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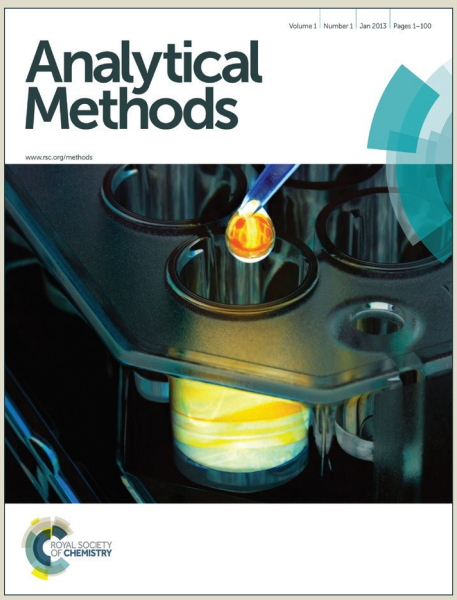

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ARTICLE TYPE

Fluorescence turn-on detection of cysteine over homocysteine and glutathion based on "ESIPT and AIE"

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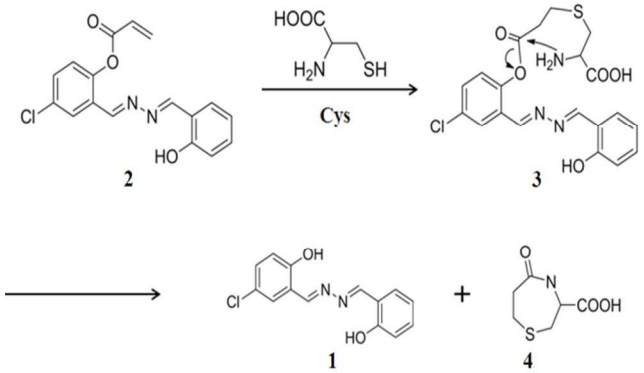
4-Chloro-2-(((2-hydroxybenzylidene)hydrazono)methyl)phenol (**1**) was reported exhibiting typical aggregation-induced emission (AIE) characteristics in our previous work. Here we introduce acryloyl to the hydroxyl moiety of **1** and developed **2** (4-chloro-2-(((2-hydroxybenzylidene)hydrazono)methyl)phenyl acrylate) for fluorescence turn-on detection of cysteine (Cys). ^{1}H-NMR and mass spectrometry data of the products revealed that reaction between **2** and Cys resulting in the formation of **1** with excited-state intramolecular proton transfer (ESIPT) and AIE property. With fluorescence enhancement detection by **2**, the linear range and detection limit for Cys were obtained as 0~30 μM (R^2 = 0.998) and 0.46 μM respectively with satisfied selectivity over homocysteine (GSH), glutathione (Hcy) and other amino acids. The method was also used for Cys detection in a serum sample.

1. Introduction

Cysteine (Cys) is one of the most abundant biothiols in living organisms, and plays important roles in biological systems[1]. It involves in protein synthesis, maintaining biological redox homeostasis, post-translational modifications, metal binding, and detoxification. Also, alterations in the level of Cys can cause many diseases. For example, elevated levels of Cys may lead to neurotoxicity, and low Cys levels is associated with slowed growth rate, hair depigmentation, edema, lethargy, liver damage, muscle and fat loss, skin lesions, and weakness[2, 3]. Therefore, it is important to develop highly sensitive and selective detection methods for Cys in biological systems.

As to the detection of biologically relative molecules, fluorescent method is still considered to be a powerful analytical tool due to its simplicity, sensitivity, selectively, and visualization. To date, michael additions[4-6], cyclization reactions with aldehydes[7-9], cleavage reactions[10-12] and others[13-18] have been utilized to construct the fluorescent probes for Cys, in which organic dyes were selected as the fluorescent units. Among these fluorescent methods for Cys detection, fluorescence probes with aggregation-induced emission (AIE) property are catching increasingly attention[19-22]. Compared with most fluorophores suffering from aggregation-caused quenching (ACQ) of their emissions at high probe concentration or in a poor solvent, fluorescence probes with AIE property as anti-ACQ materials emit strong fluorescence in "aggregate" state. However, the reported AIE fluorescence probes for Cys still have some limitations, including poor sensitivity[20, 21] and incapable of discriminating Cys with other sulfhydryls (homocysteine and glutathione)[19, 22]. Therefore, there is still a huge space to develop novel AIE fluorescent probes for the selective detection of Cys with high sensitivity.

In our previous works, a series of salicylaldehyde azine derivatives with AIE characteristics have been developed[23-27]. Two salicylaldimine moieties in these molecules were connected by N-N single bond, and the schiff base bearing o-hydroxyl group on the benzene ring was introduced to form intramolecular hydrogen bond, which allowed the excited-state intramolecular proton transfer (ESIPT) process[28, 29]. Under well-dispersed solution state, intramolecular hydrogen bonds assisted two salicylaldimine moieties to rotate freely around the N-N single bond, which resulted in nonradiative intramolecular rotation decay of excited molecules, and salicylaldehyde azine derivatives showed weak emission. Under aggregate state, intramolecular rotation of N-N single bond would be restricted and ESIPT process would be maintained, thus, strong fluorescence could be obtained.



Scheme 1 Cyclization reaction of **2** with Cys followed by hydrolysis to give the final AIE product **1**.

In this paper, we developed a new AIE probe 4-chloro-2-(((2-hydroxybenzylidene)hydrazono)methyl)phenyl acrylate (**2**) for the detection of Cys by protecting one OH-moiety of a N, N'-salicylaldehyde azine derivative, 4-chloro-2-(((2-hydroxybenzylidene)hydrazono)methyl)phenol (**1**) with acryloyl which is known as a selective reaction group of Cys[30]. As a result of destroy the hydrogen bonding and the free rotation of the N-N bond[31-35], the probe **2** showed very weak emission in 30% DMSO-water solution. Upon addition of Cys, the reaction of Cys with **2** induced hydrolysis of acrylate and resulted in **1**, thereby enabling the ESIPT process and turning on the AIE (Scheme 1). ^{1}H-NMR and mass spectrometry data of the products supported this fluorescence sensing mechanism. The method exhibited good selectivity to Cys over homocysteine, glutathione, and other amino acids, with a linear range of 0~30 μM and limit of detection as 0.46 μM. The method was then applied to the detection of Cys in a fetal bovine serum (FBS) sample.

2. Experimental

2.1 Apparatus

Fluorescence spectral measurements were recorded on a JASCO FP-6500 spectrofluorimeter (Tokyo, Japan) equipped with a xenon discharge lamp, 1 cm quartz cells. All pH tests were made with a Model pHS-3C pH meter (Shanghai, China). NMR spectra were measured using a JOEL JNM-ECA300 spectrometer operated at 300 MHz. Electrospray ionization-mass spectra were obtained on a high performance 1100 liquid chromatography-mass spectroscopy spectrometer (Agilent Technologies, Kyoto, Japan) without using the liquid chromatography part. Elemental analyses were carried on a FLASH EA1112 elemental analyzer. All of the measurements were operated at room temperature of 298 K.

2.2 Reagents

All reagents and solvents used in this paper were of analytical grade without further purification. 5-Chlorosalicylaldhyde, acryloyl chloride, salicylaldehyde hydrazone and N-ethyl maleimide (NEM) were purchased from Alfa Aesar Co. (Tianjin, China). Other reagents were purchased from Sinopharm Chemical Reagent Beijing Co., Beijing, China. Absolute dimethyl sulfoxide (DMSO) of analytical grade and deionized water were used throughout the experiment. The 10 mM stock solution of the dye reagent was prepared by dissolving the compound in DMSO.

2.3 Analytical procedure

For Cys detection, to a 5.0 mL flask with PBS buffer (10 mM, pH 7.4, 30% DMSO) containing different amount of Cys, 15 μL of the **2** stock solution was added to each flask by a pippet to obtain 30 μM **2** solution. After well mixed, the solution was allowed to stand at room temperature for 40 min. Then 2.0 mL of the solution was transferred to a 1 cm cell for fluorescence measurement by excitation/emission at 400/558 nm.

Cys detection in fetal bovine serum (FBS) was carried out as follows. FBS was reduced with triphenylphosphine to convert disulfides in FBS to free thiols, and proteins present in the sample after reduction were removed by a precipitation procedure according to the literature[36, 37]. Then FBS was diluted 30 times in PBS buffer (10 mM, pH 7.4, 30% DMSO). Then 15 μL of the **2** stock solution was added. After well mixed, the solution was allowed to stand at room temperature for 40 min. Then 2.0 mL of the solution was transferred to a 1 cm cell for fluorescence measurement by excitation/emission at 400/558 nm.

2.4 Procedures for Synthesis

4-Chloro-2-(((2-hydroxybenzylidene)hydrazono)methyl)phenol (**1**). In a 25 mL flask 0.16g (1 mmol) of 5-chlorosalicylaldhyde was dissolved in 10 mL of ethanol. Salicylaldehyde hydrazone (0.14g, 1 mmol) was then added with vigorous stirring at room temperature. After the addition, the stirred mixture was allowed to stand at room temperature overnight, and the resulting precipitation was filtered and washed by 6 mL of ethanol 3 times. After drying in vacuo a yellow product was obtained (yield, 72.5%). ^{1}H-NMR (d6-DMSO) δ (ppm): 11.16(s, 1H, OH), 11.07(s, 1H, OH), 9.02 (s, 1H, CH = N), 8.95 (s, 1H, CH = N), 7.77 (d, 1H, Phen-H, J = 2.6 Hz), 7.71 (dd, 1H, Phen-H, J_1 = 7.9 Hz, J_2 = 1.8 Hz), 7.41 (m, 2H, Phen-H). ^{13}C-NMR ($CDCl_3$) δ (ppm): 163.7, 161.0, 159.2, 157.8, 133.9, 133.1, 131.2, 129.3, 123.7, 120.5, 120.2, 119.0, 118.7, 117.1. Anal. Calcd for $C_9H_9BrO_3$: C, 61.21; H, 4.04; N, 10.20. Found: C, 61.22; H, 4.19; N, 10.21.

4-Chloro-2-formylphenyl acrylate. A mixture of 1.56 g (10 mmol) of 5-chlorosalicylaldhyde and 0.978 mL (12 mmol) of acryloyl chloride was stirred in the presence of 1.66 g (12 mmol) of K_2CO_3 in 10 mL of dry acetone for 10 h. At the end of this period, 50 mL of water was added to the mixture with vigorous stirring, and then the solvent was extracted with 3×60 mL portions of CH_2Cl_2. The combined dichloromethane extracts were then purified by flash chromatography (petroleum ether : ethyl acetate = 8 : 1) to give a white solid (yield, 71.6%). ^{1}H-NMR (d6-DMSO) δ (ppm): 10.00 (s, 1H, CH = O), 7.97 (d, 1H, Phen-H, J = 2.7 Hz), 7.84 (dd, 1H, Phen-H, J_1 = 2.7 Hz, J_2 = 8.6 Hz), 7.45 (d, 1H, Phen-H, J = 2.7 Hz), 6.59 (m, 1H, J = 17.2 Hz), 6.45 (m, 1H, J_1 = 17.2 Hz, J_2 = 10.1 Hz), 6.22 (m, 1H, J = 10.1 Hz). ^{13}C-NMR ($CDCl_3$) δ (ppm): 189.3, 164.3, 149.7, 135.7, 135.0, 131.6, 131.2, 129.7, 127.6, 126.5. Anal. Calcd for $C_{10}H_7ClO_3$: C, 57.03; H, 3.35. Found: C, 57.10; H, 3.41.

4-Chloro-2-(((2-hydroxybenzylidene)hydrazono)methyl)phenyl acrylate (**2**). In a 25 mL flask 0.21g (1 mmol) of 4-chloro-2-formylphenyl acrylate was dissolved in 10 mL of ethanol. Salicylaldehyde hydrazone (0.14g, 1 mmol) was then added with vigorous stirring at room temperature. After the addition, the stirred mixture was allowed to stand at room temperature overnight, and the resulting precipitation was filtered and washed by 6 mL of ethanol 3 times. After drying in vacuo a yellow product was obtained (yield, 61.7%). ^{1}H-NMR (d6-DMSO) δ (ppm): 10.99(s, 1H, OH), 8.89 (s, 1H, CH = N), 8.72 (s, 1H, CH = N), 8.04 (d, 1H, Phen-H, J = 1.7 Hz), 7.71 (d, 1H, Phen-H, J = 5.8 Hz), 7.67 (dd, 1H, Phen-H, J_1 = 1.9 Hz, J_2 = 6.5 Hz), 7.41 (dd, 1H, Phen-H, J_1 = 1.7 Hz, J_2 = 4.2 Hz), 7.39 (d, 1H, Phen-H, J = 4.2 Hz), 6.97 (dd, 1H, Phen-H, J_1 = 1.9 Hz, J_2 = 5.8 Hz), 6.94 (d, 1H, Phen-H, J = 6.5 Hz), 6.61 (m, 1H, J = 12.6 Hz), 6.50 (m, 1H, J_1 = 12.6 Hz, J_2 = 7.9 Hz), 6.23(m, 1H, J = 7.9 Hz). ^{13}C-NMR ($CDCl_3$) δ (ppm): 164.5, 164.0, 159.2, 156.5, 148.8, 134.8, 134.0, 132.6, 131.3, 131.2, 128.5, 128.3, 127.9, 126.2, 120.1, 118.7, 117.1. Anal. Calcd for $C_{17}H_{13}ClN_2O_3$: C, 62.11; H, 3.99; N, 8.52. Found: C, 61.28; H, 3.97; N, 8.21.

3. Results and discussion

3.1 Fluorescence turn-on detection of Cys

The AIE characteristics of **1** in DMSO/water mixed solvent had been demonstrated in our previous work and **1** showed aggregation induced emission around 558 nm in DMSO concentration less than 70%[27]. In this work we designed a new probe **2** by protecting one OH-moiety of **1** with acryloyl group. As a result of destroy the hydrogen bonding and the free rotation of the N-N bond[31-35], 30 μM of probe **2** showed very weak fluorescence at 558 nm in 30% DMSO containing PBS buffer (10 mM, pH 7.4). However, when increasing amounts of Cys were added to the solution, fluorescence intensity at 558 nm gradually increased (Fig. 1). Additionally, as indicated in Fig. S1, the fluorescence intensity of **2** was linearly proportional to Cys concentrations of 0~30 μM (y = 0.54x + 2.60, R^2 = 0.998), and the detection limit based on IUPAC (C_{DL} = 3 S_b/m)[38] was determined to be 0.46 μM (n = 10), which is comparable to other methods based on conjugate addition-cylization reaction[8, 30-32, 39-48].

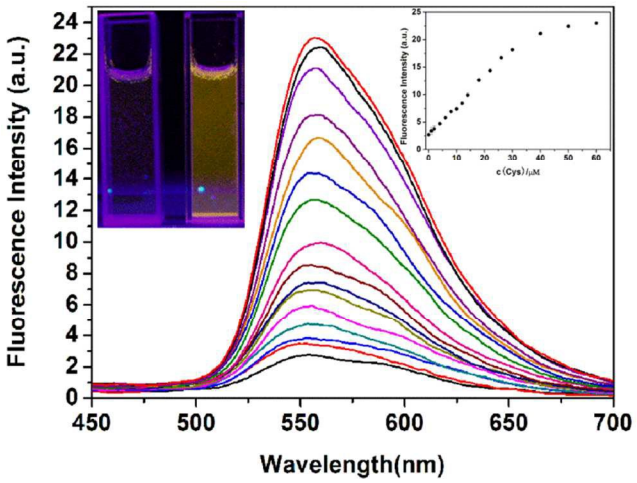


Fig. 1 Fluorescence spectra of 30 μM **2** in the presence of different amounts of Cys (from 0 to 60 μM) in PBS buffer (10 mM, pH = 7.4, 30% DMSO), λ_{ex} = 400 nm. Inset: photographs of **2** (30 μM) before and after addition of Cys (30 μM) under a UV lamp (365 nm) and the fluorescence intensity at 558 nm as a function of Cys concentration.

3.2 Mechanism of fluorescence turn-on response towards Cys

According to the literature, an intramolecular cyclization reaction between acrylate and Cys would break the acryloyl group in probe **2**[30], resulting in the formation of **1** (Scheme 1), which could light-up by intramolecular hydrogen bond formation to activate the ESIPT process and aggregation in the buffer to activate the AIE process. To further verify the fluorescence turn-on response of **2** towards Cys was due to the formation of **1**, we examined the ^{1}H-NMR spectra of **2**, **2**-Cys reaction products, and **1** at room temperature. As seen in Fig. 2, after addition of 1 equiv. of Cys in a solution of d6-DMSO/D_2O (9 : 1) with **2** for 30 min, the characteristic alkenyl proton H_a, H_b, H_c from 6 to 7 ppm disappeared completely, accompanying by signal formation in agreement with those of compound **1**, which suggested that **1** was formed after Cys added. Furthermore, mass spectral analysis of the reaction products of **2** and Cys also confirmed the formation of **1** (Fig. S2).

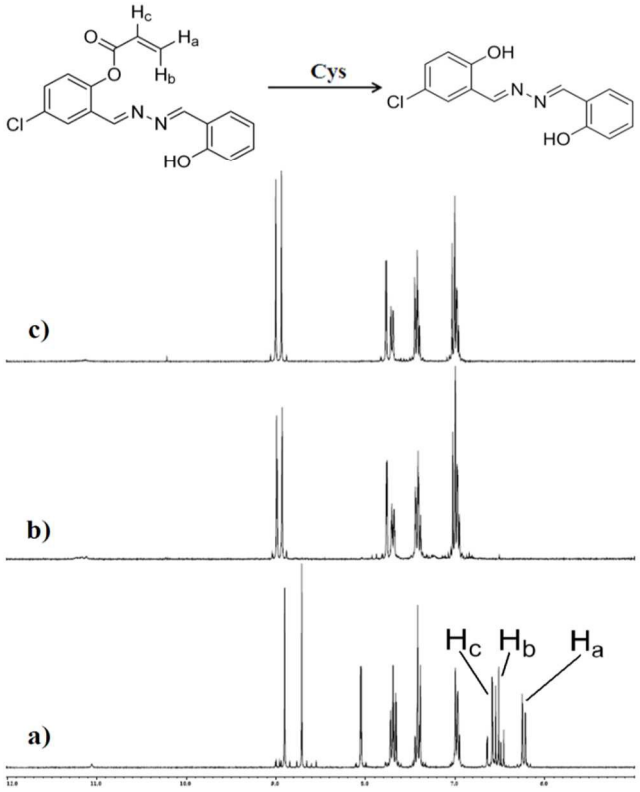


Fig. 2 Partial ^{1}H NMR spectra in d6-DMSO–D_2O (9 : 1, v/v): (a) probe **2**; (b) probe **2** with Cys (1 equiv.), 30 min; (c) compound **1**.

3.3 Optimization of the analytical condition

3.3.1 Effect of DMSO volume fraction

The mixture of 30 μM **2** and 30 μM Cys in 30% DMSO containing 10 mM PBS buffer at pH 7.4 showed maximum fluorescence intensity (Fig. 3). High DMSO volume fraction would enhance the solubility of **2**, and could promote the reaction of **2** with Cys. However, a higher DMSO volume fraction was not preferred for the fluorescence of the product **1** due to it's in a "solution" state and could not activate the AIE process (Fig. S3). Thus, 10 mM PBS buffer with 30% DMSO was chosen for Cys detection.

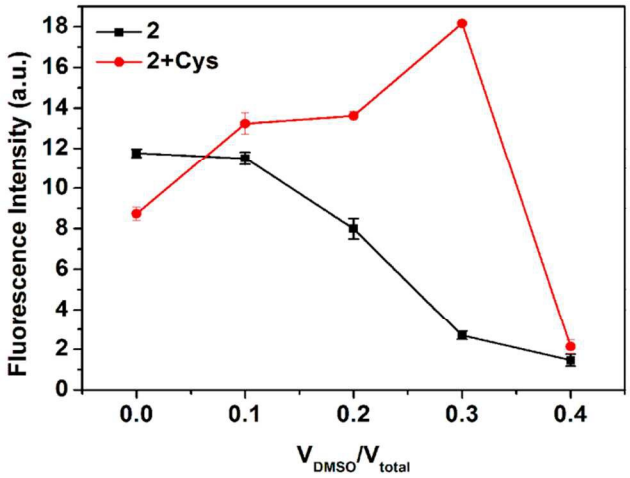


Fig. 3 Fluorescence intensity of **2** (30 μM) at 558 nm before and after addition of Cys (30μM) in PBS buffer (10 mM, pH = 7.4) with different DMSO volume fraction, λ_{ex} = 400 nm.

3.3.2 Effect of pH

Effect of pH on the detection of Cys was shown in Fig. 4. Fluorescence intensity of the mixture of 30 μM **2** and 30 μM Cys in 30% DMSO containing 10 mM PBS buffer reached its maximum at pH 7.4. Fluorescence intensity was low both in acidic and alkaline pH conditions. In acidic solution, reaction of **2** and Cys was not preferred and AIE fluorescence was low due to less **1** formed. Although alkaline conditions benefit the reaction of **2** with Cys[44], but high pH could lead to deprotonation of the hydroxyl groups of **1**. The anionic form of **1** became well-dispersed in solution, and thus AIE fluorescence was decreased (Fig. S4). Therefore, pH 7.4 was chosen in this work.

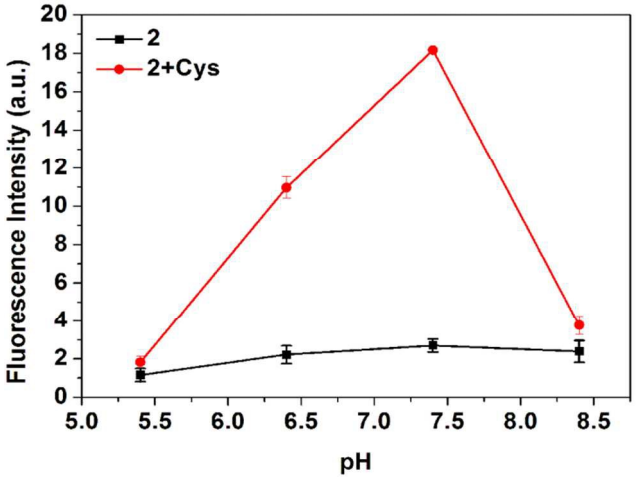


Fig.4 Fluorescence intensity of **2** (30 μM) at 558 nm before and after addition of Cys (30μM) in PBS buffer (10 mM, 30% DMSO) with different pH, λ_{ex} = 400 nm.

3.3.3 Effect of reaction time

The reaction time of **2** and Cys was investigated. Time dependence of fluorescence intensity is displayed in Fig. 5. The reaction of 30 μM **2** and 30 μM Cys was completed in 40 min. So, 40 min reaction time was chosen in this work.

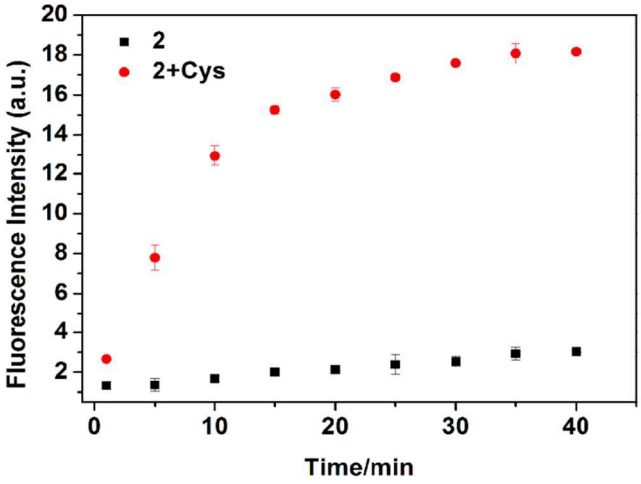


Fig.5 The effect of reaction time on the fluorescence intensity, in PBS buffer (10 mM, pH = 7.4, 30% DMSO) with 30 μM **2** and 30 μM Cys, λ_{ex} = 400 nm, λ_{em} = 558 nm.

3.4 Selectivity to Cys

To investigate selectivity, the fluorescence response of **2** toward Hcy, GSH and other amino acids(Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val) was studied. As shown in Fig. 6, there was almost no response of **2** to non-thiol amino acids, and the fluorescence intensity changes of **2** toward Hcy, GSH were also negligible. According to the literature[30], intramolecular cyclization reaction between acrylate and Hcy would produce an eight-membered ring, mass spectral analysis confirmed the existence of an eight-membered ring for the reaction of 1 mM **2** and 1 mM Hcy in DMSO (Fig. S5). While formation of an eight-membered ring with Hcy was kinetically disfavored compared with the case of Cys in which a seven-membered ring formed. Under the analytical condition in this work, it need a longer reaction time to produce eight-membered ring. So, Hcy could not induce obvious fluorescence enhancement in 40 min reaction time. As to GSH, there was only conjugate addition reaction between acrylate and GSH (Fig. S6).

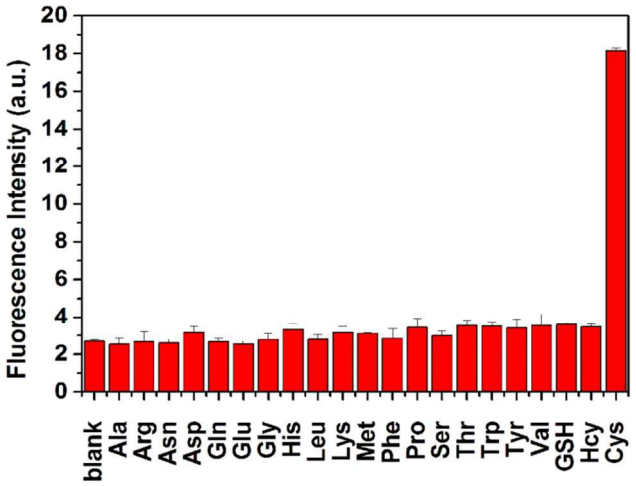


Fig.6 Fluorescence intensity of 30 μM **2** in the presence of 30 μM Cys, Hcy, GSH and other amino acids(Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val) in PBS buffer (10 mM, pH = 7.4, 30% DMSO), λ_{ex} = 400 nm, λ_{em} = 558 nm.

3.5 Interference of foreign substances

The interference of some foreign substances to 30 μM Cys (within 5% error) was tested. As shown in Table 1, most of the foreign substances displayed no significant influence on Cys detection. The major interferences were Ag^{+}, Hg^{2+} and Cu^{2+}, which might form metal coordination polymers with Cys[49].

Table 1 Interference of foreign substances[a]

substance	concentration (μM)	Response ratio[b] (%)
Na^+, K^+, Ca^{2+}, NH_4^+	3000	<-4.2
Mn^{2+}	1000	-4.0
Mg^{2+} , Al^{3+}, Ba^{2+}	500	<3.9
Fe^{3+}, Pb^{2+}, Cd^{2+}	100	<-3.4
Ag^+,Hg^{2+}, Cu^{2+}	1	<-3.2
Ala, Arg, Asn, Asp, Gln, Glu, Gly, His, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val	3000	<4.2
ATP, ADP, AMP	1000	<-1.6
ppi	500	2.0
Adenosine	3000	-1.1
Glucose	3000	-1.1
BSA(Albumin from bovine serum)	30	-1.8

[a]Conditions: 30 μM **2**, 30 μM Cys in PBS buffer (10 mM, pH = 7.4, 30% DMSO), 40 min reaction at room temperature. Excitation/emission was performed at 400/558 nm.

[b]Response ratio = $(I- I_0)/I_0$, where I_0 and I denote fluorescence intensity before and after addition of a foreign substance to the solution.

3.6 Application in real sample analysis

The proposed method was applied to Cys detection in a Cys containing sample reduced fetal bovine serum (FBS)[50]. As shown in Fig. S7, the fluorescence emission of **2** showed a significant increase with the addition of FBS, while the fluorescence intensity remained almost the same as **2** alone when FBS pretreated with the thiol blocking reagent N-ethylmaleimide (NEM). The result indicated that the fluorescent enhancement of **2** was indeed attributable to the Cys in FBS. By using standard addition method, the amount of Cys in the serum sample was determined to be 245.6 μM with satisfied recovery (Table 2). The results suggested that the proposed method could be applied in the detection of Cys in serum samples.

Table 2 The results of a fetal bovine serum sample analysis [a]

Sample	Added Cys (μM)	Measured (μM)	Recovery (%)	RSD (n=3, %)
1	0	245.6		3.2
2	50	311.2	105.3	3.5
3	100	358.7	103.8	4.3

[a] Conditions: 30 μM **2** in PBS buffer (10 mM, pH = 7.4, 30% DMSO), 40 min reaction , λ_{ex} = 400 nm, λ_{em} = 558 nm.

4. Conclusion

In summary, we have described a fluorescence turn-on probe for Cys detection based on intramolecular cyclization reaction between probe **2** and Cys as well as on the ESIPT and AIE property of the corresponding product **1**. The proposed method showed high selectivity towards Cys over thiol containing amino acids Hcy, GSH and others. Detection limit for Cys was 0.46 μM with a linear range of 0~30 μM in 30% DMSO containing 10 mM PBS buffer at pH 7.4. The method also showed satisfied results for the detection of Cys in a serum sample FBS.

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Notes and references

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† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

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