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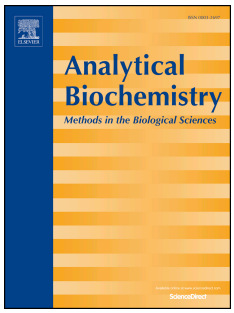

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A label-free turn on fluorescence probe for rapidly distinguishing cysteine over glutathione in water solution

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A novel label-free fluorescent chemodosimeter (C1) based on coumarin and N-(4-Aminobenzoyl)-β-alanine for the selective detection of cysteine (Cys) over glutathione (GSH) was synthesized, which was involved by click reaction of Cys to C=N of schiff base. The probe C1 featured a fast response (about 3 min), emission in the visible region and high selectivity. Addition of Cys in HEPES-NaOH solution (pH = 7.4) to C1 in water resulted in appearance of a new emission peak at 445 nm in company with remarkable enhancement of fluorescence intensity, while other amino acids did not induce any significant fluorescence change. Meanwhile, the addition reaction of Cys to C1 elicited 90.8-fold fluorescence intensity enhancement, which resulted in emission color change from orange to blue.

Keywords: fluorescence probe, turn on, coumarin, N-(4-Aminobenzoyl)-β-alanine, cysteine, Schiff base

Introduction

As one of the most important components among the bio-relevant proteins, cysteine has attracted much attention in recent years due to its crucial role in biological body, such as catalysis, heavy metal binding, protein turnover, and signal transduction. [1-4] Therefore, a number of diseases can result from abnormalities in cysteine levels. Cysteine deficiency is known to be involved in many syndromes, such as edema, lethargy, hair depigmentation, liver damage, muscle and fat loss, slowed growth, and skin lesions. [5-6] Additionally, the increased cysteine levels can lead to a higher risk of cancer, Alzheimer's, and cardiovascular diseases. [7-10] Therefore, it is of great importance to develop rapid, sensitive, selective, and quantitative analysis of the cysteine level.

To date, many strategies have been developed for the measurement of cysteine have been studied, including mass spectrometry, [11-12] high performance liquid chromatography (HPLC), [13-14] capillary electrophoresis, [15-16] optical assays, [17-18] and the electrochemical analysis. [19-20] Nevertheless, most of above-mentioned methods require professional experts, or expensive instruments, or cumbersome separation and purification procedures of samples. As a highly sensitive, non-destructive technique, fluorescence analysis has also been applied extensively for the measurement of cysteine. [21-25] However, some of them reveal some limitations in the process of detections, including the introduction of heavy metal ions, [26-28] or a long response time. More importantly, only a few sensors able to detect Cys over GSH have been reported to date. [29-31] Therefore, it is still high interest to explore low-cost and time-saving methods for measuring cysteine.

Herein we report a new turn on fluorescence probe C1 (Scheme1) based on coumarin, which can effectively discriminate Cys from GSH in aqueous solution.

Results and discussion

Selective response of probe C1 to Cys

In order to investigate the effect of various amino acids on the fluorescence spectra of C1, the amino acid Cys, His, Lys, Arg, Cys-Cys, Thr, Gly, Ala, Asp, Val, Met, Glu, Ser, GSH, Tyr and Na_2S were used to evaluate the response properties of C1 in aqueous solution (HEPES-NaOH, 10.0 mM, pH=7.4). Figure.1 shows the changes in the fluorescence spectra of C1 upon addition of various analytes. Compound C1 had a large effect only with Cys among the analytes examined. In the presence of Cys, C1 showed fluorescence enhancement (about 90.8-fold enhancement) so strong and a dramatic fluorescence color change from orange to light blue which could easily be identified by the naked eye under UV lamp during the detection process. Other analytes gave no distinct response to C1 in the fluorescence spectra. This obvious feature reveals that compound C1 has high selectivity for Cys.

Sensitivity of C1 for Cys detection

The fluorescence spectra of C1 upon titration with Cys were then performed (Fig.2). The fluorescence intensity of C1 (5.0 μM) at 445 nm was gradually increased with the addition of an increasing amount of Cys (0-11.0 μM). The enhanced intensity of C1 displayed a good linear regression relationship (Stern–Volmer equation) [32]: $y = 1.84E4[Cys]+3.50E3$ ($R^2 = 0.996$) with the concentration of Cys in the range of 0-7.0 μM based on the titration experiment (Fig.3). The limit of detection (LOD) of C1 for Cys was estimated to be as low as 0.15 μM based on $3\sigma/S$, where σ is the standard deviation of blank measurements, and S is the slope between fluorescence intensity versus sample concentration. The results indicate that the probe C1 can be used as a sensitive chemosensor for the detection of Cys in aqueous solution.

Competition experiments

To further investigate the interference of other analytes on the detection of Cys, competition experiments were performed in which various analyte (10.0 μM) were added to a solution of C1(5.0 μM) in the presence of Cys. As shown in Fig.4, all of the competing analytes tested have virtually no influence on the fluorescence detection of Cys. The result suggested that C1 could be an effective sensor for the detection of Cys in aqueous solution.

The effect of pH

The fluorescent response of probe C1 toward Cys in HEPES-NaOH buffer (10.0 mM) at different pH conditions were further investigated to apply probe C1 in complicated systems, such as environmental or biological systems. The results indicate that the response of probe C1 towards Cys was pH dependent. The fluorescence intensity of probe C1 had a relatively weak emission band at 445 nm and remained unaffected between pH levels 5.0 and 10.0, as shown in Fig.5. Upon addition of Cys, the maximal fluorescence signals were observed in the pH range of 6.6-8.0 without the interference by protons, and the stable fluorescence of the probe at a pH level of approximately 7.4 was favorable for the sensing assays of Cys in environmental and biological samples.

Response time studies

Response time is also an important property of fluorescence probe. The time course for fluorescence intensity changes of probe C1 (5.0 μM) in the presence of Cys (10.0 μM) at 445 nm (excited at 380 nm) was investigated (Fig.6). The result showed that the fluorescence response occurs immediately after addition of Cys, and the recognition interaction completed within 3 min, indicating that probe C1 might be used for the real-time monitoring of Cys conveniently.

Reaction mechanism

Coumarin was selected as the fluorophore due to its desirable photophysical properties, such as a large Stokes' shift, and visible emission wavelengths. The N atom of C=N acts as an electron donor for the photo-induced electron transfer (PET) process from C=N to coumarin ring, leading to a low fluorescence of probe C1. After adding Cys, the double bond C=N is broken, and blocking of the PET process can cause fluorescence recovery. In order to investigate the mechanism we contrastively analysed the ^{1}H NMR spectra (Fig.7). With addition of Cys to probe C1 in D_2O, the CH=N protons at 9.14 ppm (H1) of probe C1 dramatically disappear while new peaks at 3.80 ppm (H1') and 3.90 ppm (H2) were observed. The changes of these peaks should be attributed to the mercapto group of Cys added to the carbon atom of C=N group with shift from CH=N to CH-NH. Therefore, the CH=N proton (H1) shifted to high-field and a peak appeared at 3.90 ppm because of the existence of the NH proton of CH-NH.

Experimental

Apparatus and Chemicals

Melting point was determined using an SGW X-4 digital melting point apparatus (Shanghai Instrument Physical Optics Instrument Co., Ltd). 1H NMR spectrum was run on a Varian Mercury-Plus 400 NMR spectrometer using TMS as the internal standard (Varian, San Diego). Elemental analysis was taken with a Vario EL CHNS elemental analyzer (Elementar, Germany). Mass spectrum was recorded with a VG ZAB-HS double focusing mass spectrometer (Thermo Onix Ltd, UK). Fluorescence spectra, fluorescence lifetime and quantum efficiency were measured with a Fluorolog 3-TSCPC (Horiba Jobin Yvon Inc. France). All chemicals were purchased from Aladdin Industrial Corporation (China) and used without further purification. The solvents were purified using standard methods.

All solutions of C1 (5.0 μM) and various amino acid and Na_2S (10.0 μM) were prepared in HEPES-NaOH buffer solution (10.0 mM, pH 7.4) and allowed to equilibrate at room temperature for 3 minutes before spectral measurements. Every measurement was performed three times at same testing conditions in a quartz cell with a 1 cm path length with 380 nm excitation wavelength.

Synthesis of 8-formyl-7-hydroxy-4-methylcoumarin

A solution of 8-Formyl-7-hydroxy-4- methylcoumarin was prepared by the known method. [33] 7-Hydroxy-4-methylcoumarin (5.0 g, 0.0284 mol) and hexamine (10.0 g, 0.071 mol) in acetic acid (37 mL) were stirred for 5.5 h at 95 ºC. Then, hydrochloric acid (75 mL, HCl: H_2O = 84:100, v/v) was added and further heated for 45 min. After cooling, the mixture was poured into ice-water (375 mL) and extracted with ethyl acetate (150 mL×3). The organic layer was dried over

sodium sulfate and the solvent removed. The residue was purified by column chromatography on silica gel using dichloromethane as eluent to provide the product as a light yellow solid. Yield 0.9 g (15.5 %). ^{1}H NMR ($CDCl_3$, δ ppm): 2.45 (s, 3H); 6.22 (s, 1H); 6.92 (d, J = 9.2 Hz, 1H); 7.74 (d, J = 8.8 Hz, 1H); 10.63 (s, 1H); 12.23(s, 1H). MS m/z: 205$[M+H]^+$.

Synthesis of 7-hydroxy-4-methyl-8-(4′-(N-carboxylethylformamide)phenylimino)methyl-2H-1-benzopyran-2-one (C1)

8-formyl-7-hydroxy-4-methylcoumarin (0.21 g, 0.1 mmol) and N-(4-Aminobenzoyl)-β-alanine (0.20 g, 0.11 mmol) were dissolved in anhydrous ethanol (20 mL). The reaction mixture was refluxed for 2 h then the mixture was cooled to room temperature. The precipitate was filtered off, washed with cold ethanol two times and dried in vacuum to give the desired product as a yellow solid. Yield 0.34 g (85.1 %). m. p 265.4-267.2℃. ^{1}H NMR (d_6-DMSO, 400MHz, δ ppm): 2.33 (s, 3H); 2.51-2.55 (t, J = 8.0, 2H) ; 3.44-3.48 (t, J = 8.0, 2H); 6.17 (s, 1H); 6.84-6.86 (d, J = 8.0, 1H), 7.50-7.52 (d, J = 8.0, 2H); 7.70-7.72 (d, J = 8.0, 1H); 7.92-7.94 (d, J = 8.0, 2H); 8.60 (s, 1H); 9.14 (s, 1H); 12.20 (s, 1H), 14.65 (s, 1H). ^{13}C NMR (d_6-DMSO, δppm): 18.3, 33.7, 35.6, 106.0, 110.4, 110.9, 114.0, 121.2, 128.6, 130.6, 133.0, 148.5, 153.8, 154.0, 157.6, 159.0, 165.2, 165.2, 172.9.

Conclusions

In conclusion, a new label-free and Cys-specific probe was successfully developed. The probe showed a rapid response and selectivity for detection of Cys over GSH. Addition of Cys in HEPES-NaOH (pH = 7.4) to C1 in water resulted in a fluorescence intensity enhancement about 90.8-fold, while other amino acids did not induce significant emission change. In addition, it is worth noting that these fluorescence experiments were performed in a pure aqueous solution, which is different from other probes determined in organic solvents or mixed solutions, and has potential application in the detection and analysis of various Cys-related issues in biological and medical areas.

Acknowledgements

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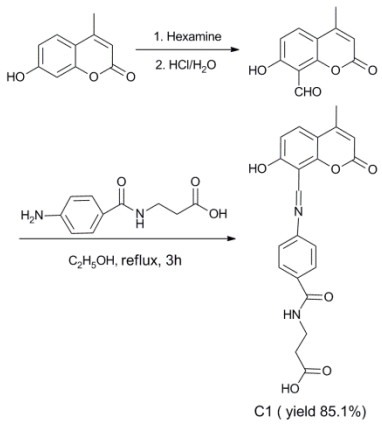
Scheme and Figures



Scheme 1 Synthetic route of compound C1

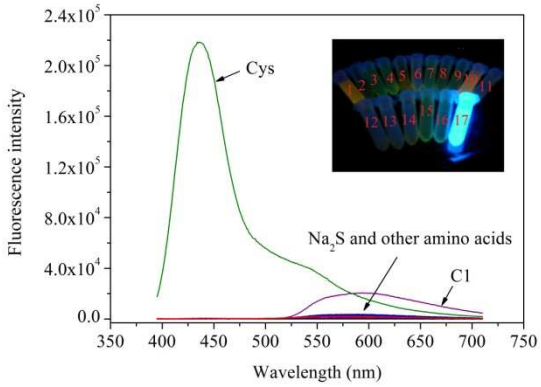


Fig.1. Fluorescence emission and fluorescence color changes (Insert) of C1 (5.0 μM) upon addition of various analytes (10.0 μM) in aqueous solution. (HEPES-NaOH, 10.0 mM, pH = 7.4, λ_{ex} = 380 nm). Analytes:1: C1, 2: His, 3: Lys, 4: Arg, 5: Cys-Cys, 6: Thr, 7: Gly, 8: Ala, 9: Asp, 10: Na_2S, 11: Met, 12: Glu, 13: Ser, 14: GSH 15: Val, 16: Tyr, 17: Cys.

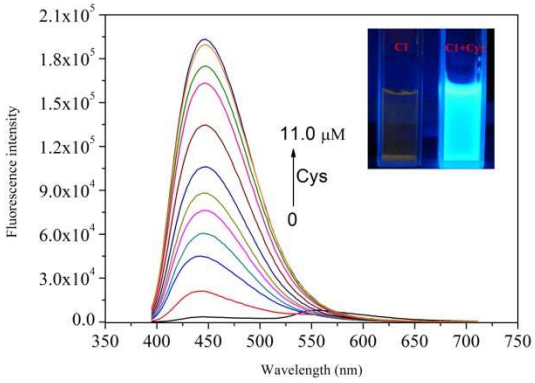


Fig.2. The change in fluorescence intensity of probe C1 (5.0 μM) at 445nm against varied concentrations of Cys from 0 to 11.0 μM in aqueous solution. Insert: The fluorescence colors of C1 (5.0 μM) and C1 (5.0 μM)+Cys (11.0 μM) in aqueous solution under 365 nm UV irradiation. (HEPES-NaOH, 10.0 mM, pH = 7.4, λ_{ex} = 380 nm).

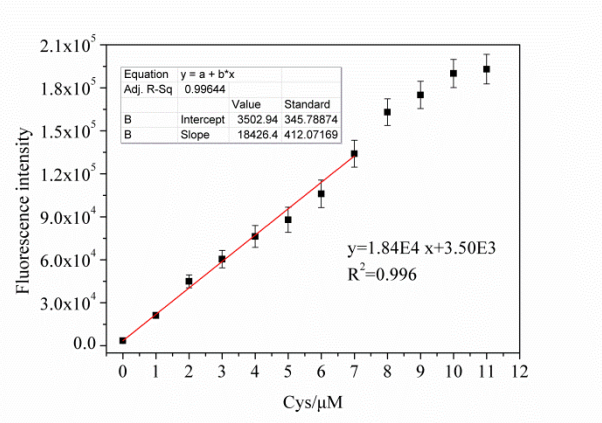


Fig.3. Calibration curve based on fluorescence intensities (445 nm) as a function of Cys concentrations in aqueous solution (HEPES-NaOH, 10.0 mM, pH = 7.4, λ_{ex} = 380 nm).

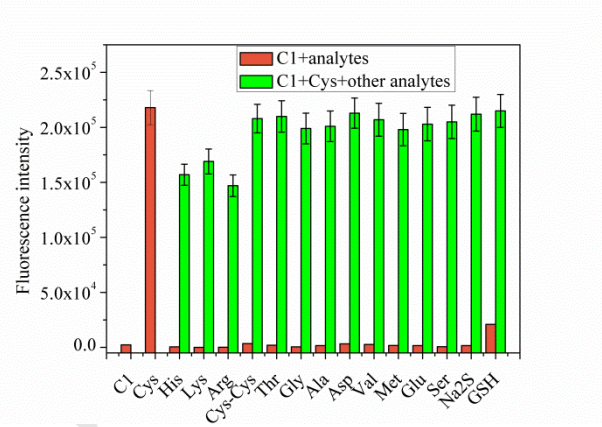


Fig.4. Changes in fluorescence intensity of C1 (5.0 μM) toward Cys (2.0 equiv.) in the presence of the competing analytes (amino acids and Na_2S, 2.0 equiv., respectively), λ_{ex} = 380 nm, λ_{em} = 445 nm, HEPES-NaOH, 10.0 mM, pH = 7.4.

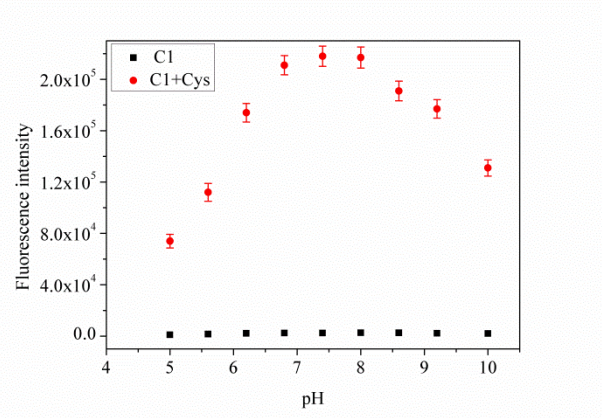


Fig.5. The fluorescence intensity (445 nm) of probe C1 (5.0 μM) with 10.0 μM Cys was obtained at different pH values (HEPES-NaOH, 10.0 mM, λ_{ex} = 380 nm).

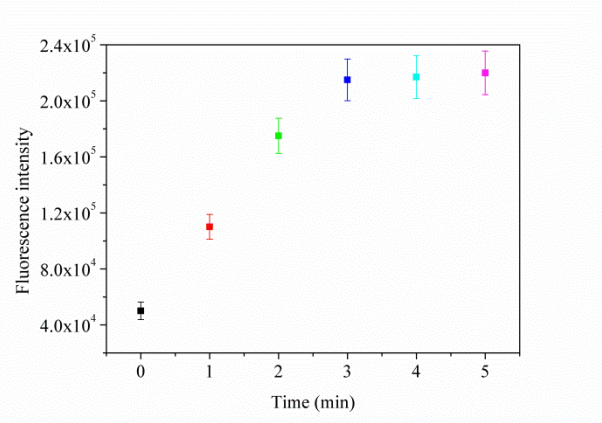


Fig.6. Time dependent fluorescence emission (445 nm) change of C1 (5.0 μM) upon addition of Cys (10.0 μM) in HEPES-NaOH buffer solution, λ_{ex} = 380 nm.

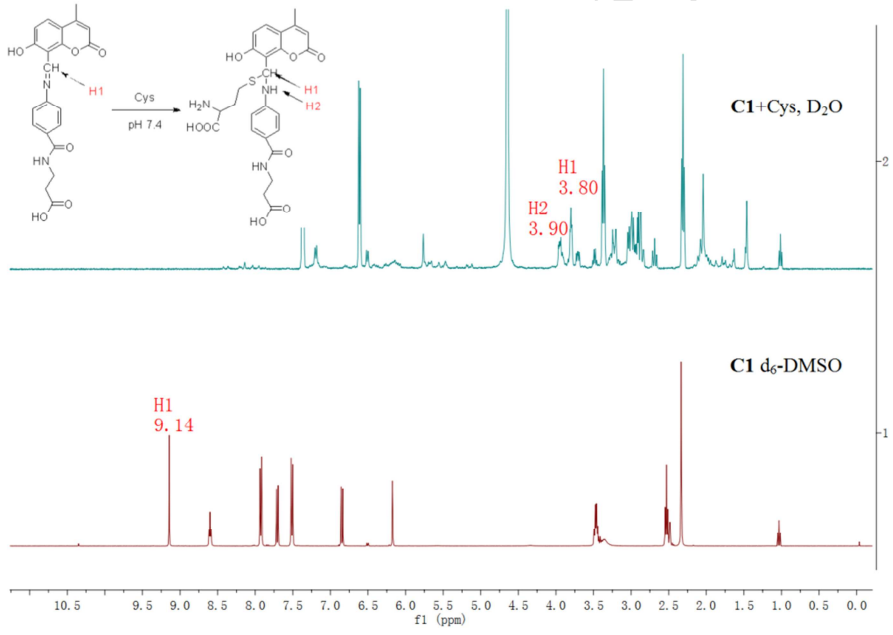


Fig.7. The comparative trial of H NMR spectra of C1 (d_6-DMSO, 400 MHz) and C1+1.0 equiv. Cys (D_2O, 400 MHz).