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ORIGINAL ARTICLE

A New Turn on Fluorescent Probe for Selective Detection of Cysteine/Homocysteine

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Abstract A new fluorescent probe (probe 1) was developed for recognition of cysteine (Cys) and homocysteine (Hcy). Probe 1 exhibited a large absorption peak blue-shift (107 nm) as well as enhanced fluorescence responses to Cys/Hcy based on cyclization of thiol containing amino acids with aldehydes, inhibiting the C=N isomerization-induced quenching process by an intramolecular hydrogen bond. The detection mechanism was proved by ^{1}H NMR and mass spectrometry analysis.

Keywords Fluorescent probe · Cys/Hcy · Ratiometric probes · Inhibiting C=N isomerization · Intramolecular hydrogen bond

Introduction

The thiol containing amino acids, such as cysteine (Cys) and homocysteine (Hcy) and glutathione (GSH) play crucial roles in numerous biological processes in living organism [1]. For example, deficiency of Cys is associated with many human diseases, such as retarted growth, hair depigmentation, edema, lethargy, liver damage, muscle and fat loss, skin lesions, and weakness [2]. An elevated level of Hcy in human plasma is a risk factor for disorders including cardiovascular diseases, Alzheimer's disease, neural tube defects, inflammatory bowel disease and osteoporosis [3]. GSH plays a critical redox homeostasis for cell growth and function [4, 5]. Therefore, the detection of biothiols in living system may help in early diagnosis and prevention of related diseases. Therefore, it is of great importance to develop efficient methods for determination of biothiols.

Among various methods for detection of biothiols, the fluorescence method based on fluorescence probe is advantageous due to its desirable features such as high sensitivity, simplicity, and potential for *in vivo* imaging [6]. Significant efforts have been devoted to construction of fluorescent probes for biothiols [7]. Many fluorescent probes involved in different detecting mechanisms have been developed for thiols detecting, such as Michael addition reaction [8–16], cyclization reaction based on aminothiols and aldehyde [17–21], cleavage reaction induced by thiols [22–30], and ligand displacement of metal complexes by thiols [31–33].

Although Cys, Hcy and GSH possess similar structures with a thiol group, they are associated with different diseases. Therefore, it is of significance to develop a fluorescent probe capable of discriminating the structural difference between these biothiols. However, the design of a highly selective detection system that can discriminate between biothiols with similar structures and reactivities is still a great challenge. In this work, we developed a new fluorescent turn-on probe (probe 1) based on 7-nitrobenz-2-oxa-1,3-diazole (NBD) fluorophore to construct a Cys/Hcy-selective sensor. NBD was chosen as the fluorophore because of its long emission wavelength, good cell permeability and applications in chemical biology and bioanalytical studies [34–36]. The aldehyde group is a specific recognition group for Cys/Hcy. It can react with Cys and Hcy to form thiazolidine and thiazinane, respectively [37, 38].

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Experimental

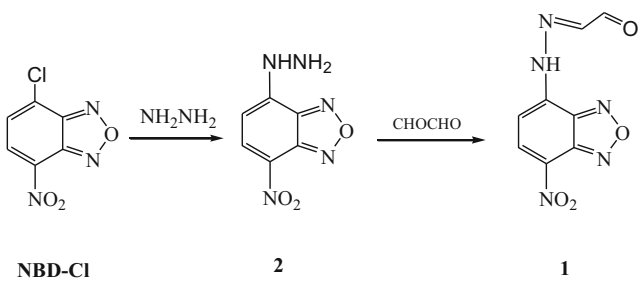
Reagents and Apparatus

Acetonitrile of spectroscopic grade and deionized water (distilled) were used throughout the experiment as solvents. All the chemicals of analytical grade for syntheses were purchased from commercial suppliers and were used without further purification. NMR spectra were recorded with a 400 MHz Varian spectrometer. Electrospray ionization mass spectra (ESI-MS) were measured on a micrOTOF-Q II system. Absorption spectra were obtained on a TU1901 ultraviolet–visible spectrophotometer. The fluorescence spectra were measured with a Cary Eclipse fluorescence spectrometer.

Syntheses

The procedure for the synthesis of probe 1 was shown in Scheme 1. Compound 2 was prepared similar to the reported procedures by using NBD-Cl and hydrazine hydrate as raw materials [39]. Hydrazine monohydrate (1.5 mL, 30 mmol) in 20 mL CH_3OH was added in dropwise to a solution of NBD-Cl (200 mg, 1 mmol) in 20 mL $CHCl_3$. The resulting solution was stirred at room temperature for 3 h. A yellowish-brown precipitate appeared gradually. It was filtrated and washed with small amount of $CHCl_3$, then dried under vacuum at 50 °C to get the desired product (140 mg, 84 %), which was used for next step without further purification.

Glyoxal, 40 % solution (*w/v*) (1 ml, 12 mmol) was added to a solution of compound 2 (195 mg, 1 mmol) in 20 mL absolute ethanol. The mixture was stirred at room temperature for 6 h. The solvent was evaporated *in vacuo*. The residue was purified by column chromatography on silica gel with ethyl acetate/hexanes (1/2, *v/v*) to afford a dark red solid of 82 mg in 35 % yield. ^{1}H NMR (DMSO-d_6, δ, ppm), 7.32(d, J=8.8 Hz, 1H, phenyl-H), 7.89 (d, J=8.0Hz, 1H, -N=CH), 8.74 (d, J=8.8 Hz, 1H, phenyl-H), 9.65 (d, J=7.6 Hz, 1H, -CHO), 13.37 (s, 1H, -NH). ^{13}C NMR (DMSO-d_6), 191.68, 145.12, 144.34, 143.86, 139.20, 136.57, 127.68, 105.77. MS (ESI-MS): m/z calculated for $C_8H_5N_5O_4$, 235.03, found: $[M-1]^+$, 233.8.



NBD-Cl 2 1

Scheme 1 General procedure for the synthesis of probe 1

Preparation of Solutions

The stock solutions (5 mM) of alanine (Ala), arginine (Arg), cysteine (Cys), glutamic acid (Glu), glutamine (Gln), glutathione (GSH), glycine (Gly), histidine (His), homocysteine (Hcy), lysine (Lys), methionine (Met), phenylalanine (Phe), serine (Ser), tyrosine (Tyr), or other thiol-containing compounds, such as mercaptopropionic acid (MPA), ethyl 2-mercaptoacetate (EMA) were prepared in deionized water. Stock solution of 1 (1 mM) was prepared in acetonitrile. PBS buffer solutions (10 mM) were prepared by using proper amount of NaH_2PO_4 and Na_2HPO_4 under adjustment by a pH S-3C meter.

In titration experiments, 1 was diluted to certain concentrations with CH_3CN-PBS (10 mM, pH=7.2, 3:1, *v/v*). Then 3 mL of 1 was put into a quartz optical cell with an optical path of 1 cm. The stock solution of each anion was added into the quartz optical cell step by step via a microsyringe and the solution was stirred for 15 min before recording the spectra.

Results and Discussion

Absorption Properties Investigation

The recognition of Cys/Hcy with 1 was investigated in a mixture of CH_3CN-PBS (10 mM, pH=7.2, 3:1, *v/v*) solution by UV–vis absorption. The solution of probe 1 displayed a distinct absorption band centered at 583 nm. Addition of 6 equiv. of non-thiol amino acids, including Arg, Phe, Met, Glu, Gln, His, Lys, Ala, Tyr, Gly, Ser, or thiol-containing compounds, such as GSH, mercaptopropionic acid (MPA), ethyl 2-mercaptoacetate (EMA), only caused a negligible change in the absorption spectra of probe 1 (Fig. 1). However, addition of Cys or Hcy resulted in decreasing of absorption peak at

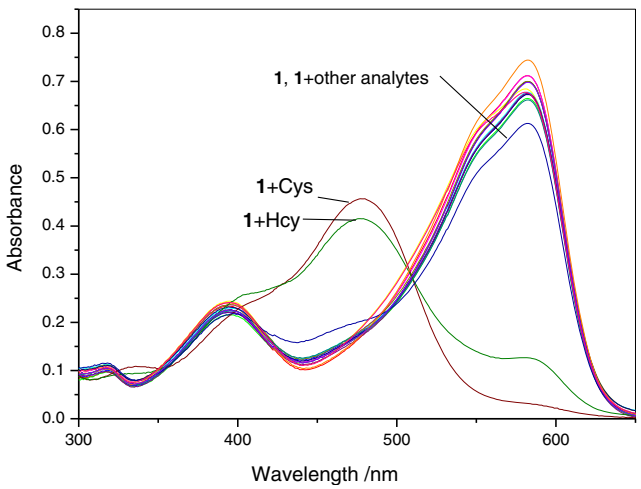


Fig. 1 Absorption spectra of 1 (30 μM) in the presence of various analytes (170 μM) in CH_3CN-PBS (10 mM, pH=7.2, 3:1, *v/v*)

Fig. 2 Photograph of 1 with addition of analytes, from left to right: blank, Glu, Arg, Phe, Met, Hcy, Gln, His, Lys, Ala, Tyr, Gly, Cys, Ser, GSH, MPA, EMA

583 nm and increasing of absorption peak at 476 nm. The results showed that the presence of most amino acids had little effect on the detection of Cys and Hcy using probe 1. The selective recognition of 1 to Cys/Hcy could also be detected by naked eye, with the color of 1-Cys or 1-Hcy turned from purple to orange, as shown in Fig. 2.

The sensing properties of probe 1 toward Cys was examined by investigating its absorption behavior. With increasing the concentration of added Cys, the absorption peak at 583 nm decreased whereas the 476 nm peak appeared and increased with a clear isosbestic point at 507 nm upon addition of Cys (Fig. 3a). The ratios of the absorbances at 476 and 583 nm varied significantly when increasing the concentration of added Cys (up to 30-fold), which was highly desirable for ratiometric probes as the sensitivity and dynamic range of ratiometric probes were regulated by the ratios. Ratiometric probes allow measurement at two different wavelengths, which could provide a built-in correction for environmental effects and improve the detection sensitivity [40–42]. The changes of absorbance at 476 and 583 nm displayed a good linear relationship with the low concentration of Cys. Figure 3b indicated the ratio of A_{476}/A_{583} was proportional to the concentration of Cys in the range 0–10 μM (A_{476} and A_{583} refer to the absorbances at 476 and 583 nm, respectively). A linear equation was obtained as A=0.229+30,365 C (C is the concentration of Cys) with R=0.998. The detection limit was calculated to be 0.31 μM. Compared to Cys, the other similar thiol-based amino acid, Hcy, has similar effect on the absorption spectral change of 1 (Fig. S4 and Fig. S5). The ratio of the absorbances at 476 and 583 nm varied less significantly than Cys when increasing the concentration of Hcy (up to 17-fold). The ratio of A_{476}/A_{583} was proportional to the Hcy concentration in the range 0–40 μM (Fig. S6). A linear equation could be obtained as A=0.2045+25284.26782 C with R=0.997. The detection limit was calculated to be 0.37 μM.

Fluorescence Titration Studies

As shown in Fig. 4a, the fluorescence of 1 was very weak in CH_3CN-PBS (10 mM, pH=7.2, 3:1, *v/v*). Upon progressive addition of Cys, the fluorescence of the solution gradually increased upon exciting at 470 nm. The emission intensities at 544 nm increased significantly with the increasing concentration of Cys. After the addition of Cys was more than 5 equivs., the fluorescence intensity at 544 nm increased slowly (up to 5.4 fold in the range from 0 to 100 μM of Cys concentration). The results could be ascribed to the Cys-triggered conversion of the aldehyde group in 1 to the thiazoline derivative, which resulted in the fluorescence recovery of the probe. A linear calibration graph of the responses of the fluorescent intensity at 544 nm to the Cys concentrations from 0 to 10 μM could be observed (insert in Fig. 4b), which indicated that

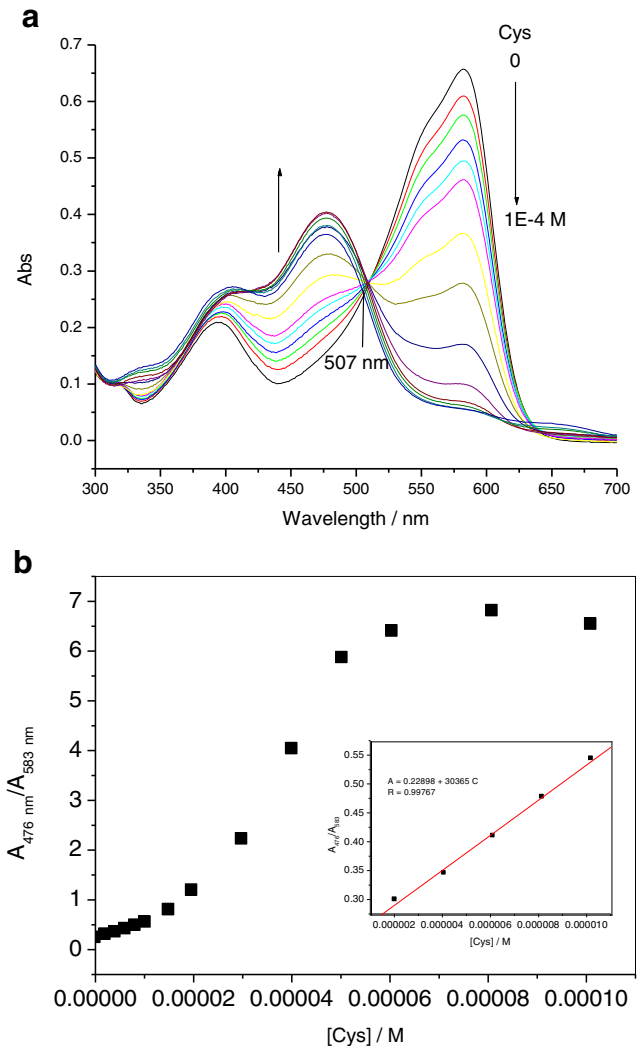


Fig. 3 **a** Absorption spectra of 1 (30 μM) in CH_3CN-PBS (10 mM, pH=7.2, 3:1, *v/v*) in the presence of Cys. **b** The absorbance ratios A_{476}/A_{583} of 1 (30 μM) with the concentration of Cys. Inset: the linear correlation for 1 against the low concentration of Cys at A_{476}/A_{583}

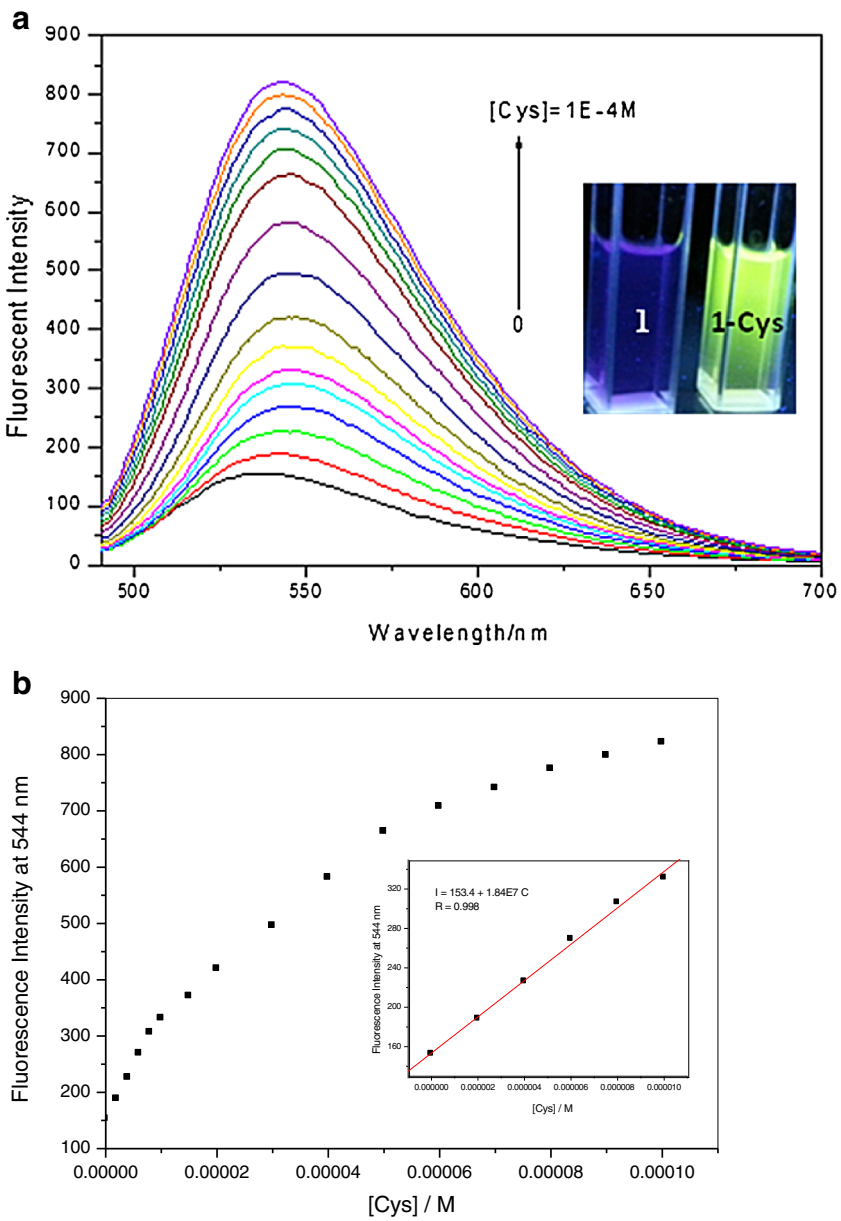


Fig. 4 **a** Fluorescence spectra of 1 (10 μM) in CH_3CN-PBS (10 mM, pH=7.2, 3:1, *v/v*) with Cys (0−100 μM). Excitation at 470 nm. Ex slit and em slit were both set at 10 nm. **b** A plot of fluorescence intensity changes of 1 at 544 nm against concentration of Cys from 0 to 100 μM. Insert: the linear correlation for probe 1 against low concentration of Cys from 0 to 10 μM

probe 1 could be potentially employed to detect Cys quantitatively. A linear equation was obtained as I=153.4+1.84×10^7 C (I represented the fluorescence intensity at 544 nm) with R=0.998. From this linear calibration graph, the detection limit of probe 1 for Cys was found to be about 3.6 nM under the test conditions. This result further proved that probe 1 was highly sensitive to Cys. The fluorescence intensity varied slightly when increasing the concentration of added Hcy (up to 1.65-fold in the concentration range from 0 to 100 μM, Fig. S7 and Fig. S8).

Selectivity Investigation

The fluorescence responses of 1 (10 μM) to various amino acids and thiol-containing compounds (17 equiv.) such as Glu, Arg, Phe, Met, Gln, His, Lys, Ala, Tyr, Gly, Ser, Hcy, Cys, GSH, MPA, EMA were also investigated. As can be seen in Fig. S9, no obvious changes in the fluorescence spectra were observed upon addition of 17 equiv. of thiol compounds and non-thiol amino acids except for Cys and Hcy. In addition, the effects of interference of the above-mentioned analytes on monitoring Cys were investigated (Fig. 5). The results verified that 1 was highly selective toward Cys/Hcy over other amino acids or thiol-containing compounds.

Response Time and pH Effect Investigation

Response time is a fundamental parameter for most reaction-based probes. The time required for the reaction of probe 1 with Cys/Hcy at 25 °C was investigated. As shown in Fig. 6,

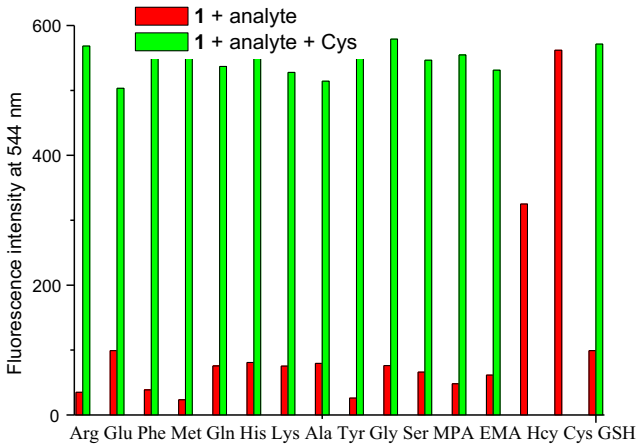


Fig. 5 Fluorescence responses of 1 (10 μM) at 544 nm in CH_3CN-PBS (10 mM, pH=7.2, 3:1, *v/v*) to various analytes (170 μM) upon exciting at 470 nm. Red bars represent fluorescence intensity of 1 with analytes. Green bars represent the subsequent addition of Cys (170 μM) to the mixture. Ex slit was set at 10 nm. Em slit was set at 2.5 nm

the fluorescence intensity at 544 nm increased with reaction time, then leveled off at reaction time greater than 15 min in both 1-Cys and 1-Hcy systems. The results revealed that the reaction of probe 1 and Cys or Hcy was completed within 15 min. Therefore, all the measurements were performed after mixing and stirring for 15 min.

The pH value of the environment around the fluorescent probe usually shows an effect on its performance toward analytes due to the protonation or deprotonation reaction of the fluorophore. The pH effect on the fluorescence intensity of 1 with and without Cys was carried out in CH_3CN: buffer (10 mM)=3:1 *v/v* (Fig. 7). The fluorescence intensity of 1 at 544 nm upon exciting at 470 nm changed little between pH 13 and 5.0, and then gradually increased from pH 5.0 to 2.5. Then the fluorescence intensity changed little at pH<2.5. The

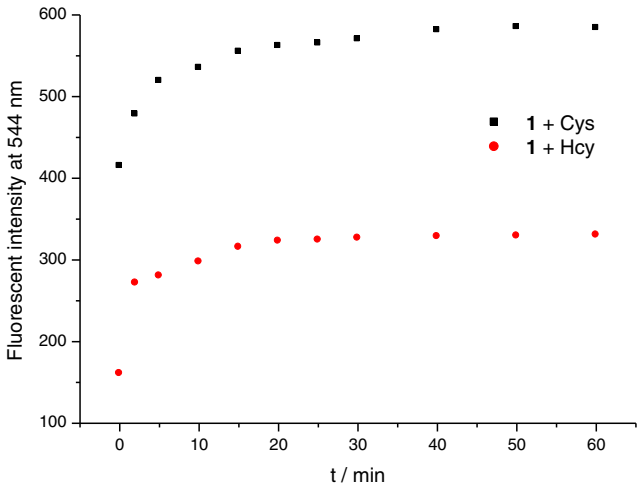


Fig. 6 The time dependence of the fluorescent intensity at 544 nm of 1 (10 μM) in the presence of Cys (170 μM) or Hcy (170 μM) in CH_3CN-PBS (10 mM, pH=7.2, 3:1, *v/v*) upon exciting at 470 nm. Ex slit was set at 10 nm. Em slit was set at 2.5 nm

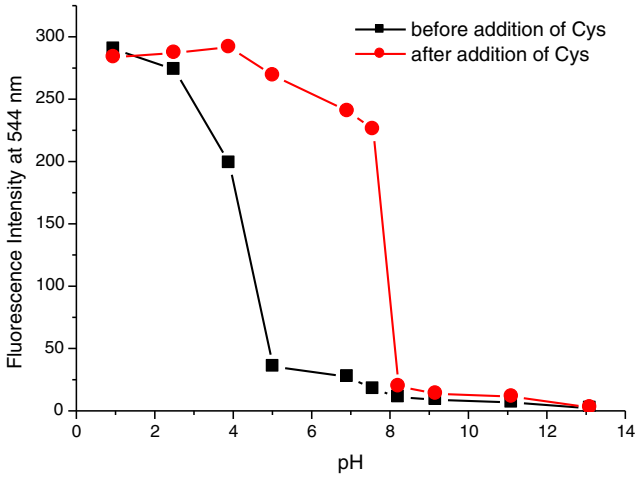
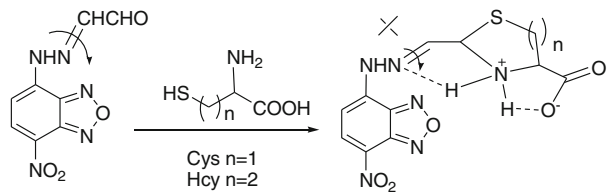


Fig. 7 Effect of pH on the fluorescence intensity at 544 nm of 1 (10 μM) in CH_3CN–buffer (10 mM) = 3:1 *v/v* upon exciting at 470 nm. Ex slit was set at 5 nm. Em slit was set at 10 nm

fluorescence intensity of 1 was very low and maintained constant at pH>7.5 before and after addition of Cys. However, in a range of pH from 5.0 to 7.5, the fluorescence intensity of 1 increased greatly in the presence of Cys. Thus the suitable pH of 1 for recognition of Cys was in the range of 5.0~7.5.

MS, ^{1}H NMR Analysis and Mechanism

In combination with the previous conclusions by other groups [17], a possible mechanism was proposed as shown in Scheme 2. The spectroscopic responses of 1 toward Cys/Hcy could be ascribed to the cyclization reaction of aldehyde group in 1 with Cys/Hcy. Due to the rapid C=N isomerization in the excited state [43–46], probe 1 showed no fluorescence. The cyclization reaction of aldehyde group in probe 1 with Cys/Hcy would result in the formation of thiazolidines/thiazinanes. The five-membered cyclic intramolecular hydrogen bond [17] between thiazolidine/thiazinane NH and the imine N atom in 1-Cys/1-Hcy would prevent the C=N bond isomerization-induced quenching, thereby causing a fluorescence enhancement of the system. The data of ESI mass spectrum provided evidence of the cyclization of Cys with probe 1 in CH_3CN/H_2O (3:1, *v/v*) solution (Figure S10 in Supplementary information). The peak at 338.34 (calcd. 338.04), corresponding to the cyclization product [1+Cys-



nonfluorescent fluorescencent

Scheme 2 Possible interaction mechanism of probe 1 and Cys/Hcy

$H_2O+H]^+$, and the peak at 360.32 (calcd. 360.03) corresponding to $[1+Cys-H_2O+Na]^+$ could be observed when Cys (3.4 mM) was added to 1 (8.5 mM), whereas free 1 exhibited a peak at 233.8, which corresponds to $[1-H]^+$ (Figure S3 in Supplementary information). The result confirmed the sensing mechanism of the probe toward Cys, which resulted in the formation of the thiazoline derivative.

The ^1H NMR spectra of 1-Cys mixture was shown in Fig. S11. The resonance signal corresponding to the aldehyde proton at 9.65 ppm disappeared. Two new peaks at 4.97 and 4.92 ppm assigned to the methine proton of the thiazolidine appeared. The peak at 5.17 nm might be assigned to the cyclic proton α to carboxylic acid. The other peaks between 8.44 and 6.38 ppm in 1-Cys mixture could also be found to shift to high field compared to the peaks between 8.74 and 7.32 ppm of 1, indicating an obvious interaction between probe 1 and Cys.

Conclusions

In summary, we have developed a fluorescent probe for the detection of Cys and Hcy over other amino acids in CH_3CN/PBS buffer (10 mM, pH 7.2). The probe displayed an enhancement in fluorescence intensity for Cys and Hcy over other amino acids in CH_3CN/PBS buffer (10 mM, pH 7.2). This probe exhibited about a 107 nm blue-shift of absorption spectra upon addition of Cys/Hcy. Importantly, this probe could detect Cys/Hcy quantitatively by ratiometric absorption method with excellent sensitivity. The spectroscopic responses of 1 to Cys/Hcy could be ascribed to cyclization of thiol containing amino acids with aldehyde group in 1, inhibiting the C=N isomerization-induced quenching process by an intramolecular hydrogen bond. The proposed mechanism was proved by MS and ^1H NMR spectra.

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