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Communication

A Highly Selective Fluorescence Sensor for Cysteine/Homocysteine and Its Application in Bioimaging

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A new near-infrared (NIR) sensitive Cys/Hcy probe based on squaraine was rationally designed and synthesized. This is a Cys/Hcy sensor with excitation in the near-infrared region and features excellent selectivity for Cys/Hcy over other amino acids and GSH.

Reactive oxygen species (ROS), which are byproducts of numerous cellular events, target proteins through the oxidation of their thiol containing amino acids to form disulfide bonds.¹ As a result, ROS serve as signaling molecules to regulate the protein structure and function, which is important for various cellular processes such as proliferation, differentiation, and apoptosis.²⁻⁴ There are three mercapto biomolecules, cysteine (Cys), homocysteine (Hcy) and glutathione (GSH), that play crucial roles in maintaining biological systems. However, abnormal levels of Cys are implicated in a variety of diseases, such as liver damage, skin lesions, and slowed growth.^{5,6} Hcy is a risk factor for Alzheimer's,⁷ folate and cobalamin (vitamin B12) deficiency,^{8,9} and cardiovascular diseases (CVD).¹⁰ The normal level of homocysteine in healthy adults is in the range of 9–13 μM in the serum,¹¹ and abnormally high levels (more than 15 μM) of homocysteine in the serum result in hyperhomocysteinemia.¹² Therefore, the development of a rapid, selective and sensitive detection method for thiols in biological samples is of significant importance.

In recent times, fluorescent molecular probes have emerged as an attractive tool for selective detection of various chemical and biological components,¹³⁻¹⁶ including thiols¹⁷⁻²¹ due to their high sensitivity and operational simplicity. The fluorescence method features its low detection limit and its potential for in vivo imaging of living cells, whereas colorimetric assay exhibits inherent merits, including naked-eye detection and its real-time in situ responses, which are suitable for the analysis of biological samples.

Even though some excellent fluorescent thiols sensors have been developed such as chromene-based probes,^{22,23} nitroolefin-based probes,²⁴ acryloyl chloride-based probes,²⁵ 2,4-dinitrobenzene-based probes,²⁶ maleimide-based probes^{17,27,28} and squaraine²⁹⁻³⁴. The squaraine is one of the most attractive chromophores for a colorimetric probe due to its excellent photophysical properties. The detection mechanism of thiols by squaraines was first clarified by Ros-Lis et al.³⁵ Being electrophilic due to its electron-deficient central four-membered ring, it has been proved to react with some nucleophilic reagents such as anion cyanide and thiols.³⁶⁻³⁹ Herein, we report a new near-infrared (NIR) sensitive Cys/Hcy probe based on the nucleophilic attack of thiols to the electron-deficient central squaraine ring of **1** (Scheme 1). The investigation shows that the changes of the probe on absorbance spectra and emission spectra after reaction with thiol could be used for the detection of

Cys/Hcy.

The synthesis of probe is summarized in Scheme 1. In a 100 mL round-bottom flask equipped with a Dean-Stark trap, a (330.24 mg, 2.0 mmol) and b (114 mg, 1 mmol) were dissolved in a solution of benzene (30 mL) and n-butanol (30 mL), and the reaction mixture was refluxed over 22 h. After cooling to room temperature, a green solid obtained was separated by filtration and washed with ethyl acetate. An $\text{H}_2\text{O}/\text{CH}_2\text{Cl}_2$ solution containing the product was allowed to evaporate slowly at room temperature for several days, and the crystals that subsequently formed were suitable for X-ray crystallography. The crystal structure shows that two benzene rings and squaraine ring are coplanar, which can be explained that the probe has strong fluorescence emission. ¹H NMR (CDCl_3 , 300 MHz): δ 12.15 (s, 2H), 7.93 (s, 2H), 6.38 (s, 2H), 6.16 (s, 2H), 3.47 (s, 8H), 1.25 (s, 12H), ¹³C NMR (75 MHz, CDCl_3): δ 183.54, 173.06, 164.88, 156.22, 133.12, 110.68, 108.16, 99.18, 46.07, 23.72, 13.33, ESI-MS m/z : [probe + H]⁺ Calcd for $\text{C}_{24}\text{H}_{28}\text{N}_2\text{O}_4$ 409.21, Found 409.25. Crystal data for $\text{C}_{24}\text{H}_{28}\text{N}_2\text{O}_4$: crystal size: 0.11 \times 0.1 \times 0.09, monoclinic, space group P121/c. $a = 9.447$ (4) \AA , $b = 7.179$ (3) \AA , $c = 15.819$ (7) \AA , $\beta = 105.464$ (7)°, $V = 1034.0$ (8) \AA^3 , $Z = 2$, $T = 173.15$ K, $\theta_{\text{max}} = 27.47$ °, 8439 reflections measured, 2350 unique ($R_{\text{int}} = 0.0444$). Final residual for 198 parameters and 2350 reflections with $I > 2\sigma(I)$: $R_1 = 0.0581$, $wR_2 = 0.1588$ and $\text{GOF} = 1.226$. (Fig. 1 and Fig. S1, ESI†)

Hcy was firstly used to examine the sensing properties of probe **1**. As shown in Fig. 2, addition of Hcy to probe **1** (5 μM) solution in CH_3CN -HEEPS buffer (10 mM, pH 9.0, 1:1, v/v) resulted in fast and distinct optical changes. In the absorption spectra, a gradual decrease of the absorption peak at 645 nm was observed against time within a few minutes (Fig. 2). Meanwhile, in the fluorescence spectra, significant fluorescence quenching at around 661 nm was observed (Fig. 3). Notably, this detection process is accompanied by apparent color changes (Fig. 2, inserted), indicating that probe **1** can be used as a naked-eye diagnostic tool for rapid detection of Hcy. Upon addition of Cys to probe, a similar phenomenon both on UV-vis spectra and fluorescence spectra can be observed (Fig. S2, ESI†). However, GSH induced a negligible response for probe **1**.

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 acid (Asp), methionine (Met), valine (Val), proline (Pro) and histidine (His), as well as biothiols (Cys, Hcy, and GSH) were tested. As shown in Fig. 4, probe **1** displays significant fluorescence quenching not only towards Hcy, but also towards Cys, which indicates that probe **1** can be used to detect

these two biothiols rapidly and simultaneously. In contrast, addition of other analytes showed almost no effect. For other thiols, such as ME (2-mercaptoethanol) and MPA (Mercaptopropionic Acid), probe showed similar fluorescence responses for ME, while MPA, like GSH, induced a negligible response (Fig. S3, ESI†). Moreover, competitive experiments showed that colorimetric and fluorescence detection of Cys/Hcy by probe 1 in the presence of various amino acids is still effective (Fig. S4, ESI†). Therefore, probe 1 has high selectivity for Cys/Hcy.

To investigate the detection limit of probe for Hcy, probe (5 μM) was treated with various concentrations of Hcy (0–80 μM) and the relative emission intensity at 661 nm was plotted as a function of the Hcy concentration (Fig. 5). The emission intensity of probe was linearly proportional to Hcy concentrations of 0–80 μM . The detection limit, based on the definition by IUPAC ($C_{\text{DL}} = 3 \text{ Sb/m}$)⁴⁰, was found to be 0.067 μM . Similarly, the detection limits were estimated to be 0.059 μM for Cys (Fig. S5, ESI†).

Time-dependent modulations in the absorbance spectra of probe 1 were monitored in the presence of 10 eq. of thiol. The kinetic study showed that the reaction was complete within 60 s for Hcy and Cys, indicating that probe reacts rapidly with these two thiols under the experimental conditions (Fig. S6, ESI†). The pH range for the determination of thiol was also studied. Fig. S7 showed the fluorescence intensity obtained for the free probe and probe-Hcy in different pH values. When the solution pH was between 2.0 and 12.0, the fluorescence emission of probe is very obvious. When pH value exceeds 12.0, the fluorescence emission of probe becomes feeble. However, when the solution pH is between 4.0 and 12.0, Hcy induced an increasing fluorescence quench for probe. To get the better optical responses to Hcy/Cys, the pH 9.0 was used for further studies.

The detection mechanism is shown in Scheme 2. It involves a nucleophilic attack of Hcy/Cys to the electron-deficient central four-membered squaric acid ring in 1. The nucleophilic attack of a thiol will break the conjugation in probe 1 and induce a color fading. To examine this plausible mechanism in detail, 2-mercaptoethanol (ME), which has the similar structure with Hcy/Cys and the same response to 1, was selected for the mechanism study. The identification of stable products in the ESI-MS analysis made it possible to propose the signaling mechanism: a peak at $m/z = 525$ corresponding to $[\text{probe} + \text{ME} + \text{K}]^+$ is clearly observed (Fig. S8, ESI†). We know the GSH has more big space structure which often results in its failure in reacting with the thiol probe. Likewise, the steric hinderance can be explained the reason why GSH did not react with the squaraine probe.

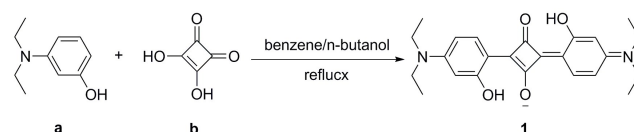
To test the capability of probe 1 to image thiols in living cells, HepG2 cells were incubated with probe 1. As shown in Fig. 6A, under selective excitation at 575 nm, HepG2 cells pretreated with N-ethylmaleimide (NEM, 20 μM , 30 min, a trapping reagent of thiol species), and then incubated with 1 (5 μM), a red fluorescence was exhibited inside the cells. Further incubated with Hcy (80 μM) for 30 min at 37 $^{\circ}\text{C}$, it was found almost no fluorescence (Fig. 6B), indicating the specific detection of Hcy by 1. These results suggest that 1 can enter cells and make fluorescent labeling.

In summary, we have demonstrated a new fluorescence probe 1 for imaging Cys/Hcy in living cells. This is a Cys/Hcy sensor with excitation in the near-infrared region and features excellent selectivity for Cys/Hcy over other amino acids and GSH. In addition, the probe features a rapid signal response time, a good linearity range and a low detection limit (0.067 μM for Hcy, 0.059 μM for Cys). Moreover, confocal lasers scanning microscopy experiments indicate that 1 can be used for

bioimaging of Cys/Hcy.

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Inserting Graphics



Scheme 1 The structure of the probe.

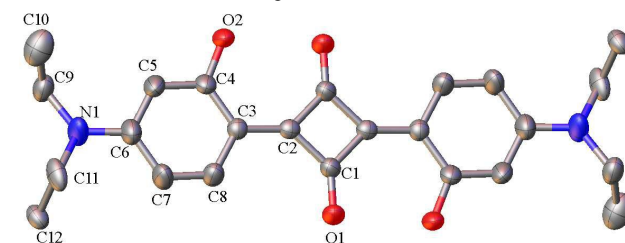


Fig. 1 The thermal ellipsoids of probe drawn at the 50% probability level.

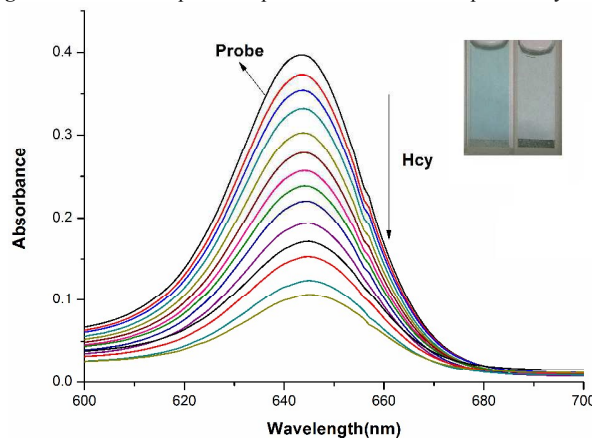


Fig. 2 UV-vis spectra of probe 1 (35 μM) in the presence of various concentrations of Hcy (0–120 μM) in CH_3CN -HEPES buffer (10 mM, pH 9.0, 1 : 1, v/v) Inset: a color change photograph for Hcy.

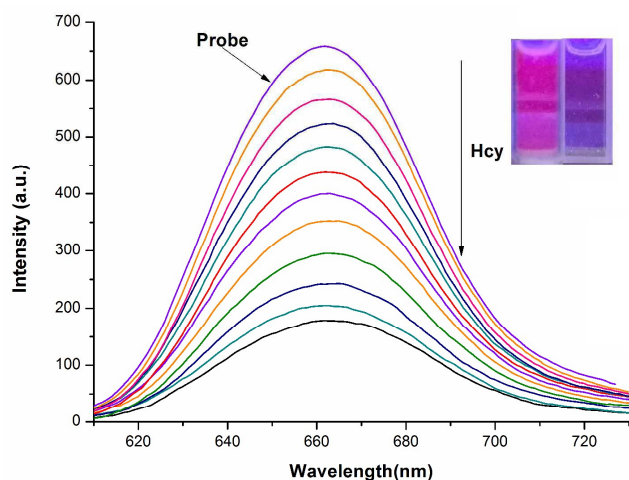


Fig. 3 The fluorescence spectra of the probe (5 μM) in the presence of various concentrations of Hcy (0–80 μM) in CH_3CN -HEPES buffer (10 mM, pH 9.0, 1:1, V/V). ($\lambda_{\text{ex}} = 575 \text{ nm}$, slit: 5 nm/5 nm).

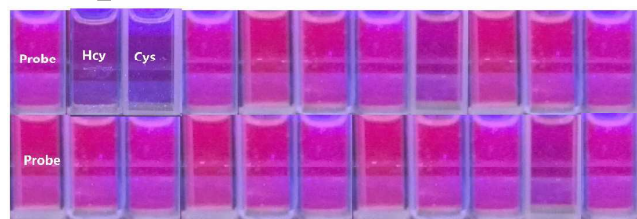
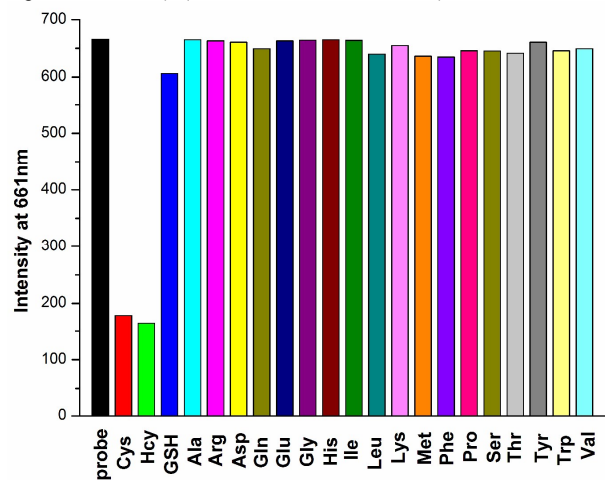


Fig. 4 Above: Optical density two-dimensional graph of the probe at 661 nm, respectively upon addition of various natural amino acids (including Hcy, Cys, Ala, Arg, Asp, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, Trp and Val). Bottom: a color change photograph for thiol and other amino acids under illumination with a 365 nm UV lamp.

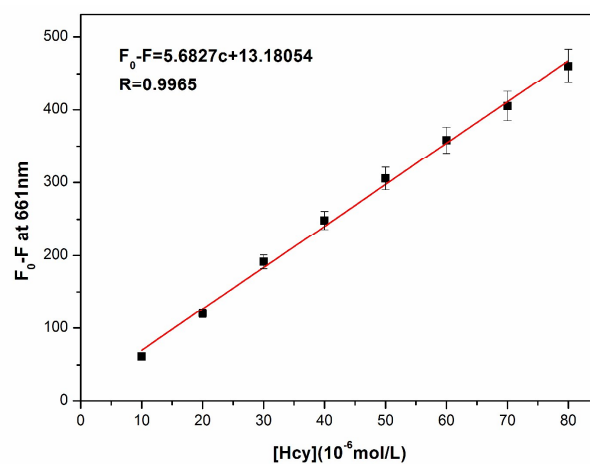
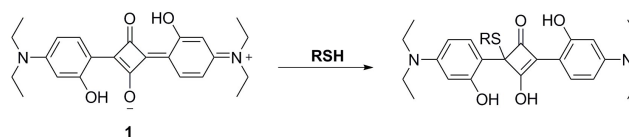


Fig. 5 The linearity of the relative fluorescence intensity versus Hcy 15 concentration.



Scheme 2. The mechanism of chemosensor.

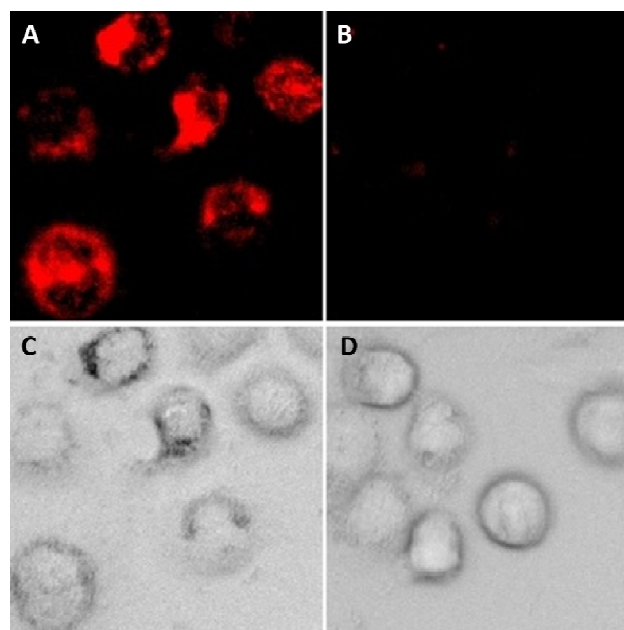


Fig. 6 Confocal fluorescence images of HepG2 cells: (A) Fluorescence image of HepG2 cells pretreated with NEM (20 μM), and then incubated with 1 μM and its bright field image (C); (B) Fluorescence image of HepG2 cells incubated with 15 μM probe after pretreated with NEM (20 μM) for 30 min at 37°C, further incubated with Hcy (80 μM) and its bright field image (D).

Notes and references

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