coumarin HOMO to the aldehyde carbonyl LUMO in **1** will be depressed in the **1**–Hcy complex, where the sp^2 hybrid carbonyl is transformed to an sp^3 hybrid carbon owing to the thiazinane ring formation. Therefore, the excited electrons in **1**–Hcy will decay by emitting a strong fluorescence as observed in the similar structural transformation (Fig. S9).†[20]

In summary, a chemodosimeter having a salicylaldehyde moiety as a reaction unit and a coumarin skeleton as a signaling unit was prepared and its fluorescence turn-on property was characterized in the presence of various amino acids. The selective recognition of **1** for Hcy and Cys over other amino acids and GSH was shown by obvious fluorescence intensity enhancement in water at biological pH, and was clearly visible to the naked eye. This enhancement in the fluorescence intensity was also observed in human blood plasma containing 10 mM of GSH.

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