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An excited state intramolecular proton transfer dye based fluorescence turn-on probe for fast detection of thiols and its applications in bioimaging

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Abstract In this study, a new fluorescent probe 2-(2'-hydroxy-5'-N-maleimide phenyl)-benzothiazole (probe **1**), was designed and synthesized by linking the excited state intramolecular proton transfer (ESIPT) fluorophore to the maleimide group for selective detection of thiols in aqueous solution. The fluorescence of probe **1** is strongly quenched by maleimide group through the photo-induced electron transfer (PET) mechanism, but after reaction with thiol, the fluorescence of ESIPT fluorophore is restored, affording a large Stokes shifts. Upon addition of cysteine (Cys), probe **1** exhibited a fast response time (complete within 30 s) and a high signal-to-noise ratio (up to 23-fold). It showed a high selectivity and excellent

sensitivity to thiols over other relevant biological species, with a detection limit of 3.78×10^{-8}M (S/N=3). Moreover, the probe was successfully applied to the imaging of thiols in living cells..

Keywords ESIPT; PET; Fluorescent probe; Thiols; Cell imaging

1. Introduction

Thiols, such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) play crucial roles in in physiological and pathological processes. For instance, a deficiency of Cys causes various health problems, such as retarded growth, lethargy, liver damage, skin lesions, etc. [1–3] At elevated levels in plasma, Hcy is a risk factor for cardiovascular disease, Alzheimer's disease [4]. GSH is played a crucial role in mammalian and eukaryotic cells [5, 6]. Thus, the development of an effective method for the detection and quantification of thiols is of great importance in physiological media for academic research and clinical applications [7, 8]. Many techniques including, high-performance liquid chromatography [9, 10], electrochemical methods [11], liquid chromatography-electrospray tandem mass spectrometry [12], and mass mpectroscopy [13, 14] have been developed for the detection of thiols. Among the various detection techniques, fluorescence–based method has become a popular approach for detection of thiols due to its high sensitivity, high temporal and spatial resolution, and noninvasiveness [15–17]. In particular, reaction-based fluorescent probes have been rapidly developed in recent years due to their high selectivity with large spectroscopic changes [18–20]. To date, a large number of fluorescent probes for thiols have been successfully developed by utilizing the strong nucleophilicity of

the thiol group to the specific reaction sites, such as an alkene or alkyne group with an electron withdrawing group[21–23], sulfonamide[24], sulfonate ester[25, 26], selenium-nitrogen bond[27, 28], disulfide bond[29, 30], nitroolefin moiety[31] and other[32–38]. Among the reactive sites, maleimides are known to react fairly selectively with thiols via a Michael addition reaction [35]. Using this general approach, maleimide derivatives based on triphenylamine [39], chromenoquinoline [40], naphthalimide[41–43], fluorescein[44], dansyl amide[45], coumarin[46, 47], and boron-dipyrromethene [48] have been developed as thiols probes. However, there are still some limitations in these fluorescence probes, including a small Stock's shifts, a long response time, and their poor water-solubility. Fluorescent probes with larger Stokes shifts and a rapid response time can diminish self-quenching and light scattering interferences, and enhance the detection sensitivity in complicated biosystems. Thus, it is of considerable interest to design and develop new fluorescent probes for thiols [49–51].

It is well known that dyes based on ESIPT mechanism have a large Stokes shifts compared to the normal fluorophores, which can be avoided the self-absorption, the inner filter effect, and improved the fluorescence analysis[52–54]. According to these, we successfully synthesized a new fluorescent probe **1** for thiols, combining 2-(2'-hydroxyphenyl)-benzothiazole (HBT) derivative which are known to exhibit an excited state intramolecular proton transfer (ESIPT) from the phenol form to the keto form results in a large Stokes shift as the fluorophore platform, and a maleimide group as the reactive site for thiols (Schemes 1 and 2). This is significantly important

to enhance the Stock's shifts and sensitivity toward thiols. The probe **1** exhibits a highly selectivity toward Cys with a larger Stokes shift and a very rapid response time in an aqueous medium.

Scheme 1 The chemical structure and synthetic routes to the probe **1**.

2. Experimental

2.1. Materials and Methods

Amino acids, 5-aminosalicylic acid, 2-aminophenol, polyphosphoric acid, maleic anhydride, and N-ethylmaleimide were purchased from Aladdin Reagents. The solutions were purchased from Shanghai Experiment Reagent Co., Ltd (Shanhai, China). All chemicals were used without further purification. All reactions were magnetically stirred and monitored by thin layer chromatography (TLC). Flash chromatography was performed using Qingdao Haiyang silica gel (200–300 mesh). The solutions of amino acids and reduced glutathione (GSH) were prepared in deionized water. The solution of N–ethylmaleimide was prepared in EtOH. Absorption spectra were recorded using a Varian Carry 4000 spectrometer. Fluorescence spectra were obtained using a Hitachi F-7000 Fluorescence Spectrophotometer, the slit width of 1.0 nm and matched quartz cells. The ^{1}H NMR and ^{13}C NMR spectra were collected on a BRUKER AVANCE III HD 600 MHz spectrometer at 600 MHz and 150 MHz NMR spectrometer, respectively. The following abbreviations were used to explain the multiplicities: s=singlet; d=doublet; t=triplet; q=quartet; m=multiplet; br=broad. Mass spectra were obtained using a

Thermo Scientific Q Exactive LC-MS/MS. The fluorescence images were obtained through EVOS® FL Auto Imaging System.

2.2. Synthesis and characterization

The synthetic routes to compounds **1** and **2** are shown in Scheme 1. The starting material **2** was prepared according to the reported procedure. Compound **2** was prepared by a simple one-step procedure in good yield from the reaction of 2-aminophenol and 5-aminosalicylic acid in polyphosphoric acid (PPA) as a catalyst [55, 56]. Condensation of **2** with maleic anhydride in refluxing glacial acetic acid afforded the desired product **1** as a yellow solid. The structures of **1** and **2** were characterized by ESI–MS, ^{1}H– and ^{13}C–NMR and the corresponding spectra are shown in Supplementary information.

2.3. Typical procedure for thiols determination

Double distilled water was used to prepare all aqueous solutions. All the measurements were made according to the following procedure. PBS buffer solution (10 mM, pH 7.4) 2 mL and the stock solution of probe **1** (2.0 mM) 2 μL were mixed in a 1 cm path quartz cuvette, followed by addition of an appropriate volume of thiols sample solution. The reaction solution was mixed and kept 30 s at room temperature, which was then transferred to measure UV-vis absorbance within the wavelength range of 250~450 nm or fluorescence intensity/spectrum with λex/em = 370/460 nm, and the excitation slit widths of 5 nm and the emission slit widths of 10 nm. In the meantime, a blank solution of probe **1** was prepared and measured under the identical conditions for stable and photostable.

2.4. Fluorescence imaging in living cells

The HeLa cell line was provided by Key Laboratory of Chemical Biology and Molecular Engineering of Ministry of Education. Cells were grown in RPMI 1640 medium supplemented with 10% FBS (Fetal Bovine Serum) and 1% antibiotics at 37 ^{o}C in humidified environment of 5% CO_2. Fluorescence imaging were acquired through an EVOS® FL Auto Cell Imaging System with a 40× oil immersion objective lens. Emission was collected at DAPI light channel (λ_{ex} = 357 nm, λ_{em} = 447 nm).

2.5 Theoretical calculation methods

Geometry of probe **1** and probe **1** + Cys were first optimized by the DFT method at the B3LYP/6-31g* level, then optimized geometries were used time dependent DFT (TDDFT) calculations for understanding the electron excitation process. All these calculations were performed with Gaussian 09 [57].

3. Results and discussion

3.1. The sensing properties of probe 1 towards Cys

As the representative of thiols, Cys was used to examine the sensing properties of probe **1**. Firstly, the solubility of probe **1** in aqueous solution was examined by UV absorption spectra with the increased concentration of probe **1**. As shown in Fig. S1, the absorbance intensity was closely related with the concentration of probe **1** from 0 to 12 μM with a good linear relationship (R^2=0.998) in PBS buffer solution, suggesting that the solubility of probe **1** in aqueous solution was 12 μM. At the same time, the molar absorption coefficient of probe **1** at 340nm was measured as ε=18900

$M^{-1}cm^{-1}$ using the Beer-Lambert Law. In addition, the stability of probe **1** was investigated using fluorescence spectrum on the aqueous probe solution. After stated in the PBS solution for 24 hours, the relative fluorescence intensity of probe **1** has no apparent change (Fig. S2a). Moreover, the photostability of probe **1** was also evaluated by continuous irradiation for 20 minutes using a 150 W Xe lamp as the light source, also the relative fluorescence intensity of probe **1** has no apparent change (Fig. S2b), indicating that probe **1** is sufficient photostability in aqueous solution. Particularly, it is almost non-fluorescent in PBS buffer solution, in which a PET process took place from the electron-donating maleimide groups to the electron-accepting the HBT derivative. The addition of Cys, however, a dramatic fluorescence intensity increase was observed in a few seconds centered at 460 nm (Fig. 1), which was in agreement with a typical PET process. The PET quenching process in probe **1** could be rationalized by density function theory (DFT) and time dependent DFT (TDDFT) calculations at the B3LYP/6-31g* level [40, 58]. As shown in Fig. 2 and Table 1, the HOMO of probe **1** was localized on HBT moiety but the LUMO was located on maleimide part, and according to TDDFT calculations, the $S_0 \rightarrow S_1$ of probe **1** was a forbidden transition due to oscillator strength (f=0.0016) based on the principle of Kasha's rule [59]. Therefore, the S_1 of probe **1** would be a dark state, the presence of maleimide part induced the non-radiative transitions, and thus probe **1** was non-fluorescen. In contrast, the allowed $S_0 \rightarrow S_1$ transition (f=0.468) in the corresponding probe **1** + Cys indicated that the species could be fluorescent. The results were in agreement with the experimental studies, which could further

prove the sensing mechanism.

Fig. 1 Changes in absorption spectra (a) and fluorescence spectrum (b) of **1** (2 μM) measured with and without 3 μM Cys in a PBS buffer solution (10 mM, pH 7.4, containing 0.1% DMSO).

Fig. 2 View of the frontier molecular orbitals (MOs) of probe 1 and generated from DFT B3LYP/6-31g* geometry optimization.

Table 1. Selected electronic excitation energies (eV) and oscillator strengths (*f*), configurations of the low-lying excited states of probe **1** and probe **1**+Cys, calculated by TDDFT//B3LYP/6-31g*, based on the optimized ground state geometries.

Encouraged by these results, the fluorescence titration studies of probe **1** (2 μM) towards different amounts of Cys were performed in a PBS buffer solution (10 mM, pH 7.4, containing 0.1% DMSO). As shown in Fig. 3, the free probe **1** was almost no fluorescent (Φ = 0.015, with quinine sulfate as a reference) [60]. Upon treatment with increasing concentrations of Cys, the fluorescence intensity of probe **1** at 460 nm gradually increased, and reached saturation when the amount of Cys was more than 3 μM. In this case, a ca. 23–fold fluorescence enhancement was observed. Moreover, a linear relationship with the Cys concentration from 0 to 3.0 μM could be obtained (Fig. 3b). Based on the definition by IUPAC (C_{DL}= 3 Sb/m) [61], the detection limit was found to be 3.78 $\times 10^{-8}$M. Upon addition of GSH to probe **1**, a similar phenomenon on fluorescence spectra could be observed (Fig. S3).

Fig. 3 (a) Changes in fluorescence emission spectrum of probe **1** (2 μM) measured with various amounts of Cys in a PBS buffer solution (10 mM, pH 7.4, containing 0.1% DMSO); (b) Fluorescence titration profile (at 460 nm) *vs.* concentration of Cys in solution for probe **1**.

3.2 The selectivity of probe 1 for Cys.

Next, we examined the fluorescence spectral changes of probe **1** (2 μM) towards natural amino acids, including thiols with mercapto groups (GSH, Hcy and Cys), and those without mercapto groups (L−alanine (Ala), L−arginine (Arg), Lasparagine (Asp), L−glutamine (Gln), L−glutamic acid (Glu), L−glycine (gly), L−histidine (His), L−isoleucine (Ile), L−leucine (Leu), L−lysine (Lys), L−methionine (Met), L−phenylalanine (Phe), L−proline (Pro), L−serine (Ser), L−threonine (Thr), L−tryptophan (Trp), L−tyrosine (Tyr), and L−valine (Val)). As shown in Fig. 4a, only thiols with mercapto groups caused a significant enhancement in fluorescence intensity and the other amino acid samples exhibited no noticeable increase of the fluorescence signal. At the same time, all the competing amino acids did not interfere the detection of Cys (Fig. 4b). In addition, the large fluorescence change of probe **1** induced by Cys was also observable by the naked eye. When probe **1** was excited at 365 nm using a UV lamp in the presence of 2.0 equiv. of various amino acids, only Cys caused a strong green fluorescence (Fig. 4c). These results indicated that probe **1** is highly selectivity towards thiols over other amino acids.

Fig. 4 (a) The fluorescence spectra of probe **1** (2 μM) upon addition of 4 μM of Cys

and various other amino acid in a PBS buffer solution (10 mM, pH 7.4, containing 0.1% DMSO); (b) Fluorescence response of probe **1** (2 μM) to 3μM of Cys in the solution containing 10μM of various amino acid; (c) Fluorescence images of probe **1** (2 μM) to various amino acids (4 μM) in PBS buffer (10 mM, pH 7.4, containing 0.1% DMSO) on excitation at 365 nm using a UV lamp.

3.3 Influences of time and pH

We also carried out the effect of reaction time on the fluorescence emission of the system (Fig. 5). The reaction of probe **1** with Cys reached equilibrium within 60 seconds after the addition of 3μM of Cys, indicating that probe reacted rapidly with Cys under the experimental conditions. In addition, the probe **1** was stable in a pH region of 2.0-12.0, and displayed good fluorescence response toward Cys in the physiological pH range, which was favorable in practical application (Fig. S4). As described above, probe **1** displayed excellent analytical property comparing with some other fluorescent probes of recent reports for the detection of Cys. The comparison data are listed in Table 2, indicating that probe **1** is promising for practical analysis.

Fig. 5 Time–dependent fluorescence intensity changes of probe **1** (2 μM) at 460 nm, upon addition of 3 μM of Cys. Conditions: PBS buffer (10 mM, pH 7.4, containing 0.1% DMSO) at 25 ^{o}C.

Table 2. Comparison of probe **1** for the detection of thiols

3.4 Proposed mechanism

To confirm the reaction mechanism, we first performed NMR studies of probe **1** in the presence of 2–mercaptoethanol (ME). Compared with the ^{1}H NMR spectrogram of probe **1**, the signal of original proton(—CH=CH—) at 7.190 ppm disappeared and appearance of new signals at 4.5-3.3 ppm which were supposed to arise due to the Michael addition of ME to the electron-deficient alkene groups in probe **1** (Fig. S5)[62]. We also studied the interaction of substance **2** and Cys using UV-vis absorption. As shown in Fig S6, **2** did not react with Cys. We reasoned that the plausible mechanism of the interaction between probe **1** and Cys was presented in Scheme 2. Further, we performed the HRMS experiment on probe **1** treated with Cys (Fig. 6), where in the peak at m/z = 444.0673 corresponding to [probe **1**+Cys + H]$^{+}$ (444.0682 calc.) was clearly observed. These results provide further evidence for the proposed mechanism.

Scheme 2 The proposed sensing mechanism of probe **1** toward Cys.

Fig. 6 HRMS charts of probe **1** treated with Cys. The peak (m/z) at 444.0673 corresponds to [probe **1** + Cys + H]$^{+}$ (Calcd for: 444.0682).

3.5 Fluorescence imaging for living cells

To value the practical utilities, we further measured the permeability and the monitoring of probe **1** toward Cys in living cells using an EVOS® FL Auto Cell Imaging System. The optical window at the DAPI channel (345–447 nm) was chosen as a signal output. As shown in Fig. 6, the HeLa cells were found to have almost no

fluorescence in the channel (Figure 7A). When the cells were incubated with probe **1** (5μM) for 30 min at 37◦C, they showed weak fluorescence as a result of the fact that probe **1** binds the cellular biothiols (Figure 7B). When the cells were pretreated with 100μM Cys and then incubated with probe **1** (5μM), they gave a stronger fluorescence (Figure 7C). When the cells were pretreated with N–ethylmaleimide (NEM, 1.0mM, a thiol blocking reagent) and then incubated with probe **1** (5μM), a remarkable decrease in fluorescence intensity was observed (Figure 7D). These results suggested that probe **1** was good cell-membrane permeability and responsive to intracellular thiols.

Fig. 7 A) Fluorescence image of Cys in HeLa cells using probe **1**.A) HeLa cells only; B) HeLa cells incubated with probe **1** (5 μM, 30 min); C) HeLa cells pre-incubated with Cys (100 μM, 30 min), and then treated with probe (5 μM, 30 min); D) HeLa cells pre–incubated with NEM (1 mM, 30 min), and then treated with probe **1** (5 μM, 30 min). Emission was collected at DAPI Light Cube (excited at 357 nm). Scale bar: 20 μm.

Conclusions

In summary, a new "turn–on" fluorescence probe **1** for thiols was exploited by coupling the reaction–based strategy with ESIPT and PET mechanism. The probe can highly selectively detect Cys with a fast fluorescence *off*–on response and an extremely low detection limit. Preliminary fluorescence imaging experiments in cells indicate its potential to probe biothiols chemistry in biological systems.

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Biographies

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Fig. 1 Changes in absorption spectra (a) and fluorescence spectrum (b) of **1** (2 μM) measured with and without 3 μM Cys in a PBS buffer solution (10 mM, pH 7.4, containing 0.1% DMSO).

Fig. 2 View of the frontier molecular orbitals (MOs) of probe 1 and generated from DFT B3LYP/6-31g* geometry optimization.

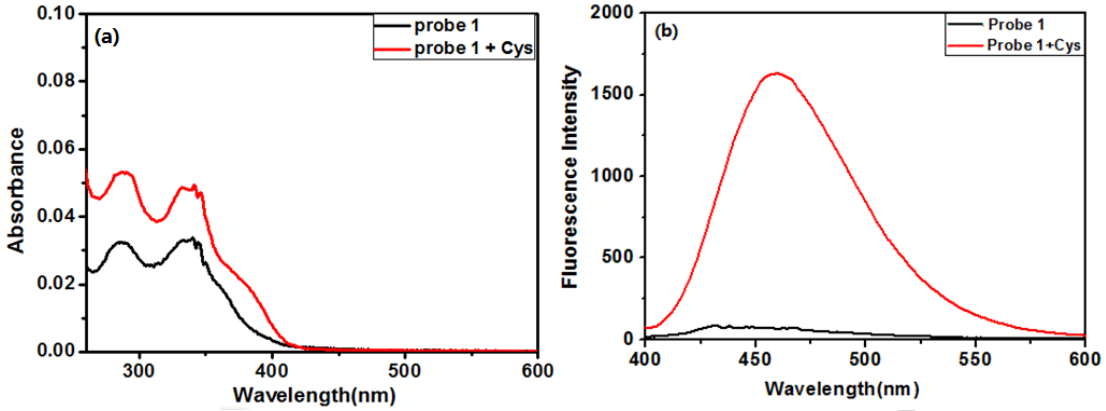
Fig. 3 (a) Changes in fluorescence emission spectrum of probe **1** (2 μM) measured with various amounts of Cys in a PBS buffer solution (10 mM, pH 7.4, containing 0.1% DMSO); (b) Fluorescence titration profile (at 460 nm) *vs.* concentration of Cys in solution for probe **1**.

Fig. 4 (a) The fluorescence spectra of probe **1** (2 μM) upon addition of 4 μM of Cys and various other amino acid in a PBS buffer solution (10 mM, pH 7.4, containing 0.1% DMSO); (b) Fluorescence response of probe **1** (2 μM) to 3μM of Cys in the solution containing 10μM of various amino acid; (c) Fluorescence images of probe **1** (2 μM) to various amino acids (4 μM) in PBS buffer (10 mM, pH 7.4, containing 0.1% DMSO) on excitation at 365 nm using a UV lamp.

Fig. 5 Time−dependent fluorescence intensity changes of probe **1** (2 μM) at 460 nm, upon addition of 3 μM of Cys. Conditions: PBS buffer (10 mM, pH 7.4, containing 0.1% DMSO) at 25 ^{o}C.

Fig. 6 HRMS charts of probe **1** treated with Cys. The peak (m/z) at 444.0673 corresponds to [probe **1** + Cys + H] $^{+}$ (Calcd for: 444.0682).

Fig. 7 A) Fluorescence image of Cys in HeLa cells using probe **1**.A) HeLa cells only; B) HeLa cells incubated with probe **1** (5 μM, 30 min); C) HeLa cells pre-incubated with Cys (100 μM, 30 min), and then treated with probe (5 μM, 30 min); D) HeLa cells pre−incubated with NEM (1 mM, 30 min), and then treated with probe **1** (5 μM, 30 min). Emission was collected at DAPI Light Cube (excited at 357 nm). Scale bar: 20 μm.



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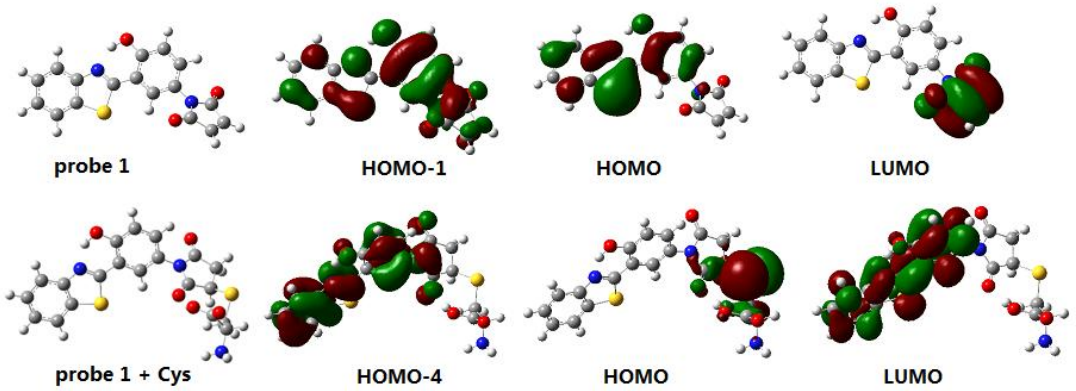

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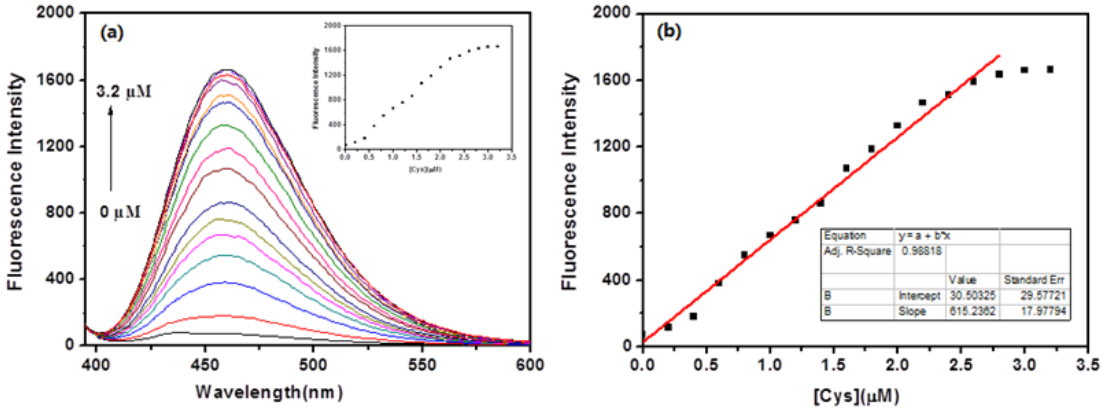

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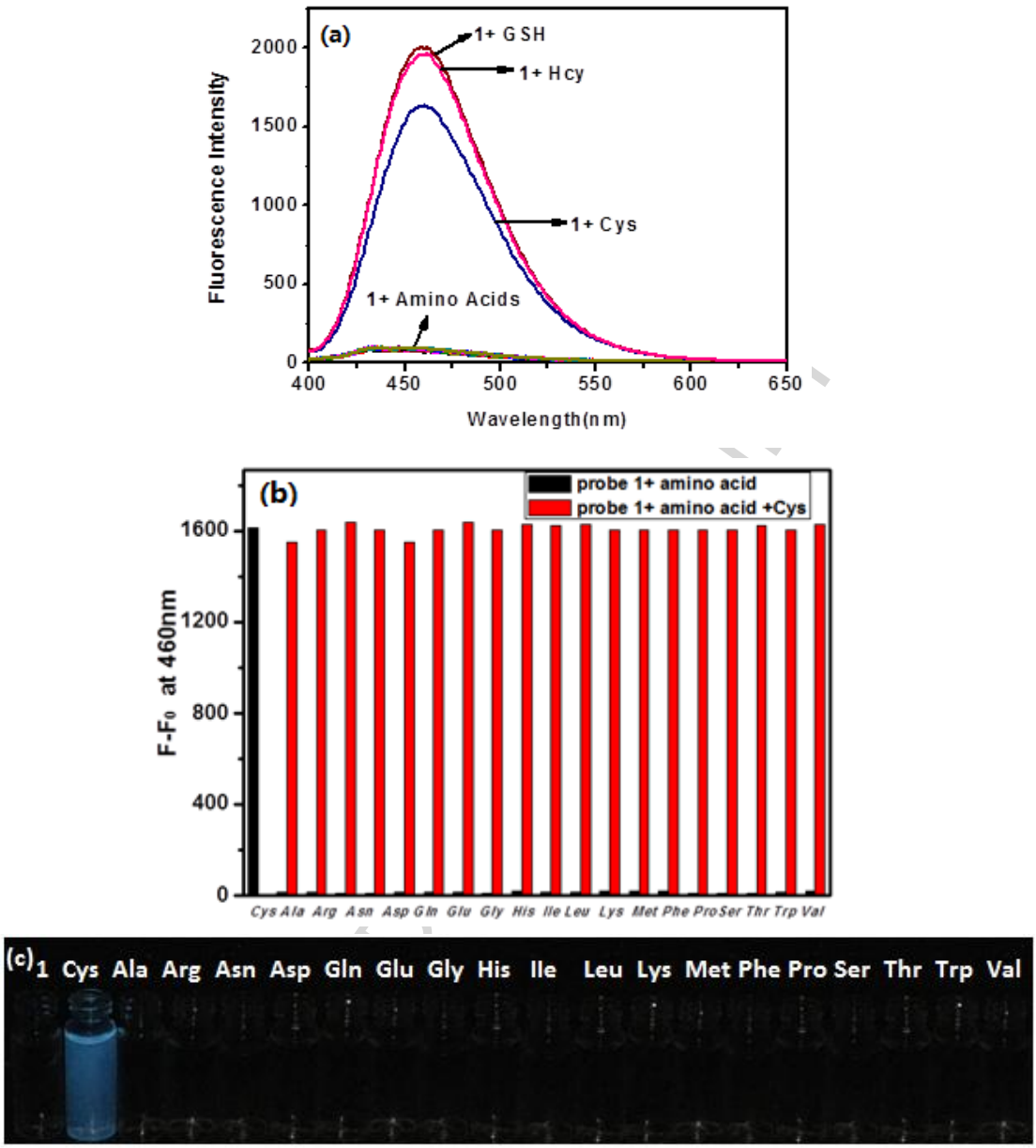

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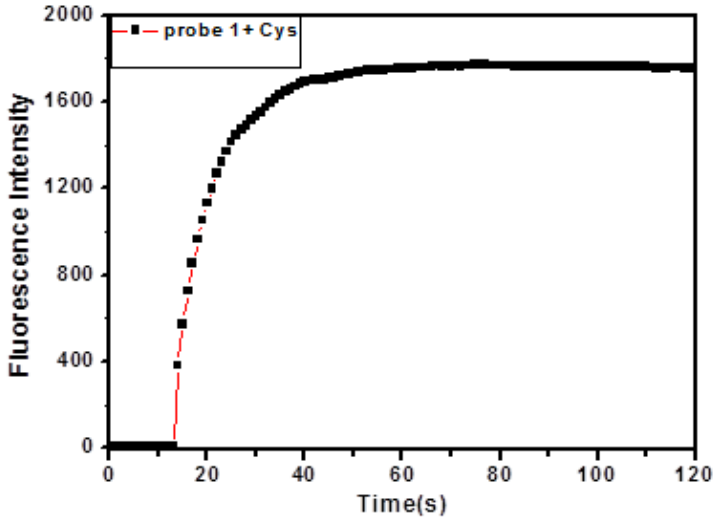

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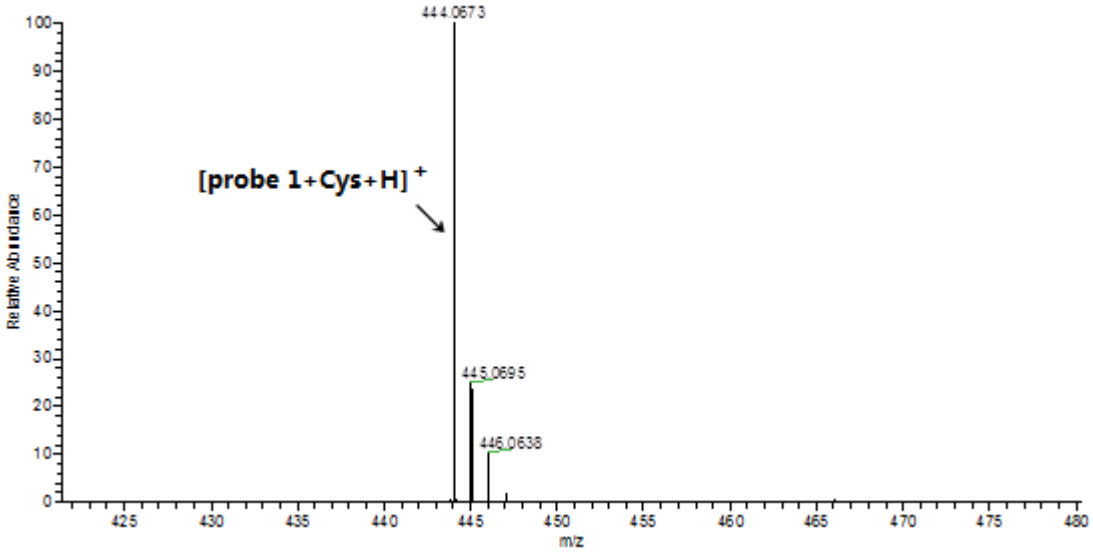

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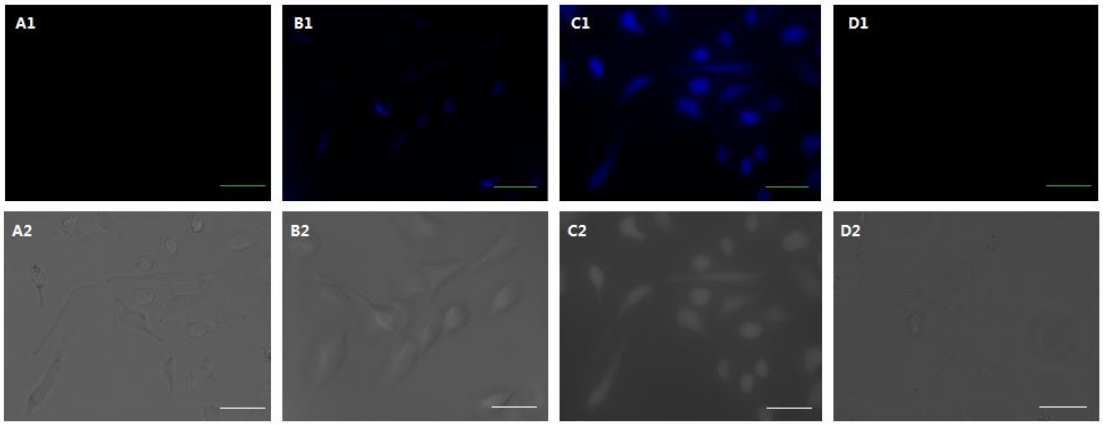

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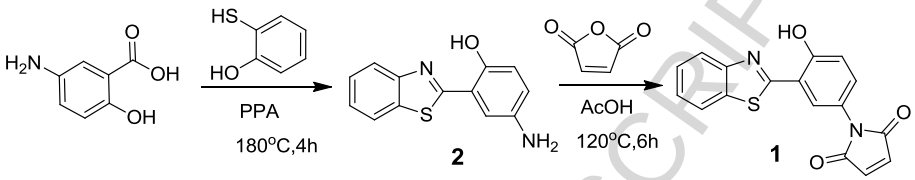
Scheme 1 The chemical structure and synthetic routes to the probe **1**.

Scheme 2 The proposed sensing mechanism of probe **1** toward Cys.



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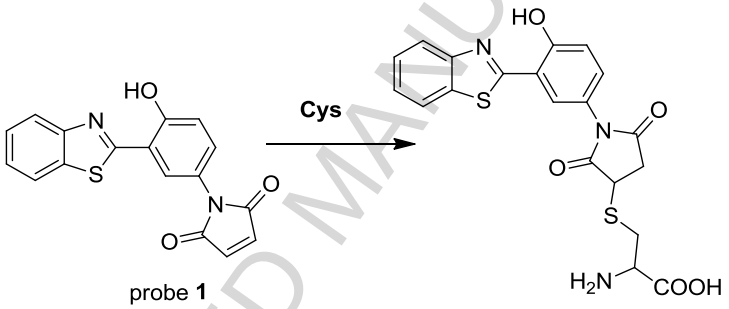

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Table 1. Selected electronic excitation energies (eV) and oscillator strengths (*f*), configurations of the low-lying excited states of probe **1** and probe **1**+Cys, calculated by TDDFT//B3LYP/6-31g*, based on the optimized ground state geometries.

Table 2. Comparison of probe **1** for the detection of thiols

Table 1. Selected electronic excitation energies (eV) and oscillator strengths (*f*), configurations of the low-lying excited states of probe **1** and probe **1**+Cys, calculated by TDDFT//B3LYP/6-31g*, based on the optimized ground state geometries.

Molecule	States	Energy Level (eV)[a]	Wavelength (nm)	*f*[b]	Transition Weights[c] CI coefficient[d]
Probe 1	$S_0 \rightarrow S_1$	2.55	485	0.0016	HOMO→LUMO 0.6944 HOMO-1→LUMO 0.1033
	$S_0 \rightarrow S_2$	3.20	387	0.0008	HOMO-1→LUMO 0.6007
Probe 1 + Cys	$S_0 \rightarrow S_1$	3.70	335	0.4680	HOMO→LUMO 0.6869
	$S_0 \rightarrow S_2$	4.29	289	0.3500	HOMO-4→LUMO 0.5466 HOMO→LUMO 0.1205

[a] Only the selected low-lying excited states are presented. [b] Oscillator strength. [c] Only the main configurations are presented. [d] The Configuration coefficients are in absolute values.

Table 2. Comparison of probe **1** for the detection of thiols

Reference	Response time	Stokes shift	Detection limit	Analyte
Dyes Pigments, 2016 [39]	2.5 min	110nm	0.13 μM	Cys
Chem. Commun., 2012 [40]	20 min	49 nm	1.94×10^{-2} μM	Cys
RSC Adv, 2013, [49]	30 min	75 nm	-----	Cys
Dyes Pigments, 2013 [31]	1 min	21 nm	0.2 μM	Cys
This work	0.5 min	90 nm	3.78×10^{-2} μM	

An excited state intramolecular proton transfer dye based fluorescence turn-on probe for fast detection of thiols and its applications in bioimaging

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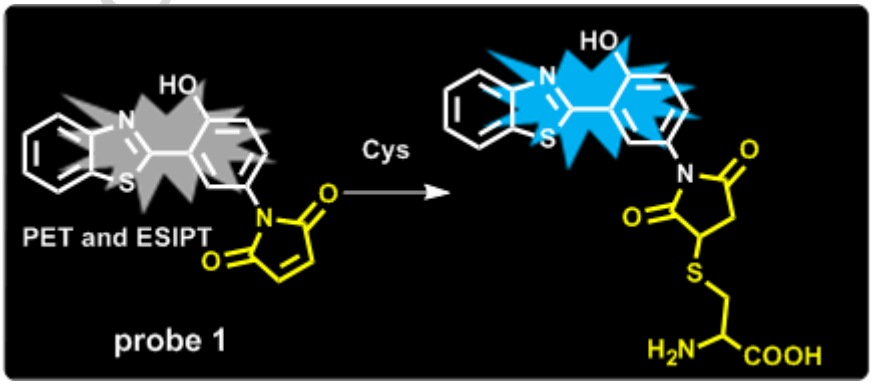

Graphical abstract

Highlights

• A new fluorescent probe **1** based on a 2-(Benzothiazol-2-yl) phenol (HBT) derivative was synthesized and characterized.
• The limit of detection of probe **1** was as low as 3.78×10^{-8} M for Cys.
• The probe **1** exhibited a large large Stock's shifts in aqueous solution.
• The probe **1** was used as practical probe for imaging of Cys in living cell.