ChemComm

Cite this: *Chem. Commun.*, 2012, **48**, 8341–8343

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A fluorescein-based probe with high selectivity to cysteine over homocysteine and glutathione†‡

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Received 1st June 2012, Accepted 27th June 2012
DOI: 10.1039/c2cc33932c

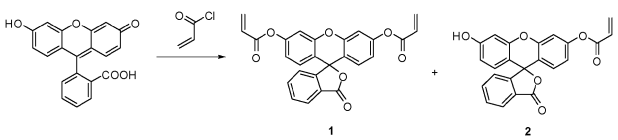
A fluorescent probe based on fluorescein displays excellent selectivity and sensitivity for cysteine and its application for bio-imaging is described.

Intracellular thiols like cysteine (Cys), homocysteine (Hcy) and glutathione (GSH) play critical roles in the regulation of important cellular processes.[1] For instance, GSH maintains the reduced state of proteins and protecting the cells against reactive oxygen species (ROS), drugs or heavy metal ions.[2] Hcy, is situated at a critical regulatory branch point in sulfur metabolism, and can be remethylated to methionine through methionine synthetase with methylenetetrahydrofolate as a cofactor in the methionine cycle.[2] Cys is a precursor amino acid of GSH and both are taken up by food or are formed as a metabolic product of Hcy.[3] Alteration of intracellular thiol concentration results in many diseased states. Elevated levels of homocysteine and cysteine are considered to be associated with cardiovascular disease.[4] A deficiency of thiols causes various health problems such as retarded growth, hair depigmentation, lethargy, liver damage, muscle and fat loss, and skin lesions.[5] Therefore, the detection and discrimination of thiol-containing molecules in biological samples are of great importance. In the past few years, various fluorescent probes for thiols based on different mechanisms have been exploited,[6–10] including Michael addition,[7] cyclization reaction with aldehyde,[8] cleavage reaction by thiols,[9] and others.[10]

Owing to the similarity in structure and reactivity of Cys, Hcy and GSH, fluorescent sensors that could discriminate the three thiols were surprisingly scarce so far.[11] The Strongin group and Li group provided the pioneer works to specifically detect Hcy.[11a,b] The Tseng group reported fluorosurfactant-capped gold nanoparticles as a sensor for the highly selective detection of cysteine.[11c] Besides, the first fluorescence-enhanced chemodosimeter discriminating Cys from Hcy and GSH was also reported by Peng *et al.*[11d] Most recently, Strongin *et al.* developed an ingenious method, where an acrylate moiety was used to discriminate Cys and Hcy by the different relative rates in an intramolecular cyclization reaction between acrylate and thiols.[11e] Herein, we report a fluorescein-based probe **1** bearing two acrylate moieties for the selective recognition of Cys over Hcy and GSH. We speculate that the double acrylate moieties can effectively enhance the selectivity of the probe to Cys compared with the single acrylate-containing analogue.

The fluorescein derivates **1** and **2** required for these studies were synthesized as shown in Scheme 1. Fluorescein was reacted with acryloyl chloride in the presence of triethylamine in CH_2Cl_2 to afford **1** and **2** in 75% and 78% yield, respectively. The experimental details and characterization data for **1** and **2** are given in Electronic Supplementary Information (ESI‡).

Fig. 1 shows the fluorescence and absorbance changes that **1** undergoes upon the addition of various analytes, including Cys, Hcy, GSH, Gly, Phe, Ser, Glu, Lys, Arg, His, Ala, Gln, Met and Tyr. After incubation with 10 equiv. of Cys for 10 min, the probe **1** displays a remarkable fluorescence enhancement at 515 nm and a new absorption band at around 490 nm in ethanol-phosphate buffer (20 mM, pH 7.4, 2 : 8 v/v). The analytes without thiols (Phe, Ser, Glu, Arg, Ala, His, Lys, Gln, Gly, Tyr and Met) induced little changes in fluorescence intensity and UV-vis spectra under the same conditions. Interestingly, the addition of Hcy and GSH did not show as remarkable a fluorescence enhancement and colorimetric change as those induced by Cys (Fig. S1, see ESI‡), even though mercapto groups were also contained in the two thiols. By contrast, the solution containing **2**, bearing a single acrylate group, did not show a considerable selectivity to Cys



Scheme 1 Synthesis of sensors **1** and **2**.

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† We note that after our work had been completed, an independent work entitled "A colorimetric and fluorescent chemodosimeter for discriminative and simultaneous quantification of cysteine and homocysteine" was published, L. Wang, Q. Zhou, B. Zhu, L. Yan, Z. Ma, B. Du and X. Zhang, *Dyes and Pigments*, 2012, **95**, 275–279. We were unaware of this article at the time of submission.

‡ Electronic supplementary information (ESI) available: experimental details; synthesis and characterization of **1** and **2**; spectroscopic data; Job's plots of the reaction between **1** or **2** and Cys; mass spectra of **1** or **2** reacting with Cys. See DOI: 10.1039/c2cc33932c

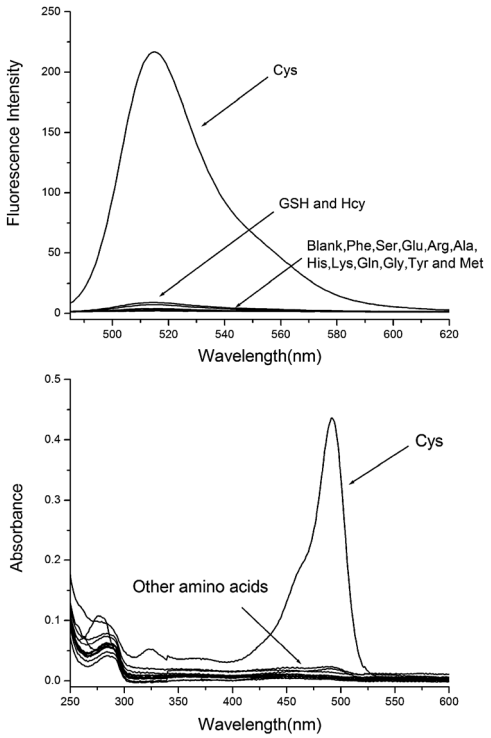


Fig. 1 Top: Fluorescence spectra of **1** (5 μM) upon incubation with various analytes (50 μM) for 10 min in ethanol-phosphate buffer (20 mM, pH 7.4, 2 : 8 v/v) (λ_{ex} = 478 nm, slit: 1.5 nm/1.5 nm). Bottom: UV/Vis absorption spectra of **1** (10 μM) in ethanol-phosphate buffer (20 mM, pH 7.4, 2 : 8 v/v) in the presence of 100 μM analytes including Cys, Hcy, GSH, Phe, Ser, Glu, Arg, Ala, His, Lys, Gln, Gly, Tyr and Met.

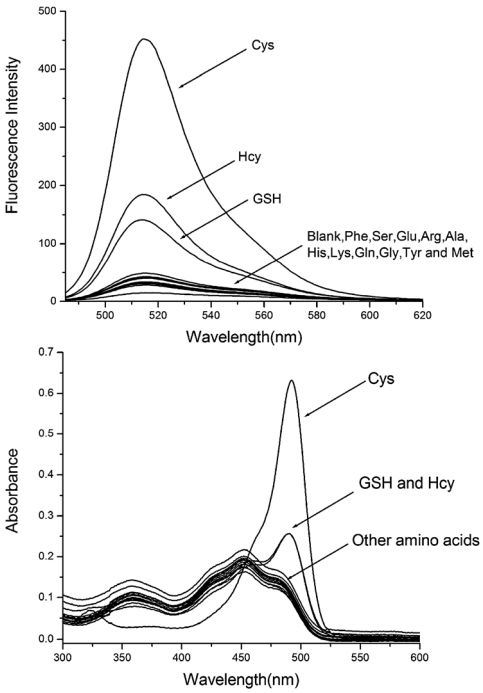
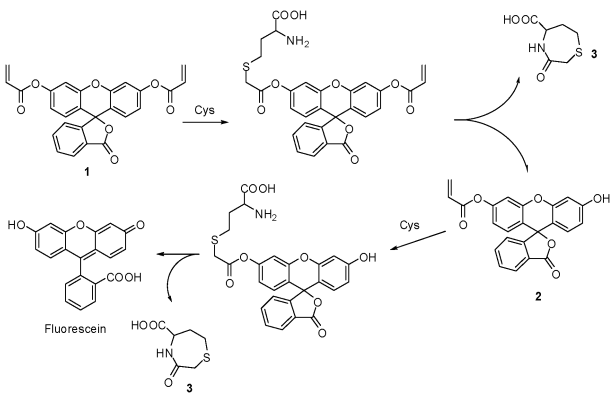


Fig. 2 Top: Fluorescence spectra of **2** (5 μM) upon incubation with various analytes (50 μM) for 10 min in ethanol-phosphate buffer (20 mM, pH 7.4, 2 : 8 v/v) (λ_{ex} = 478 nm, slit: 1.5 nm/1.5 nm). Bottom: UV/Vis absorption spectra of **2** (10 μM) in ethanol-phosphate buffer (20 mM, pH 7.4, 2 : 8 v/v) in the presence of 100 μM analytes including Cys, Hcy, GSH, Phe, Ser, Glu, Arg, Ala, His, Lys, Gln, Gly, Tyr and Met.

in that Hcy and GSH also induced great changes in the fluorescence and absorption spectra (Fig. 2). To investigate detection limit of **1** for Cys, **1** (1 μM) was treated with various concentrations of Cys (0–500 nM) (Fig. S2a, see ESI‡). The fluorescence intensity at 515 nm was plotted as a function of the Cys concentration (Fig. S2b, see ESI‡). The fluorescence intensity of **1** was linearly proportional to Cys concentrations of 0–500 nM, and as low as 77 nM concentration of Cys was detected by using **1** with a signal-to-noise ratio of 3. Similarly, the detection limit of **2** for sensing Cys was evaluated to be 121 nM (Fig. S2c and S2d, see ESI‡). The effect of pH on the fluorescence response of **1** or **2** to Cys was investigated. The results indicated that both **1** and **2** are useful for detecting Cys under neutral and basic conditions (Fig. S3, see ESI‡). Time-dependent fluorescence intensity assays show the reactions between **1** or **2** and Cys (10 equiv.) were almost complete within 15 min and 10 min, respectively (Fig. S4, see ESI‡).

For the detection mechanism, the addition of thiol to the acryloyl group in the probe followed by the cleavage of an ester bond to form the fluorescein is likely to be responsible for the fluorescence enhancement and UV-vis spectral change (Scheme 2). To examine this plausible mechanism, the stoichiometry of a binding event between thiol and **1** or **2** was initially determined. The results obtained from Job's plots show the 1 : 2 stoichiometry for the reaction between **1** and Cys and 1 : 1 stoichiometry for the reaction between **2** and Cys,



Scheme 2 A plausible mechanism for the reaction between **1** and Cys.

respectively (Fig. S5, See ESI‡). Mass spectrometry analysis of a product obtained from the reaction of **1** or **2** with Cys in CH_3CN also supports the formation of fluorescein and a cyclized product **3**. A peak at 331.1 corresponding to the resulting fluorescein and a peak at 174.0 corresponding to the cyclization product **3** were clearly observed in the mass spectra (Fig. S6, see ESI‡). Compared to **2**, the higher selectivity of **1** to Cys over Hcy and GSH should be attributed to the dual addition-cleavage processes in the reaction between **1** and Cys.

In order to further demonstrate that the permeability and the monitoring of thiols in living cells, confocal microscopy experiments were carried out. When PC-12 cells were

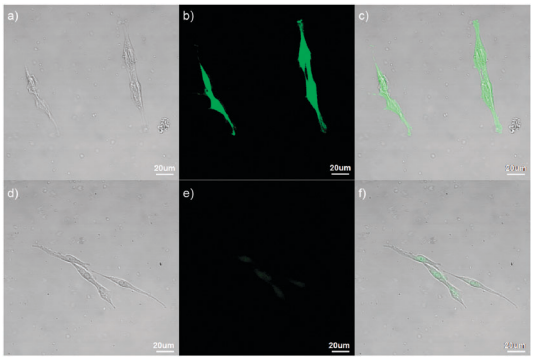


Fig. 3 Phase contrast and fluorescence images of PC-12 cells. Top: PC-12 cells were treated with 50 μM of **1** for 30 min; bottom: PC-12 cells were pre-incubated with 500 μM NEM for 50 min and then treated with 50 μM of **1** for 30 min (a and d, phase contrast images; b and e, fluorescence images; c and f, merged images).

incubated with **1** (50 μM), strong fluorescence was exhibited inside the cells (Fig. 3, top). However, in a control experiment, cells were pre-treated with an excess (0.5 mM) of the thiol-reactive *N*-ethylmaleimide (NEM), a trapping reagent for thiol species, followed by treatment with **1**. The confocal microscopic images did not show a significant fluorescence signal (Fig. 3, bottom). This confirms the specificity of **1** for thiols over other analytes in living cells.

In summary, we have developed a new fluorescein-based fluorescent probe for the detection of cysteine with high selectivity and sensitivity. The fluorescence enhancement and UV-vis spectral change are attributed to the addition of thiol to acryloyl group in the probe followed by the cleavage of ester bond to form the fluorescein. Compared with the single acrylate-containing fluorescein derivate **2**, the double acrylate-containing sensor **1** indicated a remarkable enhancement in selectivity to Cys over Hcy and GSH, owing to the dual addition-cleavage processes in the reaction between **1** and Cys.

This work was supported by grants from NSFY of China (21002049), the Natural Science Foundation of the Jiangsu Higher Education Institutions of China (Grant No. 10KJB150005), and the Opening Project of State Key Laboratory of Chemo/Biosensing and Chemometrics of Hunan University.

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