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Communication

Selective Fluorescent Detection of Cysteine over Homocysteine and Glutathione by a Simple and Sensitive Probe

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A simple fluorescent probe able to selectively and sensitively detect cysteine (Cys) with an excellent dose-dependent relationship between fluorescence intensity and concentration of Cys from 0 to 100 μM has been designed and synthesised.

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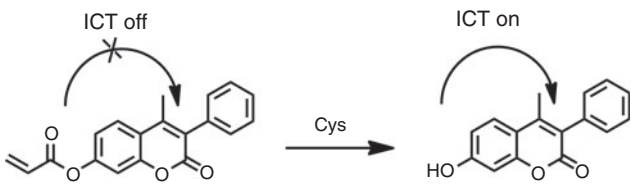
Biothiols, such as cysteine (Cys) and homocysteine (Hcy), as well as glutathione (GSH), are abundant in organisms and play important roles in a variety of physiological processes such as redox homeostasis and cellular functions.[1] Cys serves as the precursor of GSH and Coenzyme A. Furthermore, it carries out crucial roles in protein function, metabolism, detoxification, and cellular functions.[2] However, an abnormal level of Cys is associated with many health disorders. For example, low levels of Cys are considered to be related to hair depigmentation, oedema, liver damage, muscle and fat loss, weakness, hematopoiesis reduction, leucocyte loss, psoriasis, and skin lesions.[3] Elevated levels of Cys have been linked to cardiovascular complications, neurotoxicity, and Parkinson's and Alzheimer's disease.[4] Therefore, the rapid and reliable detection of Cys is of great importance in clinical applications.

Among various detection techniques, fluorescence detection has attracted much attention on account of its simplicity, excellent selectivity and sensitivity, and low cost, as well as simplicity of operation. To date, a great diversity of fluorescence probes for Cys has been designed and synthesised on the basis of different mechanisms,[5] such as Michael addition,[6] cyclisation with aldehyde,[7] conjugate addition–cyclisation,[8] cleavage of sulfonamide and sulfonate esters,[9] and cleavage of S–S bonds.[10] However, distinguishing Cys from Hcy and GSH is difficult because of their structural similarity.[11] Based on a Michael addition reaction mechanism, it's difficult to distinguish between Cys, Hcy, and GSH because the nucleophilic activity of the sulfhydryl group shows almost no difference.[12] Meanwhile, some probes have been designed and synthesised that act with photo-induced electron transfer and intramolecular proton transfer mechanisms. However, they also cannot differentiate between Cys, Hcy, and GSH.[13] Up to now, although a few fluorescence probes have been reported to distinguish Cys over Hcy and GSH,[8] there are still some limitations in their practical application. For example, they require the use of surfactants, long response times, and have relatively poor selectivity and sensitivity.[14] Thus, the development of a fluorescent probe is still needed to selectively detect Cys content.

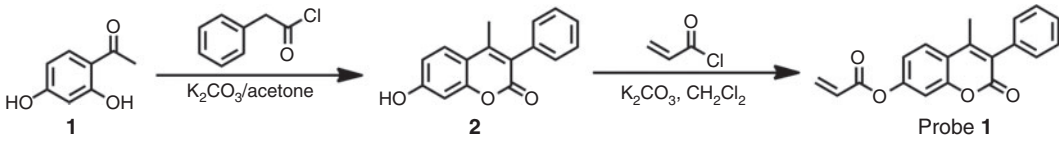
Coumarin, as a natural dye, has attracted much attention in the scientific community. When coumarin is excited by light, intramolecular charge transfer (ICT) occurs. The ICT process can be enhanced by an electron-donating group at the 7-position. In addition, a substituent group either at the 3- or 4-position also improves the ICT process via resonance and inductive effects.[15] Therefore, in this work, we designed and synthesised probe **1** (4-methyl-2-oxo-3-phenyl-2*H*-chromen-7-yl acrylate) by methyl substitution at the 4-position and phenyl substitution at the 3-position of coumarin. The ICT has been characterised (Scheme 1). Probe **1** can discriminate Cys from other thiols, even Hcy and GSH, in a short time. On the other hand, it is worth mentioning that there is an excellent dose-dependent relationship between the fluorescence intensity and concentration of Cys from 0 to 100 μM.

First, a base-catalysed Kostanecki condensation was used to synthesise 7-hydroxy-4-methyl-3-phenylcoumarin (**2**) from commercial reagents 2,4-dihydroxyacetophenone and phenylacetyl chloride.[16] Subsequently, compound **2** was reacted with acryloyl chloride to obtain **1** in 24 % yield (Scheme 2; for details and characterisation, see the Supplementary Material).



Scheme 1. Intramolecular charge transfer (ICT) process.

With probe **1** in hand, initially, its UV-vis absorption and emission spectra were measured in aqueous buffered solution (pH 7.4 phosphate buffered saline (PBS), containing 10 % CH_3CN). Upon the addition of Cys (100 μM), the maximum absorption of probe **1** (10 μM) was observed to undergo a red-shift from 315 to 380 nm (Fig. S1, Supplementary Material). Next, we investigated the fluorescence behaviour of probe **1** in the presence and absence of Cys (Fig. 1). A significant turn-on fluorescence response at 455 nm was observed with the addition of Cys. However, probe **1** exhibited a negligible fluorescence intensity in the presence of other thiol-analytes. Thus, probe **1** was capable of detecting Cys selectively. Subsequently, we carried out quantitative analysis for Cys and the results showed there was an excellent dose relationship between fluorescence intensity and concentration from 0 to 100 μM ($R^2 = 0.9983$) (Fig. 2a). This outstanding linear range is wider than most previous reports. The fluorescence detection limit of Cys was evaluated ($3s/m$, $n = 20$)[17] to 1.6×10^{-8} M, which can satisfy the detection of Cys under physiological conditions (Fig. S2, Supplementary Material). Lastly, the quantum yield of probe **1** was measured as $\Phi = 0.089$ and 0.015 in the presence or absence of Cys.[18]



Scheme 2. Synthesis of probe **1**.

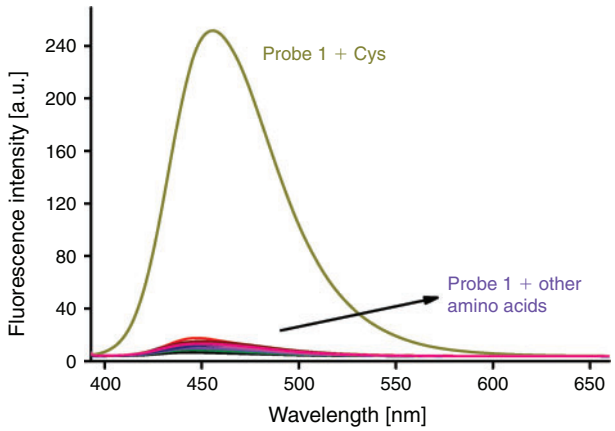


Fig. 1. Fluorescence spectra of probe **1** (10 μM) in aqueous buffered solution (pH 7.4 PBS, containing 10 % CH_3CN) with various amino acids (Cys, Hcy, GSH, *N*-acetyl-Cys, Phe, Ala, Glu, Lys, Pro, Trp, Thr, Val, Ile, Asp, Ser, Gly, DL-Met, Leu, L-Met, His, 100 μM) and incubation for 6 min at room temperature, $\lambda_{ex} = 380$ nm. Each experiment was performed in triplicate.

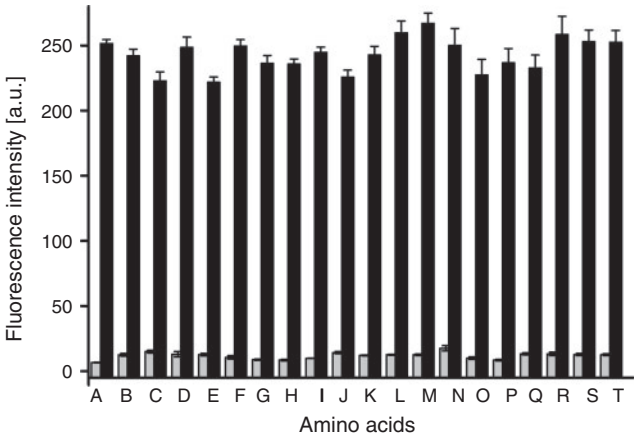


Fig. 3. Fluorescence intensity of probe **1** (10 μM) in the presence of various analytes in aqueous buffered solution (pH 7.4 PBS, containing 10 % CH_3CN), $\lambda_{ex} = 380$ nm. Grey bar: probe **1** + analytes; black bar: probe **1** + Cys + analytes; A: blank; B: Hcy; C: GSH; D: *N*-acetyl-Cys; E: Phe; F: Ala; G: Glu; H: Lys; I: Pro; J: Trp; K: Thr; L: Val; M: Ile; N: Asp; O: Ser; P: Gly; Q: DL-Met; R: Leu; S: L-Met; T: His, 100 μM. Each experiment was performed in triplicate.

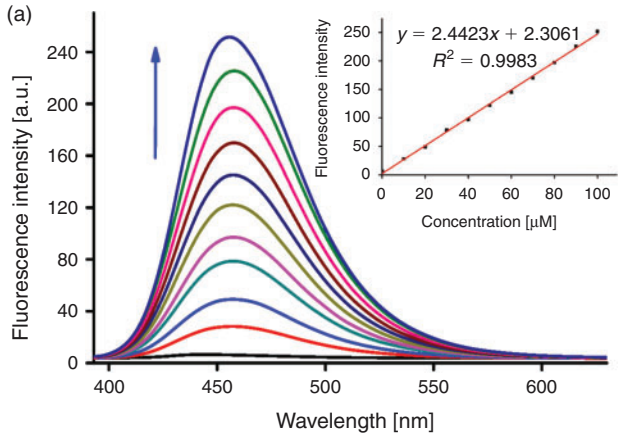
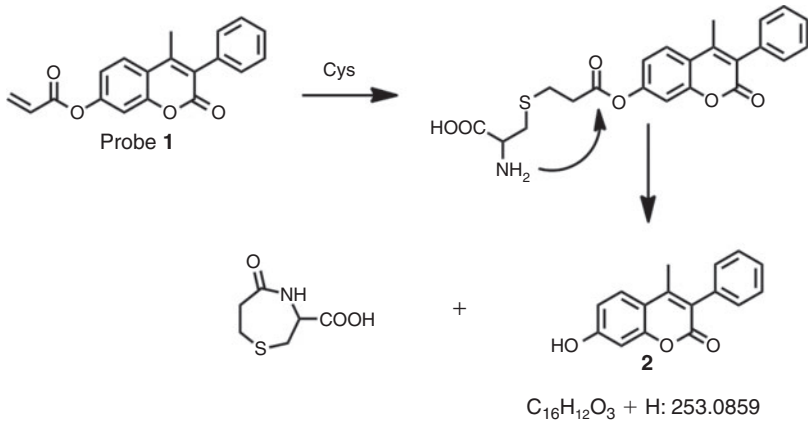


Fig. 2. (a) Emission spectra of probe **1** (10 μM) with increasing concentration of Cys (from 0 to 10 equiv.) in aqueous buffered solution (pH 7.4 PBS, containing 10 % CH_3CN), $\lambda_{ex} = 380$ nm. Inset: dose-dependent relationship between fluorescence intensity and concentration of Cys. (b) Time-dependent fluorescence intensity of probe **1** (10 μM) in the presence of Cys, Hcy, and GSH (100 μM). Each experiment was performed in triplicate.

To investigate the interaction between probe **1** and Cys, Hcy, and GSH, the time-dependent fluorescence intensity response was measured (Fig. 2b). Probe **1** exhibited insignificant fluorescent intensity which did not change as the time increased. Thus, this result suggests probe **1** is stable in aqueous buffered solution (pH 7.4 PBS, containing 10 % CH_3CN). Moreover, the fluorescent intensity increased gradually with the increase of reaction time towards Cys and approached a plateau within 25 min, which is faster than in previous reports.[14d-f] However, with the increase of reaction time towards Hcy and GSH, although the fluorescent intensities were increased, this enhancement is far lower than for Cys. Meanwhile, we estimated the pseudo-first-order rate constant (k') in aqueous buffered solution (pH 7.4 PBS, containing 10 % CH_3CN) at room temperature. The pseudo-first-order rate constants for Cys, Hcy, and GSH were measured as $k' = 1.4 \times 10^{-2}$, 4.0×10^{-4}, and $3.0 \times 10^{-4}\,s^{-1}$, respectively (Fig. S3, Supplementary Material). A difference of two orders of magnitude for the reaction rates leads to probe **1** selectively detecting Cys.

Encouraged by these results, we further evaluated the influence of other amino acids on the selective detection of probe **1** towards Cys. As shown in Fig. 3, a 38-fold enhancement in fluorescence intensity was quantified for Cys compared with the starting probe fluorescence. Markedly, when other amino acids were added, the fluorescence intensity of probe **1** for Cys had no obvious change. Notably, GSH and Hcy also did not induce a change in the fluorescence intensity of probe **1** for Cys. Thus, this result showed that probe **1** could detect Cys selectively under biological conditions. The effect of pH on fluorescence intensity was also explored (Fig. S4, Supplementary Material). First, probe **1** exhibited a very weak fluorescence intensity which showed little change over pH 2–9. However, with the



Scheme 3. Proposed reaction mechanism between probe **1** and Cys.

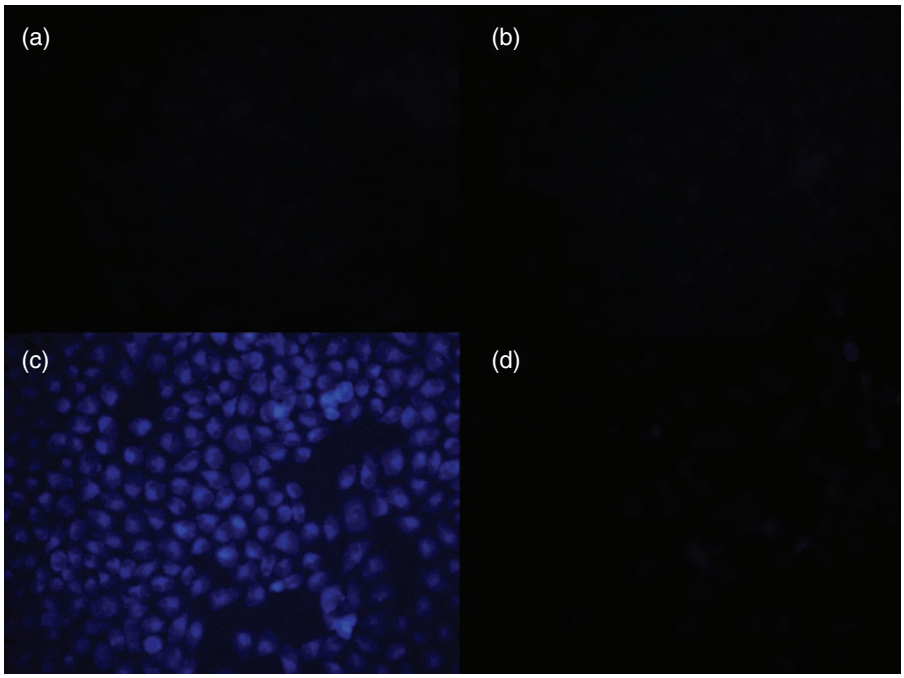


Fig. 4. Fluorescence images of probe **1**. (a) Hela cells. (b) Cells were incubated with DMSO for 1 h. (c) Hela cells were incubated with probe **1** (10 μM) for 1 h. (d) Hela cells were incubated with probe **1** (10 μM) for 1 h after pre-incubation with NEM (1 mM) for 30 min. $\lambda_{ex} = 380$ nm, scale bar = 40 μm.

addition of Cys, the fluorescence intensity showed evident enhancement over pH 7–9. Therefore, probe **1** can be used for the detection of Cys in the physiological pH range.

To explore the reaction mechanism, we analysed the mixture of probe **1** and Cys by high-resolution mass spectrometry (HRMS) whereby a peak at *m/z* 258.0856 (compound **2**, $[M+H]^+$) was clearly identified (Fig. S5, Supplementary Material). Moreover, we analysed the mixture of probe **1** and Cys by HPLC, the results of which also evidenced formation of compound **2** from the reaction between probe **1** and Cys (for analysis method, see the Supplementary Material). Based on the above a probable reaction mechanism between probe **1** and Cys is given in Scheme 3. First, conjugation of Cys to the α,β-unsaturated carbonyl moiety and subsequent intramolecular cyclisation produced compound **2** which leads to the remarkable fluorescence turn-on response of probe **1** in aqueous buffered solution (pH 7.4 PBS, containing 10 % CH_3CN).[8]

Finally, to explore the practical application of probe **1**, we incubated probe **1** with different concentrations of calf serum at 37°C. The quantitative results indicated that the fluorescence intensity increased with an increase of calf serum content (Fig. S6, Supplementary Material). In addition, we investigated the application of using probe **1** to determine the presence of Cys. The cytotoxic activity of probe **1** was measured in Hela cells by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) method[19] before bioimaging. Results showed that probe **1** (20 μM) didn't induce obvious cell death in 24 h (Fig. S7, Supplementary Material). Therefore, probe **1** (10 μM) can be safely used in cell systems in 1 h. Subsequently, we investigated the imaging of probe **1** in HeLa cells (Fig. 4). When Hela cells were incubated with probe **1** (10 μM) for 1 h, the cells presented moderately blue fluorescence. However, with pre-incubation of *N*-ethyl maleimide (NEM, 1 mM) for 30 min, the blue fluorescence didn't occur. Thus, probe **1** is capable of detecting Cys in living Hela cells by biological imaging.

In conclusion, in this work, we have designed and synthesised probe **1** on the basis of substituents at the 3/4-position of coumarin. Interestingly, probe **1** displays a remarkable fluorescence enhancement over a physiological pH range upon the addition of Cys. This provides the necessary conditions for the probe to be applied to cell systems. There is only a single methene difference between Cys and Hcy in their molecular structures, which confers difficulty for selective detection of Cys. However, results evidenced that probe **1** possesses satisfactory selectivity and sensitivity for detection of Cys. Meanwhile, there is an excellent dose-dependent relationship between fluorescence intensity and concentration of Cys from 0 to 100 μM which provides the conditions for quantitative detection of Cys in a practical application. In addition, the fluorescence intensity of probe **1** responded well to the calf serum. More importantly, its lower detection limit and bioimaging potential make probe **1** a perfect candidate for application in biological systems.

Supplementary Material

Synthesis and characterisation of probe **1** and other experimental details are available on the Journal's website.

Acknowledgements

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