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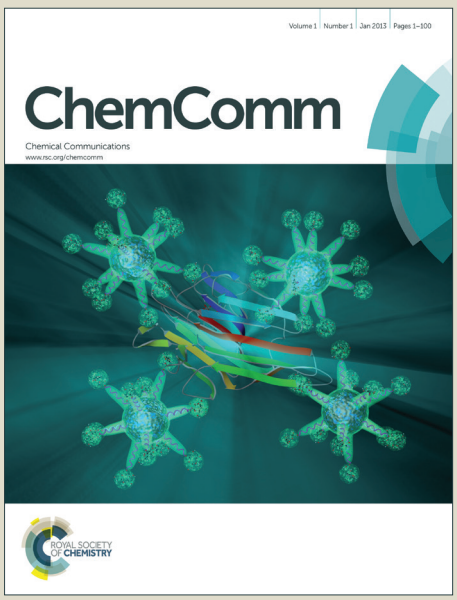

ROYAL SOCIETY OF CHEMISTRY

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Cite this: DOI: 10.1039/c0xx00000x

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ARTICLE TYPE

A low dose, highly selective and sensitive colorimetric and fluorescent probe for biothiols and its application for bioimaging†

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Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX

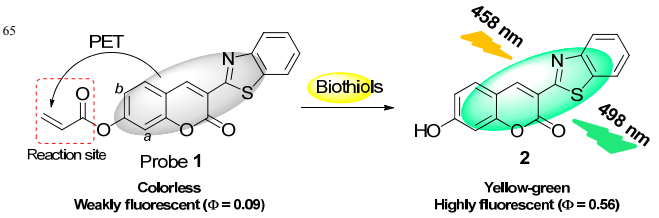
DOI: 10.1039/b000000x

A new colorimetric and fluorescent probe was reported, which can be used in a very low dosage (< 20 nM) for rapid, highly selective and sensitive detection of biothiols.

Biothiols such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) play key roles in biological systems, and abnormal level of these biothiols is related to a number of diseases. For example, Cys deficiency is associated with many syndromes such as slow growth in children, hair depigmentation, edema, lethargy, liver damage, loss of muscle and fat, skin lesions, and weakness.[1] Glutathione deficiency is also involved in many diseases such as liver damage, leukocyte loss, cancer, AIDS and neurodegenerative diseases.[2] Therefore, the rapid, convenient, selective and sensitive detection of trace amounts of these biothiols is of great importance.

Among various detection methods, fluorescent detection have proven to be some of the most convenient due to its simplicity, low cost, high sensitivity and great potential for intracellular bioimaging. Accordingly, significant efforts have been devoted in the past decade to develop fluorescent probes for biothiols.[3] These probes are mainly reaction-based, in which different mechanisms such as Michael addition,[4] cyclization reaction with aldehyde,[5] cleavage reaction such as cleavage of sulfonamide and sulfonate esters by thiols,[6] nucleophilic substitution,[7] disulfide exchange reaction[8] and others[9] are explored. These developments greatly advanced the research for optical detection of biothiols, however, many of them suffer from long response time,[5a-b,6b,7c-d,8a-c] low sensitivity,[5a,8a-b] complicated synthesis work,[4c,7e,8c,9c] or need short UV light excitation.[4a-b,6b,9c] In addition, considering organic fluorescent probe itself and its products after detection are potential contaminants to biological samples, probes are best used in low dose. So far, the dosage of most reported biothiol probes are used in a 5-20 μM level, which is already approximate to or higher than the normal level of Hcy (5-15 μM) found in plasma or serum.[10] Although a few probes can be used in a lower level (1-5 μM),[5b,11] these probes suffer from either long response time (2 h),[5b] or low sensitivity,[11a-b] or requirement of a surfactant to speed up the detection process.[11c-d] Thus, new thiol probes with improved properties are still expected to be developed.

Herein, we would like to report a new colorimetric and fluorescent dual probe, which can be used in a very low dosage (< 20 nM) for rapid, highly selective and sensitive detection of biothiols (probe **1** in Scheme 1). This probe uses 3-benzothiazolyl-7-hydroxycoumarin (compound **2**) as the fluorophore and an acrylate moiety as the fluorescence quencher and the reaction site. Compound **2** was chosen as the fluorophore because this dye has several appealing optical properties such as large absorption extinction coefficients, high fluorescence quantum yields, and both excitation and emission in visible region.[12] Since current efforts found that acrylate-functionalised probes are most selective for Cys,[13] probe **1** was initially expected to be a Cys-selective probe. However, probe **1** in this work was found to be an excellent sensor for all these three biothiols. Notably, this probe exhibits several meritorious features for sensing of biothiols: (i) high selectivity and sensitivity with a low detection limit (12 nM for Cys); (ii) rapid detection process (within a few minutes) with highly apparent colorimetric and fluorescent dual signal changes; (iii) can be used in a very low dosage (< 20 nM) as well as excellent performance in living cell imaging.



Scheme 1. Structure of probe **1** and sensing of biothiols.

The synthetic route for probe **1** is outlined in Scheme S1 (ESI†). Briefly, probe **1** can be easily prepared in good yield (80 %) simply by treatment of **2** with acryloyl chloride in dry dichloromethane under basic conditions at room temperature. The structure of probe **1** was confirmed by NMR, IR and HR-MS spectroscopy. Coumarin **2** was prepared by the literature method.[12] It is worth noting that the whole synthetic process is simple, as no column chromatography is needed for the purification of both **2** and probe **1**. Detailed synthetic procedures and structure characterizations are given in the ESI.†

Cys as one of the typical biothiols was first used to examine the sensing properties of probe **1**. In initial studies, we used 10 μM of probe **1**. As shown in Fig. 1, addition of 5 equiv of Cys to the probe **1** (10 μM) solution in DMSO-PBS buffer (20 mM, pH 7.4, 1:1, v/v) resulted in fast and distinct optical changes. In the absorption spectra, a gradual decrease of the absorption peak at 367 nm and a progressive increase of a more intensified absorption band around 460 nm with a well-defined isosbestic

point at 410 nm were observed against time within a few minutes (Fig. 1a). Meanwhile, in the fluorescence spectra, significant fluorescence enhancement around 498 nm was observed (Fig. 1b). Notably, this detection process is accompanied by highly apparent color changes (Fig. 1, inserted), indicating that probe **1** can be used as a naked-eye diagnostic tool for rapid detection of Cys. Kinetic experiment showed that the reaction of probe **1** with 5 equiv of Cys can be completed at about 5 min (Fig. S1, ESI†), which further proved the rapid detection process. In addition, we used TLC, ^{1}H NMR and Mass spectrum to analyse the isolated product from the mixture of probe **1** and Cys (Fig. S2-S5, ESI†), and the results indicate that the fluorophore **2** was produced. Thus, according to the previously reported conjugate addition–cyclization reaction mechanism for acrylate-functionalised Cys probes,[13] the sensing mechanism of probe **1** for Cys was proposed in Scheme S2 (ESI†). Clearly, the above experiments indicate that probe **1** can be a rapid, colorimetric and fluorescent sensor for Cys in aqueous solution under mild conditions.

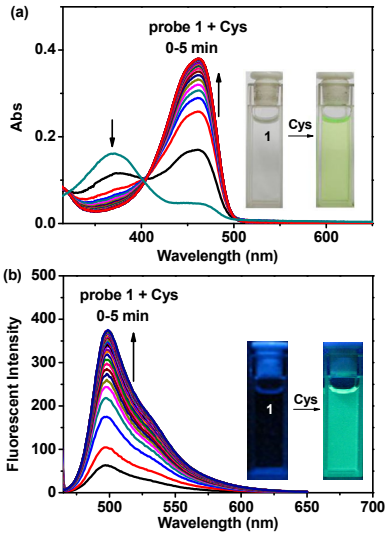


Fig. 1 UV-vis spectra changes (a) and fluorescent spectra changes (b) of probe **1** (10 μM) against time in the presence of Cys (50 μM) in DMSO-PBS buffer (20 mM, pH 7.4, 1:1, v/v) at 37 °C. Color and emission color changes are inserted, respectively. For fluorescent measurement, λ_{ex} = 458 nm, $d_{ex} = d_{em}$ = 2.5 nm.

In order to examine the selectivity of probe **1**, the optical responses of probe **1** to various natural amino acids including phenylalanine (Phe), glutamic acid (Glu), threonine (Thr), glutamine (Gln), tryptophan (Trp), alanine (Ala), arginine (Arg), glycine (Gly), lysine (Lys), leucine (Leu), serine (Ser), isoleucine (Ile), tyrosine (Tyr), aspartic (Asp), methionine (Met), and histidine (His), and various potential interfering anions such as F^-, Cl^-, Br^-, I^-, NO_3^-, NO_2^-, AcO^-, SCN^-, CO_3^{2-}, SO_4^{2-}, SO_3^{2-}, $S_2O_3^{2-}$, and CN^-, and amines such as $H_2NCH_2CH_2NH_2$, $HOCH_2CH_2NH_2$, $C_6H_5CH_2NH_2$, and $C_6H_5NH_2$, as well as biothiols (Cys, Hcy, and GSH) were tested. As shown in Fig. 2a, probe **1** displays significant fluorescence enhancement not only to Cys, but also to Hcy and GSH, which indicates that probe **1** can be used to detect these three biothiols rapidly and simultaneously. In contrast, addition of other analytes showed almost no effect. Moreover, competition experiments showed that colorimetric and fluorescent detection of biothiols (exampled by Cys) by probe **1** in the presence of various amino acids, anions and amines is still effective (Fig. 2b, and Fig. S6-7, ESI†). Therefore, probe **1** has high selectivity for biothiols.

The reactivity difference of probe **1** for Cys, Hcy, and GSH was also examined. As shown in Fig. S8 (ESI†), although there is little difference, probe **1** shows high reactivity and gives fast and distinct fluorescence signal changes to all these three biothiols, which is very different with the reported acrylate-functionalised probes because these probes are generally Cys-selective.[13] This is probably because there are no substituents on the carbons (a and b shown in Scheme 1) neighboured to the acrylate moiety for probe **1**, while the most reported acrylate-functionalised probes have one or two substituents on these carbon positions. As a result, the acrylate in probe **1** has less steric effect, which may enable probe **1** to show faster kinetics for all these three biothiols with less selectivity between each other. This can be evidenced by the fact that a recently reported mono acrylate-functionalised fluorescein-based probe,[13b] which is very similar to probe **1** (no substituents neighboured to the acrylate moiety), also showed faster kinetics and less selectivity for Cys than other acrylate-functionalised probes[13a,13c-f] with more substituents neighboured to the acrylate moiety. Therefore, we established an acylate-functionalised probe for rapid detection of Cys, Hcy and GSH simultaneously, or any one of them when the other two are not present.

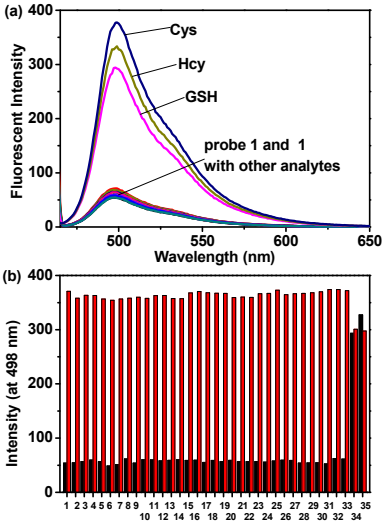


Fig. 2 (a) Fluorescence responses of probe **1** (10 μM) to various analytes (Cys, Hcy, and GSH were used 50 μM, others are used 100 μM). (b) Fluorescence intensity responses of probe **1** (10 μM) at 498 nm for Cys (50 μM) in the presence of various analytes (100 μM). Black bars represent the addition of a single analyte. Red bars represent the subsequent addition of Cys to the mixture. Analytes 1-35: 1. none, 2. F^-, 3. Cl^-, 4. Br^-, 5. I^- 6. NO_3^-, 7. NO_2^-, 8. AcO^-, 9. SCN^-, 10. CO_3^{2-}, 11. SO_4^{2-}, 12. CN^-, 13. SO_3^{2-}, 14. $S_2O_3^{2-}$, 15. Ala, 16. Glu, 17. Thr , 18. Trp, 19. Phe, 20. Gln, 21. Gly, 22. Lys, 23. Arg, 24. Ile, 25. Asp, 26 .Leu, 27. Ser, 28. Met, 29. His, 30. $H_2NCH_2CH_2NH_2$, 31. $HOCH_2CH_2NH_2$, 32. $C_6H_5CH_2NH_2$, 33. $C_6H_5NH_2$, 34. GSH, 35. Hcy. Data was collected 15 min after addition of each analyte in DMSO-PBS buffer (20 mM, pH 7.4, 1:1, v/v) at 37 °C.

The optical spectra changes of probe **1** (10 μM) were also measured with different concentrations of Cys to check its sensitivity for biothiols, and the results show that optical changes start to saturate when more than 2 equiv of Cys was added to the probe **1** solution (Fig. S9 and S10, ESI†). This indicates that

probe **1** is very sensitive to Cys. Since probe **1** itself shows low background fluorescence and compound **2** is highly fluorescent (Φ = 0.56), we then tested the fluorescent detection of Cys using much lower concentration of probe **1**. As shown in Fig. S11 (ESI†), when probe **1** was used at a 1 μM level, detection of Cys still can be very efficient. In this case, upon addition of 5 μM of Cys, significant fluorescent enhancement was also observed within 10 min (Fig. S11a). The observed pseudo-first-order rate constant k_{obs} was determined to be about 0.286 min^{-1} and 0.189 min^{-1} for probe **1** (1 μM) with 5 μM and 1 μM of Cys, respectively, indicating that both reactions at low concentrations of probe and Cys are fast (Fig. S11b). In addition, as shown in Fig. 3a, noticeable fluorescent enhancement can be observed even when Cys was present at a submicromolar level (0.5 μM) in 15 min, and saturation in the fluorescence enhancement was observed when more than 3.5 equiv of Cys was added. Moreover, the fluorescence intensity change of probe **1** at 498 nm was found linearly proportional to Cys concentrations in a range of 0–3 μM, and the detection limit of probe **1** (1 μM dose) for Cys was calculated to be about 12 nM. This value is much lower than that of the most reported biothiol probes, indicating that probe **1** is highly sensitive for Cys. It is worth noting that detection of Cys can be still effective even when the concentration of probe **1** is further reduced. As shown in Fig. 3b and Fig. S12 (ESI†), even the concentration of probe **1** is reduced to as low as 15 nM, noticeable fluorescent signal changes can be still observed when Cys is present either in a 150 nM level or even in a 15 nM level. This result clearly indicates that probe **1** can be used in a very low dosage to detect Cys with high sensitivity. To our knowledge, a fluorescent probe, which can be used in such a low dosage to detect such a low concentration of biothiols, was impressive and unprecedented.

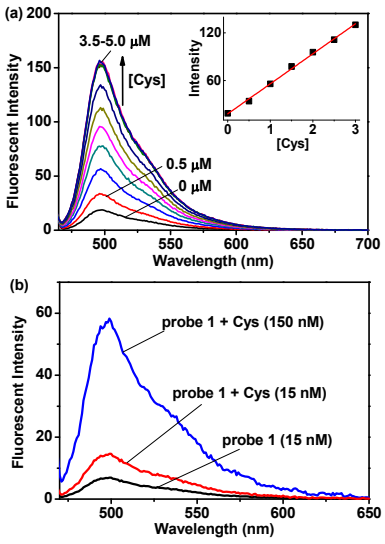


Fig. 3 (a) Fluorescence spectra changes of probe **1** (1 μM) upon addition of different concentrations of Cys. Final concentration of Cys: 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 μM. Each spectrum was obtained 15 min after Cys addition. λ_{ex} = 458 nm, slit width: (2.5, 5). Insert: Linear relationship of fluorescence intensity at 498 nm as a function of Cys concentration (0-3 μM), y = 17.8854 + 37.8980 × [Cys] with R^2 = 0.9970. (b) Fluorescence spectra changes of probe **1** (15 nM) upon addition of Cys (15 nM or 150 nM) in DMSO-PBS buffer (20 mM, pH 7.4, 1:1, v/v) at 37 °C. Each spectrum was obtained 60 min after Cys addition. λ_{ex} = 458 nm, slit width: (10, 10).

It was also found that probe **1** can work over a wide pH range. As shown in Fig. S13 (ESI†), although probe **1** is likely to be hydrolysed under basic conditions (pH > 8.5), the fluorescence of probe **1** itself slightly changes from pH 3.0 to 8.5. However, in the presence of Cys, the emission enhancements of probe **1** were observed over a wide pH range and reached maximum without further variation at pH 7.0-8.5. This result suggests that probe **1** can work over a wide pH range but best under physiological pH.

To demonstrate the practical utility of probe **1** in biological samples, the imaging of intracellular biothiols was tested in living cells. As shown in Fig. 4, when HeLa cells were incubated with probe **1**, the cells started to show strong green fluorescence (B1 and C1), even when 1 μM of probe **1** was used (B1). In contrast, HeLa cell itself showed no fluorescence (A1), and as another control experiment, when HeLa cells were treated with N-ethylmaleimide (NEM), a known thiol trapping reagent prior to incubation with probe **1**, no fluorescence was observed (D1). Since using NEM as a control can be found in many biothiol probes for imaging of cellular biothiols,[5a-b,6a-c,7d,11b-c] this result indicates that the strong fluorescence shown in B1 and C1 is coming from cellular biothiols. It should be also noted that the acrylate group in probe **1** can be potentially removed by hydrolytic enzymes insider cells, however, the result of our NEM-treated experiment (D1) also indicates that the acrylate group of probe **1** is not removed by intracellular enzymes, otherwise it will also show strong fluorescence. Therefore, probe **1** can be used to detect biothiols in living cells. Importantly, it can be used in a low dosage (1 μM) to give strong fluorescence, which is a great advantage. In fact, not all the fluorescent probes can be used in a low dosage for bioimaging. For example, a recently reported acrylate-based probe,[13b] which uses the highly fluorescent fluorescein as the fluorophore and can be used at a 5 μM level for Cys detection *in vitro* with high sensitivity, however, it needs to be used at a 50 μM level for cell imaging.

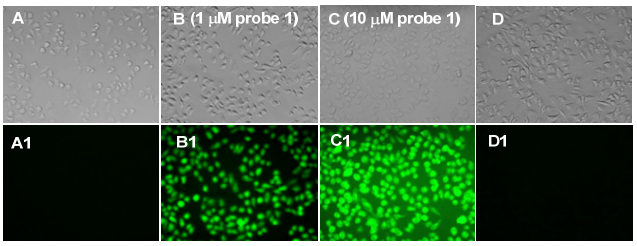


Fig. 4 Imaging of biothiols by probe **1** in living HeLa cells. Top: bright field images. A: HeLa cells; B and C: HeLa cells were incubated with probe **1** (1 μM and 10 μM, respectively) for 60 min. D: HeLa cells were pre-incubated with 500 μM NEM and then treated with probe **1** (10 μM) for 60 min. Bottom: A1, B1, C1 and D1 are fluorescence images of A, B, C and D, respectively.

In summary, we developed a low dosage colorimetric and fluorescent dual probe for biothiols. This probe can be easily prepared and shows several appealing sensing properties including high sensitivity, high selectivity, and fast response for biothiols in aqueous solution under mild conditions, together with distinct colorimetric and fluorescent turn-on signal changes. Notably, this probe can be used in a very low dosage (< 20 nM) to detect biothiols efficiently. In addition, this probe can be also used in a low dosage for cell imaging studies, thus making this

probe holds great potential for biological applications.

We thank the National Natural Science Foundation of China (Grants Nos. 21172086 and 21032001) for financial support.

Notes and references

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† Electronic Supplementary Information (ESI) available: [experimental, synthesis and structure characterizations, additional UV and fluorescence data]. See DOI: 10.1039/b000000x/

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