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# A fluorescein-based fluorescence probe for the fast detection of thiol

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ABSTRACT

A new turn-on fluorescent probe for the selective detection of thiol over other amino acids was synthesized. Probe possesses the widely-used thiol-selective 2,4-dinitrobenzenesulfonyl (DNBS) group which can react with thiol and release the fluorescein which has strong fluorescence. Fluorescein, a well known xanthene fluorescent dye, has two states at different environment. Fluorescein is in the state of spirocyclization when connected with 2,4-dinitrobenzenesulfonyl (DNBS) group which has no fluorescence. However, it is in the state of open form when it reacts with thiol which has a strong fluorescence. The transition of the two states can be used to selectively detect thiol and the color can change from colorless to yellow which can be differentiated by naked eyes. Upon the titration of thiol, the absorption band at 454 nm rises gradually and the fluorescence emerges at 521 nm and the detection limit can be as low as 0.16 μM. All of such good properties prove it to be a good sensor for the selective detection of thiol and it shows a potential use in bioimaging applications.



## Introduction

Biological thiols such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH), play essential roles in maintaining the appropriate redox status in physiological and pathological processes.[1,2] Generally, the abnormal level of cellular thiols has been related with numerous diseases such as psoriasis, slowed growth, liver damage, leukocyte loss, cancer, and AIDS.[3,4] Cysteine (Cys), one of the most abundant biothiols in living organisms, plays important roles in biological systems[5] and has been associated with neurotoxicity. Its abnormal level can cause hair pigmentation, retardation in growth, liver damage, and skin lesions.[4] Elevated level of Hcy is closely related to Alzheimer's disease,[6] cardiovascular,[7] neural tube defects, complications during pregnancy, and osteoporosis. GSH, the most abundant intracellular thiol with the concentration of millimolar range,[8] plays crucial roles in many cellular functions such as xenobiotic metabolism, maintenance of intracellular redox activity, and gene regulation.[9–11] An abnormal level of GSH is directly associated with cancer, aging, heart problems, and other ailments. Accordingly, the design and synthesis of a sensor for the detection of thiol is of significant importance.

Considering the vital important roles in human body, great efforts have been made for the detection of thiol.[12,13] Fluorescent probe, famous for its high sensitivity, relatively simple analysis protocols,[14] and far less expensive, has got much attention for the detection of metal ions,[15,16] hydrion,[17] anion,[18,19] and especially popular for the detection of biomolecule[20–22] in recent years. Lin[23] et al. designed a new NCL-mechanism-based ratiometric fluorescent probe with specificity toward aminothiols. The new fluorescent probe is capable of ratiometric detecting of aminothiols in newborn calf and human serum samples and is also suitable for ratiometric fluorescent imaging of aminothiols in living cells. Zhu[24] et al. applied fluorescein as fluorescent reporter, a 7-aminocoumarin as photo-trigger and a thiol-removable energy acceptor. Such doubly locked probes have bright prospect, promising biochemically and optically labeling cells with spatial precision for monitoring dynamic processes of cells in vitro or in vivo. Li[25] et al. used naphthalimide as the fluorescence source for the detection of thiol. The synthesized two naphthalimide-based fluorescent probes provided high on/off signal ratios and exhibited good selectivity toward thiols over other analytes and they were identified as good sensors for the detection of thiols both in living cells and in rabbit plasma samples.

Fluorescein is widely used as fluorescent sensors in biological systems.[26–28] Its relatively high molar absorption coefficients and quantum yields, high-intensity emission peaks, relative inertness under physiologically relevant conditions, and relative nontoxicity makes it an excellent dye for the design of sensors. On the other hand, fluorescence spectroscopy has become a powerful method for sensing and imaging trace amounts of samples because of its simplicity,[29,30] sensitivity, fast response times, and its application for not only in vitro assays but also in vivo imaging studies.[31,32]

As we all know, fluorescein exhibits strong fluorescence when it is in the state of open form while it has non-fluorescence when it is in the spirocyclic form. The spirocyclic form of fluorescein is

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colorless and nonfluorescent due to the break of the π-conjugation, whereas the ring opening form shows strong spectroscopic signals in both the absorption and fluorescence spectra because of the π-conjugation. Using the transformation of the two states can achieve the goal of testing different species. Here, we synthesized an asymmetric fluorescein-based probe for the detection of thiol using 2,4-dinitrobenzenesulfonyl (DNBS) group as the detection group. When it is connected with DNBS, the fluorescent probe shows either colorless in the absorption band nor non-fluorescence in the emission band because of the formation of spirocyclic form. However, it shows a color of yellow and a strong fluorescence which can be differentiated by naked eyes when it is treated with thiol. The detection limit can be as low as 0.16 μM.

## Experiment

### Chemicals and instrumentals

Fluorescein was purchased from Energy Chemical and used directly without any purification. The other chemicals were of the highest grade available and were used without further purification. All employed solvents were analytically pure and were employed without any further drying or purification.

Reactions were monitored by TLC using Merck Millipore DC Kieselgel 60 F-254 aluminum sheets. $^1$H NMR and $^{13}$C NMR spectra were recorded on Brucker AM-400 MHz instruments with tetramethylsilane as internal standard. Low-resolution ESI mass analyses were performed on a Waters LCT Premier XE spectrometer. UV–vis absorption spectra were recorded on a SHIMADZU UV–Vis spectrophotometer. Fluorescence spectra were measured on a SHMADZU RF-5301PC Fluorescence spectrophotometer.

### Preparation and characterization of 2

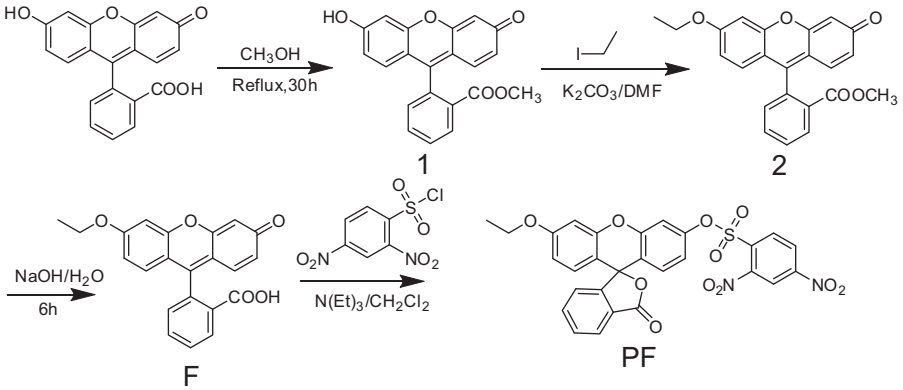
Fluorescein methyl ester **1** was synthesized according to the reported literature.[33,34] Then, compound **2** was synthesized according to the method of the reported Letter.[35] Compound **1** (1.6 g) and $K_2CO_3$ (0.5 g) were dissolved in 15 mL DMF. 0.5 mL iodoethane was added to the above solution. The whole mixture was heated at 65 °C for 6 h under stirring. After being cooled to room temperature, the solution was added to 80 mL 5% sodium chloride solution under stirring. The precipitates were collected and dried in vacuum. The targeted compound is isolated by flash column chromatography on silica gel using dichloromethane/methanol (20:3, v/v) for elution. The target products were in the color of yellow and in the state of solids. $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 8.25 (d, $J$ = 8 Hz, 1H), 7.71 (m, 2H), 7.30 (m, 1H), 6.89 (m, 3H), 6.74 (dd, 1H), 6.58 (m, 1H), 6.51 (d, $J$ = 2 Hz, 1H), 4.14 (m, 2H), 3.63 (d, $J$ = 8 Hz, 3H), 1.47 (m, 3H) (Fig. S1). $^{13}$C NMR $\delta$ 185.74, 165.59, 163.70, 159.17, 154.45, 151.16, 134.56, 132.71, 131.14, 130.39, 129.56, 128.93, 117.34, 114.66, 114.06, 106.58, 100.59, 77.40, 77.08, 76.76, 64.52, 52.42, 14.54 (Fig. S2). Yield 1.2 g.

### Preparation and characterization of F

Compound **3** was synthesized according to our previous work.[36] Compound **2** (1 g) was dissolved in 15 mL methyl alcohol and 20 mL sodium hydroxide solution at the concentration of 2 M was added to the above solution. The whole mixture was stirred overnight in room temperature. After that, 20 mL water was added and the pH was adjusted to 7 using HCl. The precipitates were filtered and dried in vacuum. The targeted compound is isolated by flash column chromatography on silica gel using dichloromethane/methanol (20:3, v/v) for elution. The compounds were in a yellow and in the state of solids. $^1$H NMR (400 MHz, $d_6$-DMSO) $\delta$ 10.17 (s, 1H), 8.03 (dd, 1H), 7.80 (m, 1H), 7.72 (t, $J$ = 4 Hz, 1H), 7.28 (m, 1H), 6.92 (m, 1H), 6.68 (m, 3H), 6.57 (d, $J$ = 8 Hz, 2H), 4.06 (m, 2H), 1.39 (m, 3H) (Fig. S3). $^{13}$C NMR $\delta$ 168.64, 159.51, 151.78, 135.61, 130.10, 128.98, 126.02, 124.62, 123.97, 112.73, 110.80, 109.40, 102.17, 101.12, 82.71, 63.60, 39.88, 39.67, 39.46, 39.25, 39.05, 14.42 (Fig. S4). HRMS: 361.1082, calcd for: 301.1076 (Fig. S7). Yield 0.8 g.

### Preparation and characterization of PF

Probe PF was synthesized according to the methods of the reported papers.[37,38] Compound F (0.15 g) was dissolved in 15 mL anhydrous dichloromethane and kept in ice bath under stirring. Then, triethylamine (150 μL) was added to the above solution. 2,4-Dinitrobenzenesulfonyl chloride (0.133 g) was dissolved in 5 mL anhydrous dichloromethane and added to the above solution dropwise in 30 min. The whole mixture was stirred in ice bath for another 30 min and stirred in room temperature for 4 h. After that, the solvents were evaporated and the product was purified by flash column chromatography on silica gel using dichloromethane/alcohol (100:1, v/v) as the eluent. The probe was in the color of light yellow and in the state of solids. $^1$H NMR (400 MHz, $CDCl_3$) $\delta$ 8.66 (d, $J$ = 4 Hz, 1H), 8.52 (dd, 1H), 8.25 (d, $J$ = 12 Hz, 1H), 8.02 (d, $J$ = 8 Hz, 1H), 7.67 (m, 2H), 7.19 (d, $J$ = 4 Hz,1H), 7.15 (d, $J$ = 8 Hz, 1H), 6.90 (m, 1H), 6.80 (d, $J$ = 8 Hz, 1H), 6.75 (dd, 1H), 6.65 (m, 2H), 4.05 (q, 2H), 1.43 (t, $J$ = 4 Hz, 3H) (Fig. S5). $^{13}$C NMR $\delta$ 169.02, 161.03, 152.55, 152.13, 151.88, 149.41, 135.38, 134.08, 133.23, 130.10, 128.96, 126.73, 126.35, 126.29, 123.89, 120.49, 119.47, 117.19, 112.81, 110.93, 110.26, 101.35, 81.87, 77.36, 77.05, 76.73, 63.99, 14.62 (Fig. S6). HRMS: 591.0715, calcd for: 591.0710 (Fig. S8). Yield 0.1 g.




**Scheme 1.** The synthesis procedure of the probe PF.

## Results and discussion

### Synthesis

The synthetic routine of probe PF is outlined in Scheme 1. We can get PF with a relatively high yield by four steps. Fluorescein methyl ester **1** was synthesized according to the reported literature. Compounds **2** and F were synthesized in high yields and F displays excellent fluorescence. Probe PF was conveniently synthesized via the condensation of F with 2,4-dinitrobenzenesulfonyl chloride in dichloromethane, and its structure was confirmed by $^{1}$H NMR, $^{13}$C NMR, HRMS spectra. Regulation of the spirocyclization of fluorescein dyes can achieve the goal of detecting thiol. PF shows nonfluorescence because of the spirocyclization of

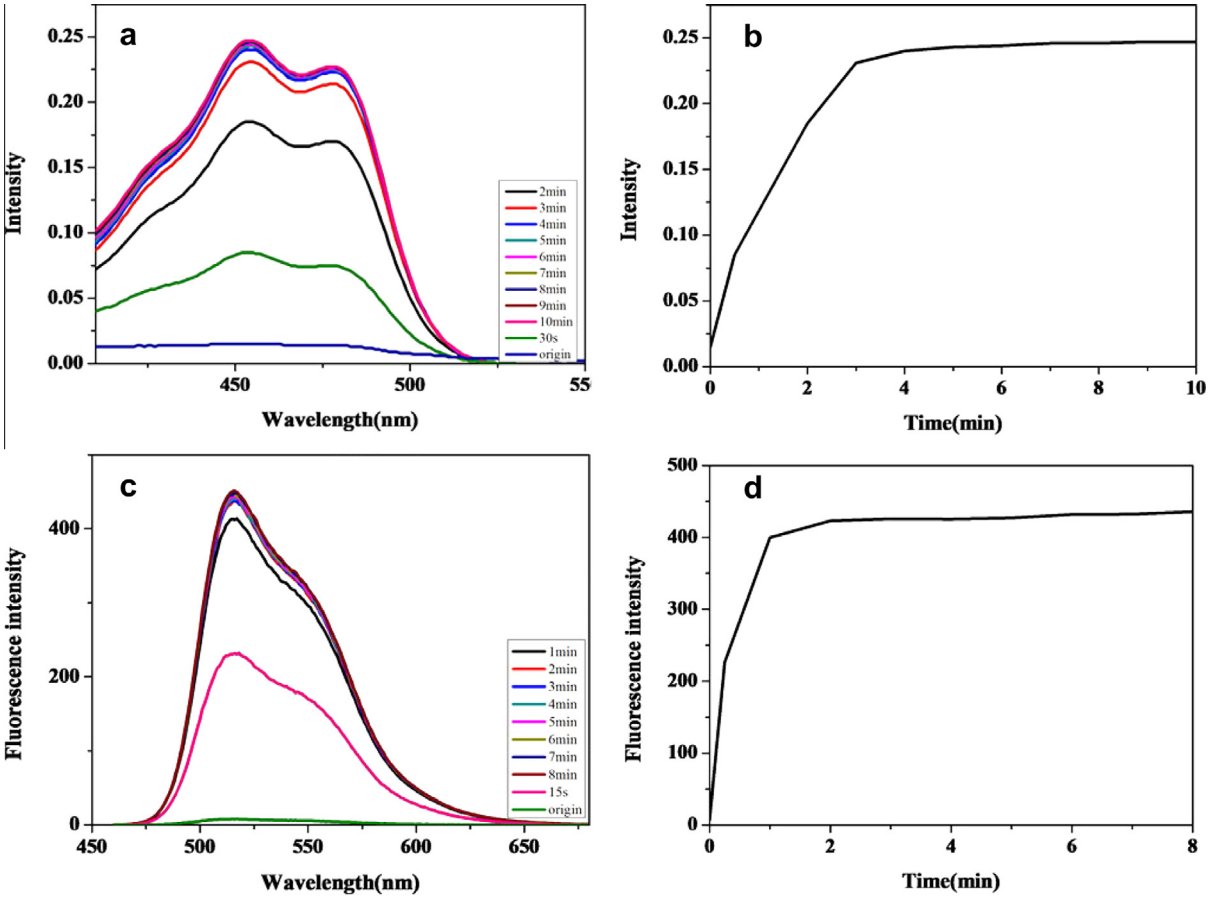



**Figure 1.** (a) Time-dependent absorption changes of probe PF with adding 5 equiv thiol. (b) The changes of absorption intensity at 454 nm in the time range of 0–10 min. (c) Time-dependent fluorescence intensity changes of probe PF after adding 5 equiv thiol. (d) The fluorescence intensity changes at 521 nm excited by 454 nm in the time range of 0–8 min. The spectroscopic properties of Probe PF were tested in aqueous phosphate buffered saline (PBS) solutions (10 mM, pH = 7.4) containing 30% DMSO in the concentration of $10^{-5}$ M.

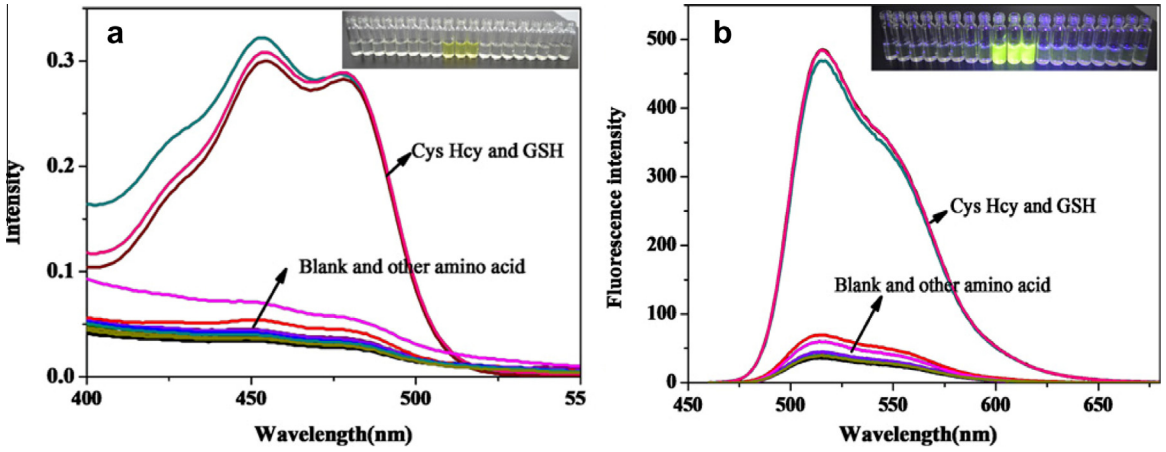



**Figure 2.** (a) Absorption of probe PF in the presence of different amino acids including Ala, Arg, Asp, Gln, Glu, Gly, His, Ile, Met, Phe, Pro, Ser, Thr, Trp, Cys, Hcy and GSH. The inset picture is the color changes after adding different amino acids. (b) Fluorescence intensity of probe PF in the presence of different amino acids excited by 454 nm. The tested amino acids are the same the absorption. Other amino acids are added 100 equiv to the probe and Cys, Hcy and GSH are added 5 equiv. The inset picture is the fluorescence changes after adding different amino acids. The spectroscopic properties of Probe PF were tested in aqueous phosphate buffered saline (PBS) solutions (10 mM, pH = 7.4) containing 30% DMSO in the concentration of $10^{-5}$ M.

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fluorescein dyes while the strong fluorescence appears when it react with thiol. The spectroscopic properties of Probe PF were all tested in aqueous phosphate buffered saline (PBS) solutions (10 mM, pH = 7.4) containing 30% DMSO in the concentration of $10^{-5}$ M.

### The response time toward thiol

To evaluate when will the probe get saturated after adding 5 equiv thiol, the time-dependent absorption and fluorescence intensity changes of probe PF after adding 5 equiv thiol is tested. The probe is in the concentration of $10^{-5}$ M which is dissolved in aqueous phosphate buffered saline (PBS) solutions (10 mM, pH = 7.4) containing 30% DMSO. 5 equiv of thiol are added to the solution and the absorption changes are shown in Figure 1. It can be seen in Figure 1a that probe PF has nearly no absorption at 454 nm before adding thiol. However, the absorption at 454 nm increases sharply in one minute with the adding of 5 equiv of thiol. After that, the absorption intensity at 454 nm increases gradually with the time going on. The absorption changes at 454 nm versus time is collected and drawn in Figure 1b. It can be seen that the probe gets to saturation in 10 min. Likewise, the fluorescence is also tested. 5 equiv thiol is also added to the solution and the fluorescence is collected. The fluorescence has the same tendency with the adding of the tested species. It can be found in Figure 1c that the probe PF has nearly nonfluorescence before adding thiol. However, the fluorescence intensity at 521 nm increases sharply in one minute after the addition of thiol and continuing increasing with the time going on. The relationship between fluorescence intensity and time is shown in Figure 1d. It can be found that the probe gets to saturated in 10 min. The color changes from colorless to yellow which can be differentiated by naked eyes and the fluorescence changes from none to strong yellow fluorescence.

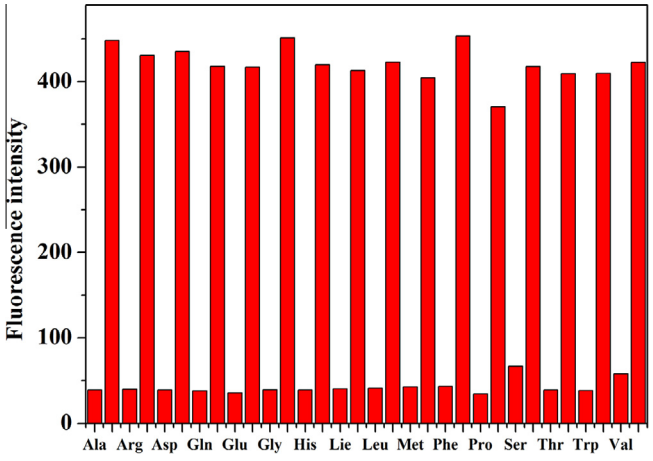


**Figure 3.** Effect of amino acids. The fluorescence intensity changes of probe PF in the presence of 100 equiv of various of amino acids. The short bar in the left is the fluorescence intensity of probe PF with other amino acids in the concentration of 1 μM. The amino acids including Ala, Arg, Asp, Gln, Glu, Gly, His, Ile, Met, Phe, Pro, Ser, Thr and Trp. The high bar in the right is the fluorescence intensity of probe PF with the adding of 5 equiv thiol to the above solutions. The spectroscopic properties of probe PF were measured in aqueous phosphate buffered saline (PBS) solutions (10 mM, pH = 7.4) containing 30% DMSO in the concentration of $10^{-5}$ M.

### The selectivity toward different amino acids

It is very important to selectively detect certain analytes for a probe. The selectivity toward different analytes is tested and is shown in Figure 2. Ala, Arg, Asp, Gln, Glu, Gly, His, Phe, Met, Ile, Pro, Ser and Thr are used as the sources of other amino acids and GSH, Cys and Hcy are used as the tested species. All the analytes are dissolved in distilled water. The probe is in the concentration of $10^{-5}$ M which is dissolved in aqueous phosphate buffered saline (PBS) solutions (10 mM, pH = 7.4) containing 30% DMSO. 100 equiv of other amino acids and 5 equiv of Cys, Hcy and GSH are added, respectively. The spectra are collected after 10 min and the fluorescence spectra are collected with the excitation at 454 nm. It can be seen from the absorption in Figure 2a that the absorption spectra have little changes with the adding of 100 equiv other amino acids. However, the absorption at 454 nm increases obviously with the adding of Cys, Hcy and GSH and the color changes from colorless to yellow which can be differentiated by naked eyes (Fig. 2a). Similarly, the fluorescence spectra have the similar trend as the absorption spectra. It can be found in Figure 2b that nearly no fluorescence can be seen after the addition of other amino acids when excited by 454 nm while probe PF shows strong yellow fluorescence after the addition Cys, Hcy and GSH. It has the emission band at 521 nm. All these data demonstrate that the probe PF has a good selectivity toward thiols over other amino acids. Thus, the probe has thiols specificity.

### The effect to the probe of different amino acids

However, only thiols specificity for a good probe is not enough. Many amino acids are mixed together whether they are

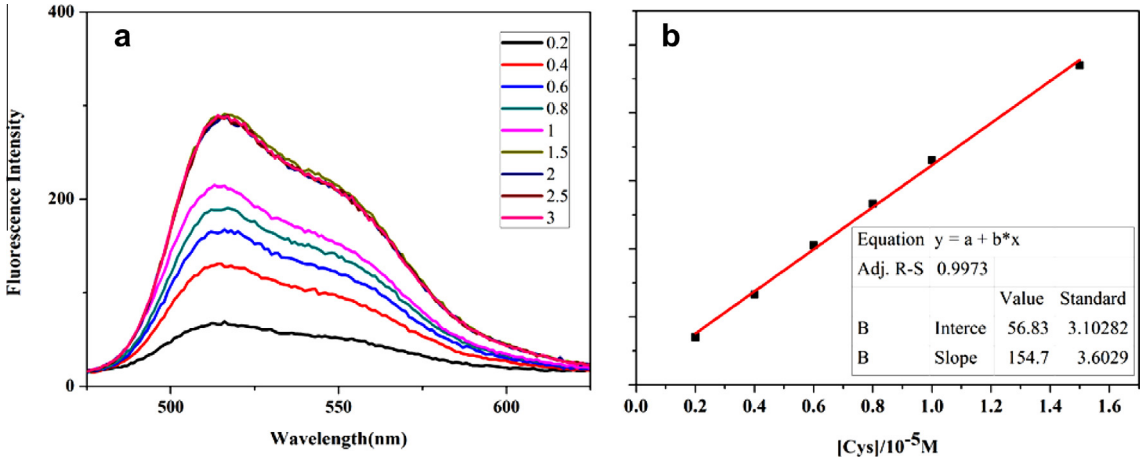


**Figure 4.** (a) Fluorescence intensity changes with adding different equivalent of thiol with excitation at 454 nm taken after 10 min. (b) Fluorescence intensity changes versus concentration of thiol at 521 nm with excitation at 454 nm taken after 10 min The spectroscopic properties of Probe PF were tested in aqueous phosphate buffered saline (PBS) solutions (10 mM, pH = 7.4) containing 30% DMSO in the concentration of $10^{-5}$ M.

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in human bodies or in other environments. Therefore, it is very important for the detection of thiols without the interference of other amino acids, especially when the other amino acids are in high quantity. Then, the effect of other amino acids is investigated. First, the probe PF is dissolved in phosphate buffered saline (PBS) solution (10 mM, pH = 7.4) containing 30% DMSO in the concentration of $10^{-5}$ M. 100 equiv of different amino acids are added to the above solution respectively. The fluorescence spectrum is tested and collected after 10 min, which is shown in Figure 3. The fluorescence intensity after adding 100 equiv of other amino acids is corresponding to the left bar in the figure. All the solutions show nearly nonfluorescence. 5 equiv of thiols are added to the above solutions respectively. The fluorescence is investigated and collected after 10 min as shown in Figure 3. The fluorescence intensity after adding 5 equiv of thiol is corresponding to the right bar in the figure. It can be seen that the probe PF can still detect thiol even with adding quantity of other amino acids. All this proved that this sensing for thiols is hardly interfered by other amino acids as can be seen in Figure 3. There's little influence to the detection of thiols even with quantity of other amino acids.

## Determination of the detection limit

To calculate the detection limit, titration of thiols (0–6 μmol) to the probe PF solution is tested. Probe PF is dissolved in phosphate buffered saline (PBS) solutions (10 mM, pH = 7.4) containing 30% DMSO in the concentration of $10^{-5}$ M. 0.4 μmol, 0.8 μmol, 1.0 μmol, 1.5 μmol, 2.0 μmol, 4.0 μmol and 6.0 μmol thiols are added to the solution respectively. Upon the titration of thiols, the fluorescence intensity at 521 nm increases gradually as shown in Figure 4. The detection limit was calculated based on the method reported in the literatures.[37,39] The detection limit is calculated by the equation: Detection limit = $3\sigma/k$. Where $\sigma$ is the standard deviation of blank measurement, $k$ is the slop between the fluorescence intensity versus thiols concentration. The fluorescence emission spectrum of PF was measured ten times to obtain the standard deviation $\sigma$ of blank measurement. To get the plot, the fluorescence intensity at 521 nm is plotted as a concentration of thiols as shown in Figure 4b. And the detection limit is calculated to be 0.16 μM.

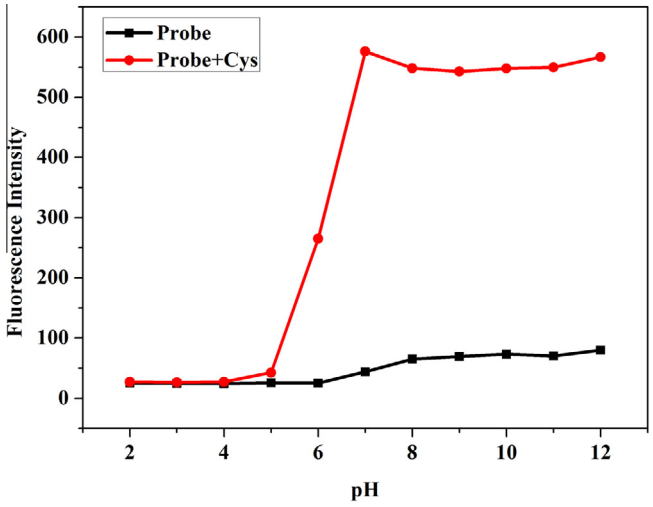


**Figure 5.** pH effect on the fluorescence behavior of probe PF (black line) and probe PF with Cys (red line). The fluorescence spectrum is collected with the excitation at 454 nm taken after 30 min. The spectroscopic properties of Probe PF were tested in aqueous phosphate buffered saline (PBS) solutions (10 mM, pH = 7.4) containing 30% DMSO in the concentration of $10^{-5}$ M.

## pH effect to the probe and probe PF

As we all know, it is very important for a fluorescent probe to be stable in a wide pH range. At least, it must be stable in physiological environment because the pH is varied in a wide range in nature and it also has slight variation in human bodies. Ester group is susceptible to pH change, both acidic and basic media can promote the hydrolysis of the ester group. pH effects on the fluorescence behavior of Probe (PF) and Probe PF with Cys are shown in Figure 5. A wide pH range from 2 to 12 is adjusted using phosphate buffered saline (PBS) solutions. Then, probe PF is dissolved in the prepared phosphate buffered saline (PBS) solutions using 30% DMSO as good solvent and then 5 equiv of Cys is added to the above solutions. The spectra of probe and probe PF with Cys are recorded after 10 min and the fluorescence spectra are collected as shown in Figure 5. It can be seen that Probe PF is stable in a wide pH range from 2 to 12. This proves that Probe PF can be used in physiological

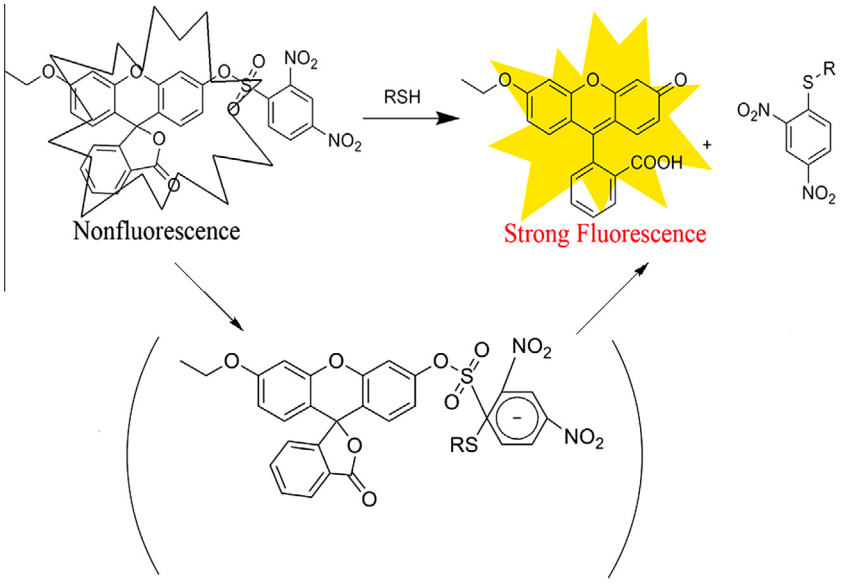


**Figure 6.** Proposed response mechanism of Probe PF to thiol.

environment. What's more, Probe PF with Cys in a wide pH range is also tested. In a pH range of 7–12, it can detect Cys, which can be used in physiological environment. The detection of thiol is a process of nucleophilic aromatic substitution and nucleophilic reaction is affected by pH. In a pH range of 2–6, the nucleophilic reaction is prevented. As a result, the probe cannot detect thiol.

## The proposed mechanism for the detection of thiols

It has been reported by Maeda's group[40] and others[41,42] that thiols could selectively cleave sulfonate ester which was a process of nucleophilic aromatic substitution as shown in Figure 6. To verify our design principle of probe PF for thiols, the reaction mechanism was studied by mass spectra. The fluorescein exhibits no fluorescence when connected with DNBS because the fluorescein is in the state of spirocyclic form which breaks the π-conjugation, whereas the ring opening form shows strong spectroscopic signals in the fluorescence spectra by reacting with thiols as shown in Figure 6. To further prove the mechanism of the reaction, the reaction of probe PF with Cys was conducted under the same conditions and the products were subjected to mass spectral analysis and $^1$H NMR analysis. The peak at *m*/*z* 361.1082 corresponding to compound **1** was observed which proved the proposed mechanism (Fig. S9). The $^1$H NMR has further proved the reaction mechanism as shown in Figure S10.

## Conclusions

In summary, we report a turn-on fluorescent probe for the detection of thiols which has visible color changes that can be differentiated by naked eyes. Probe PF has good selectivity toward thiols over other amino acids. The probe shows nonfluorescence when connected with 2,4-dinitrobenzensulfonyl (DNBS) group because the probe is in the state of spirocyclization. When the probe reacted with thiols, 2,4-dinitrobenzenesulfonyl (DNBS) group left and the fluorescin is exposed which had strong fluorescence. The detection limit of probe PF reaches to be as low as 0.16 μM. All good properties prove it to be a good sensor for the selective detection of thiol and it shows a potential use in bioimaging applications.

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## Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tetlet.2016.04.068.

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