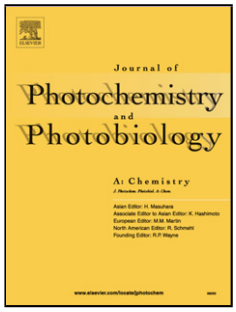

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A simple but effective fluorescent probe with large Stokes shift for specific detection of cysteine in living cells

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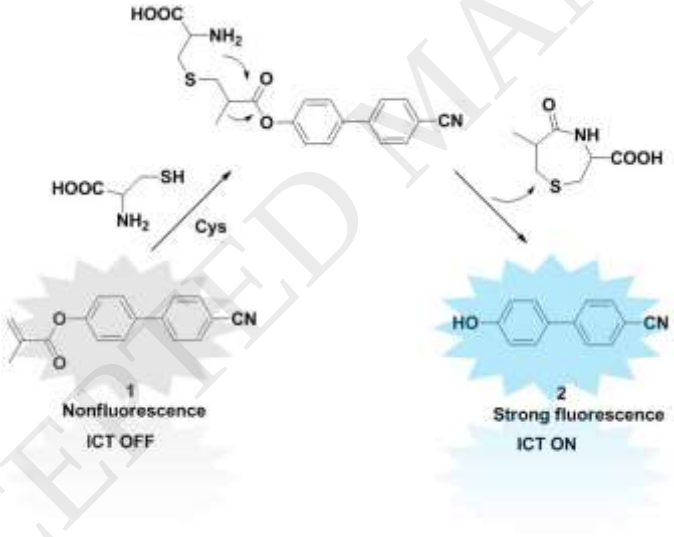
Graphic abstract



Highlights

- A 4'-hydroxybiphenyl-4-carbonitrile-based fluorescence probe has been successfully developed for highly selective, sensitive and rapid detection of Cys.
- The probe displayed a large Stokes shift (180 nm) in the detection of Cys.
- The probe could be used to detect and image both exogenous and endogenous Cys in living A549 cells

Abstract

A novel 4'-hydroxybiphenyl-4-carbonitrile-based fluorescent probe, **1**, for selective detection of cystein (Cys) over homocystein (Hcy) and glutathione (GSH) was developed. This probe had simple structure and could be easily synthesized with good yield from commercially available materials. Moreover, probe **1** showed a remarkable large Stokes shift (180 nm) and displayed a rapid (5 min) and highly sensitive response (the detection limit was 0.15 μM) for Cys with fluorescence turn-on signal changes (142-fold fluorescence enhancement). Importantly, probe **1** could be used to detect and image both exogenous and endogenous Cys in living A549 cells.

Keywords: Stokes shift; Fluorescent probe; Cell imaging; Cystein

1. Introduction

The small-molecular-weight thiols including cystein (Cys), homocystein (Hcy) and glutathione (GSH), is essential and the most plentiful amino acid in many physiological and pathological activities [1-3]. Alterations of the levels of these specific bio-thiols are implicated in a number of diseases. Although they have the similar chemistry structures, it is significant different to play fundamental roles in biological processes [4-8]. Among them, Cys is one of semi-essential amino acid relevant to amino acid transport, protein synthesis, cellular detoxification and metabolism in living systems [9-12]. It is reported that Cys deficiency could result in slow growth, hair pigmentation, edema, lethargy and liver damage. Moreover, the heighten level of Cys is associated with neurotoxicity [13-17]. Therefore, it is of great importance to monitor the level change of intracellular Cys for biochemical and clinic research.

Fluorescent detection using fluorescent probes has been recognized as one of the most powerful tools for the investigation of reactive species in living systems due to its simple manipulation, high sensitivity and the capability of the real-time detection [18-22]. In the past decade, various fluorescence probes have been designed and synthesized for the detection of biothiols [23-33]. These probes for the Cys sensing involve mainly the following three strategies: 1) cylization with aldehydes [34-36], 2) native chemical ligation [37], 3) conjugation addition with α, β–unsaturated carbonyl

[38-40]. Although these probes have made a significant contribution to the biothiols, there are still some limitations existing in the research. On one hand, most of them have a problem distinguishing Cys from GSH and Hcy because they have similar structure and reactivity. On the other hand, many of these reported fluorescent probes also show relatively small Stokes shift (<100 nm). As is well known, with the exception of an excellent selectivity, a large Stokes shift is also a very important attribute of a fluorophore because fluorescent dyes with large Stokes shifts can improve the detection sensitivity by avoiding fluorescence quenching originated from self-absorption and fluorescence errors [41-42].

On account of above considerations, we presented a novel sensitive and selective fluorescent probe **1** with a large Stokes shift (180 nm) for the imaging of Cys in living cells. This probe **1** utilized 4'-hydroxybiphenyl-4-carbonitrile, dye **2**, as the fluorescent platform, and employed methacrylate group as the response site for Cys. After the treatment of the probe **1** with Cys, an obvious cyan fluorescence signal was observed. Moreover, the probe **1** exhibited high selectivity to Cys over other bio-analytes such as GSH and Hcy. In particular, the biological applications were demonstrated that the probe **1** could be successfully applied for the imaging of Cys in living A549 cells.

2. Experiment

2.1. Materials and equipment

Unless otherwise stated, all reagents were purchased from commercial suppliers and

used as received. All accurate mass spectrometric experiments were performed on a Waters ® Xevo G2-S QTof™ mass spectrometer (Waters, Milford, MA, USA). Solvents were purified by standard methods prior to use. Twice-distilled water was used throughout all experiments. NMR spectras were recorded on a BRUKER 600 spectrometer, using TMS as an internal standard. UV-Vis absorption spectra were measured using a Shimadzu UV-2450 spectrophotometer. Uncorrected emission spectra were recorded at room temperature on a Shimadzu RF5301PC. Cell imaging was performed with a Zeiss LSM710 microscope. TLC analysis was performed on silica gel plates and column chromatography was conducted over silica gel (mesh 200–300), both of which were obtained from Qingdao Ocean Chemicals. pH measurements were carried out with a PHS-3C pH meter.

2.2. General procedure for spectral measurements

The stock solution of the probe **1** was prepared at 1 mM in DMSO. The solutions of various common amino acid (Ala, Gly, Asp, Arg, Gln, Lys, Met, Phe, Asn, Leu, Ser, His, Glu, IIe, Trp, Pro, Tyr, Val, Thr, Hcy, GSH, Cys) and ionic salt (NaCl, $CaCl_2$, $FeCl_3$, $MgCl_2$, $ZnCl_2$, NaClO, Na_2SO_4, $NaHSO_3$, NaOAc, Na_2CO_3, $NaNO_3$, Na_3PO_4) were prepared in twice-distilled water. The 3 mL for test solution was prepared by placing 0.03 mL of probe **1** stock solution and appropriate testing analyte in 1.8 mL of buffer soultion (7.4, 50 mM PBS). The resulting solution was shaken well and incubated for 5 min at room temperature before recording the spectra. For fluorescence measurements,

the excitation wavelength was 340 nm, the excitation slit widths and emission slit widths were both 5 nm.

2.3. Cell culture and confocal microscopy imaging

A549 cells were incubated in a 6-well plate in Dulbecco's modified Eagle's medium (DMEM) at 5% CO_2 and 37℃ for 24 h. Before the experiments, A549 cells were washed with PBS buffer three times. For the endogenous Cys experiment, A549 cells were incubated with probe **1** (10.0 μM) for 30 min at 37 °C, and then fluorescence imaging was performed after washing the cells with PBS buffer three times. For the exogenous Cys experiment, A549 cells were pre-incubated with 1.0 mM N-ethylmaleimide (NEM) for 30 min, and then incubated with probe **1** (10.0 μM) for 30 min at 37 °C. Finally, the cells incubated with Cys (100.0 μM) for 30 min at 37 °C. All the imaging was performed by using a Zeiss LSM710 microscope.

*2.2. Synthesis of probe **1***

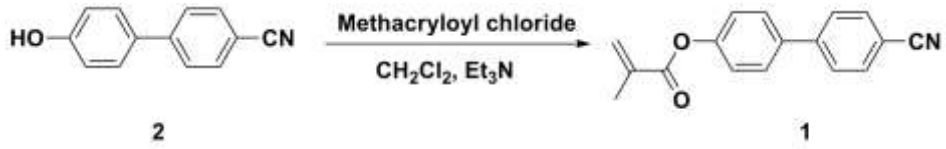
To a stirred solution of dye **2** (48.8 mg, 0.25 mmol) and triethylamine (10 μL) in 5 mL dry dichloromethane was added methacryloyl chloride (83 mg, 0.80 mmol). The resulting reaction mixture was allowed to stir at room temperature for 5 h and subsequently quenched with 20 mL of water. The resulting solution was extracted twice with 15 mL dichloromethane. After drying the mixture

over anhydrous Na_2SO_4, the drying agent was removed by filtration and the solvent was removed by distillation. The obtained residue was purified by flash chromatography on silica gel (CH_2Cl_2 as eluent) to yield probe **1** (59.9 mg, 91.1%). ^{1}H NMR (600 MHz, DMSO) δ 7.94 (d, *J* = 8.5 Hz, 2H), 7.91 (d, *J* = 8.4 Hz, 2H), 7.83 (d, *J* = 8.6 Hz, 2H), 7.33 (d, *J* = 8.6 Hz, 2H), 6.32 (s, 1H), 5.93 (s, 1H), 2.03 (s, 3H). ^{13}C NMR (150 MHz, DMSO) δ 165.68, 151.58, 144.22, 136.35, 135.67, 133.36, 128.80, 128.46, 128.04, 123.05, 119.32, 110.56, 18.53. HRMS (EI) m/z calcd for $[C_{17}H_{13}NO_2 + H]^+$: 264.1025, Found: 264.1017.

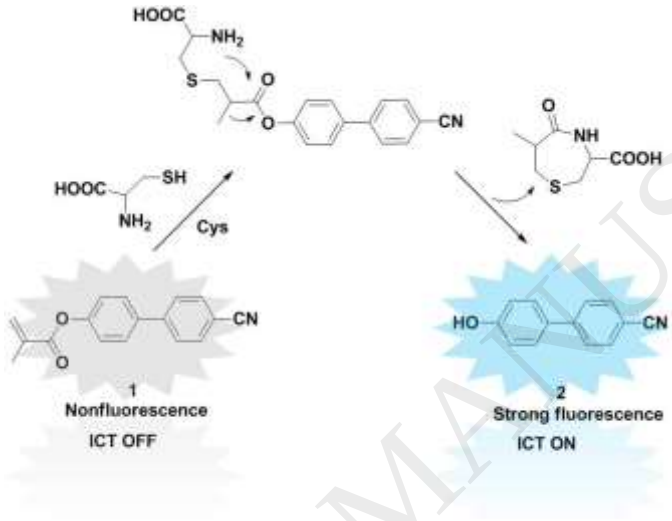
3 Results and discussion

*3.1. Design and synthseis of probe **1***

Probe **1** was designed to use 4'-hydroxybiphenyl-4-carbonitrile (dye **2**) as the fluorophore and methacrylate group as the response site (Scheme 1). Through introducing an electron-withdraw group (methacrylate group) which can attenuate the electron-donating ability of the 4'-hydroxy group, the internal charge transfer (ICT) effect of the fluorophore was weakened and resulted in the quenching of fluorescence. On the basis of the Cys-triggered addition-cyclization reaction [43], the methacrylate moiety can be cleaved to afford the 4'-hydroxy group and restored ICT effect of the fluorophore (Scheme 2). With these considerations in mind, a novel ICT-based fluorescent probe **1** for Cys was synthesized via only one step. The structure of probe **1** was confirmed by ^{1}H NMR, ^{13}C NMR and HRMS spectra (Fig. S3-S5).



Scheme 1 Synthetic route to probe **1**



Scheme 2 The proposed response mechanism of probe **1** to Cys.

3.2. Spectroscopic studies

Initially, we investigated the absorption and emission spectra of dye **2** and probe **1** in 50.0 mM PBS buffer (pH =7.4, containing 40% DMSO) at room temperature. Under this condition, dye **2** (10.0 μM) exhibited maximum absorption at 297 nm and strong fluoresces (λ_{em} = 477 nm). It's noteworthy that dyes **2** featured a large Stokes shift (180 nm), which can reduce the interference from incident light (Fig. 1). However, probe **1** (10.0 μM) showed an absorption band with a maximum at 276 nm and almost no fluorescence at 477 nm. When

treated with Cys, the solution of probe **1** showed a significant fluorescence which was identical to that of dye **2** (Fig. S1), suggesting that the addition of Cys resulted in the cleavage of methacrylate moiety in probe **1** to released dye **2**. To under the sensing process better, the fluorescence response of probe **1** (10.0 μM) to various concentrations of Cys were conducted (Fig. 2). With the increasing concentration of Cys (from 0.0 to 200.0 μM), the fluorescence intensity continuously increased. And a maximized enhancement (142-fold) was obtained when 20.0 equiv. of Cys was added. As displayed in Fig. 3, the plot between the fluorescence intensity ($I_{477\ nm}$) and the concentration of Cys (from 0.0 to 10.0 μM) presented a good linearity (R^2=0.9974). The limit of detection (LOD) was measured to be 0.15 μM on the basis of the definition of three times the deviation of the blank signal (S/N = 3). These results demonstrated that this turn-on probe **1** had a high sensitivity for Cys detection.

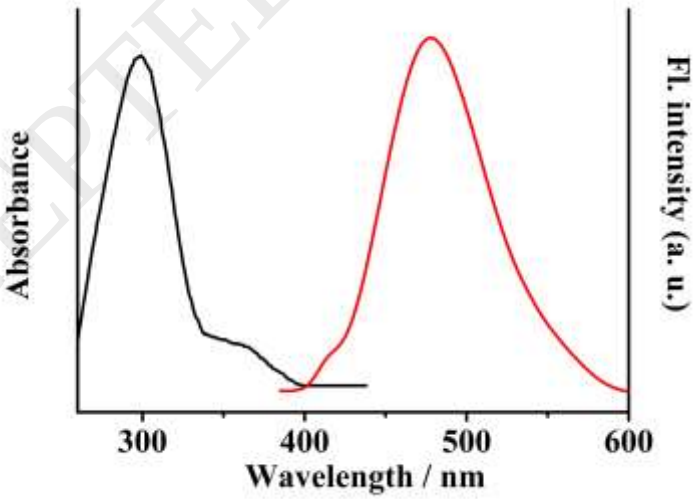


Fig. 1 UV-vis absorption (black) and fluorescence (red) spectra of dye **2** in PBS buffer.

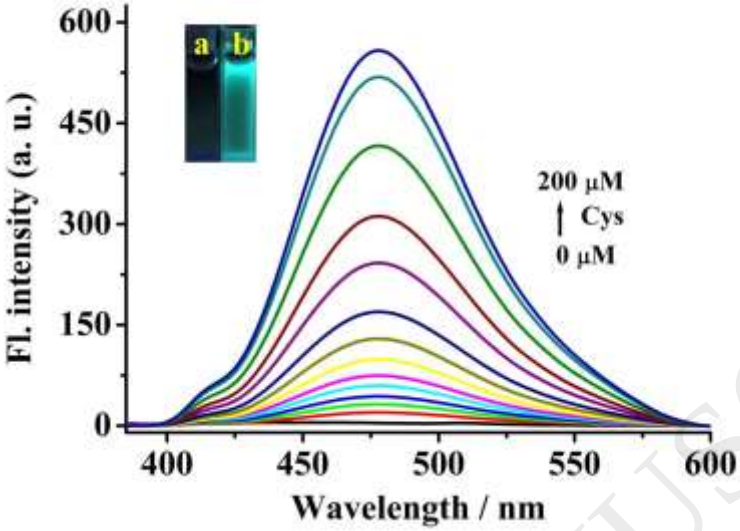


Fig. 2 Fluorescence response of probe **1** (10.0 μM) upon the addition of Cys (0.0–200.0 μM) in PBS buffer. Inset: the corresponding fluorescence images of probe **1** (10.0 μM) in the absence (left) and presences (right) of Cys (200.0 μM) under a 365 nm UV lamp.

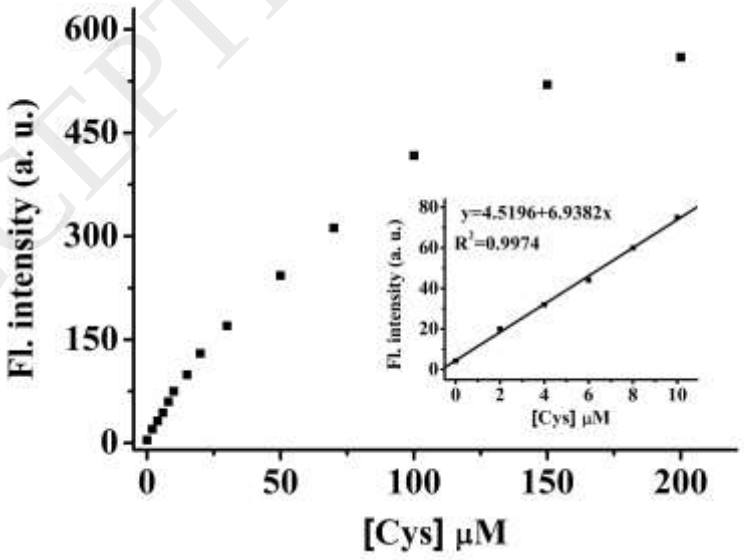


Fig. 3 Fluorescence intensity of probe **1** (10.0 μM) at 477 nm as a function of Cys concentration (0.0–200.0 μM) in PBS buffer. Inset: the linear relationship between fluorescence intensity and Cys at low concentrations.

3.3. Selectivity studies

In order to examine whether probe **1** can selectively detect Cys among other physiological amino acids, bio-relevant anions and ions, we then investigated the fluorescence spectra of probe **1** in the presence of other species (Fig. 4, Fig. S2). The addition of 20.0 equiv. of physiological amino acids (Ala, Gly, Asp, Arg, Gln, Lys, Met, Phe, Asn, Leu, Ser, His, Glu, IIe, Trp, Pro, Tyr, Val, Thr, Hcy, GSH), anions (ClO^-, SO_4^{2-}, HSO_3^-, AcO^-, CO_3^{2-}, NO_3^-, PO_4^{3-}) and ions (Na^+, Ca^{2+}, Fe^{3+}, Mg^{2+}, Zn^{2+}) did not significantly change the fluorescence of probe **1**. In contrast, when probe **1** was treated with 20.0 equiv. of Cys, a dramatic fluorescence response was observed. Moreover, competitive experiment revealed that the coexistence of these relevant species almost had no effects on the performance of probe **1** in the detection of Cys. From the above results, it could be concluded that probe **1** showed excellent selectivity towards Cys over other species.

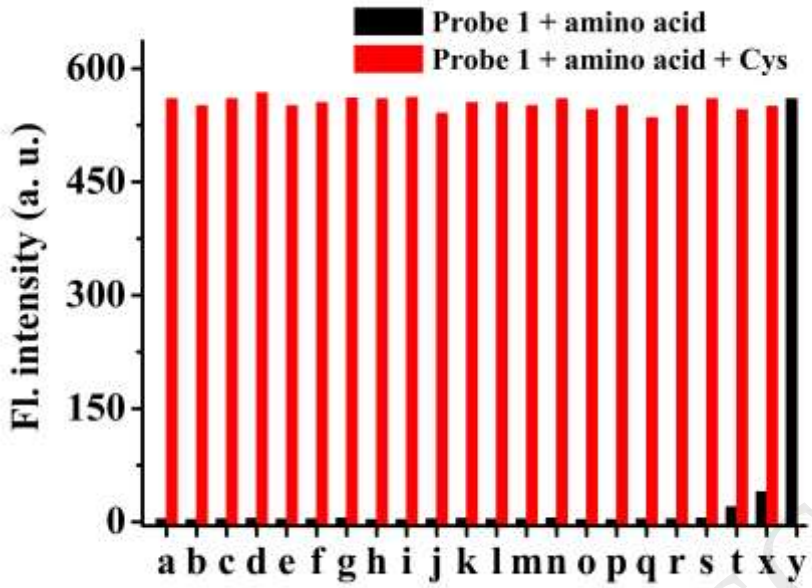


Fig. 4 The fluorescence intensity at 477 nm of probe **1** (10.0 μM) upon the addition of the various common amino acid (a-y: 200.0 μM for Ala, Gly, Asp, Arg, Gln, Lys, Met, Phe, Asn, Leu, Ser, His, Glu, IIe, Trp, Pro, Tyr, Val, Thr, Hcy, GSH, Cys) (black). The fluorescence intensity at 477 nm of probe **1** (10.0 μM) to Cys (200.0 μM) with the competition amino acid in PBS buffer (a-x: 200 μM for Ala, Gly, Asp, Arg, Gln, Lys, Met, Phe, Asn, Leu, Ser, His, Glu, IIe, Trp, Pro, Tyr, Val, Thr, Hcy, GSH) (red).

3.4. Effect of reaction time

The time-dependent fluorescence spectra of probe **1** (10.0 μM) in the presence of different amounts of Cys (0.0 μM, 6.0 μM, 20.0 μM, 50.0 μM, 100.0 μM, 200.0 μM) were studied by measuring the fluorescence intensity at 477 nm along with the time. As depicted in Fig. 5, upon the treatment with 20.0 equiv.

of Cys, the fluorescence intensity increased immediately and leveled off with 5 min. However, negligible changes in the fluorescence intensity at 477 nm were detected in the absence of Cys for the same interval of time. These findings indicated that this probe **1** could be acted as a rapid response fluorescent probe to detect Cys.

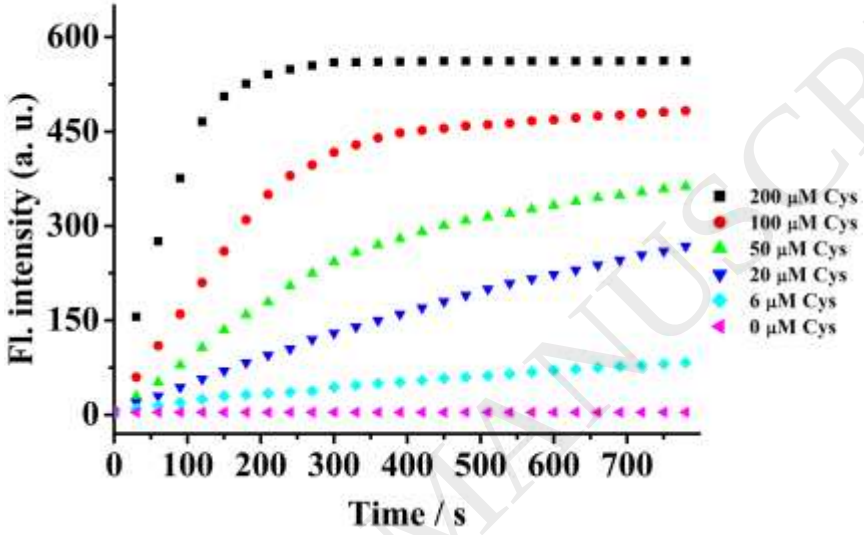


Fig. 5 Time-dependent fluorescence intensity of probe **1** (10.0 μM) at 477 nm reacted with the different concentration of Cys (0.0, 6.0, 20.0, 50.0, 100.0, 200.0 μM) in PBS buffer.

3.5. Effect of pH

The pH effect on the fluorescence spectra of probe **1** (10.0 μM) toward Cys (200.0 μM) was also investigated in aqueous solution. As shown in Fig. 6, the fluorescence intensity of probe **1** remained constant while the pH changes from 2 to 12, which indicated that probe **1** itself was pH-insensitive. On the contrary,

in the presence of Cys, the fluorescence intensity at 477 nm was intensively increased with the pH changes from 6 to 9. These results suggested that probe **1** had potential to be used in physiological environment.

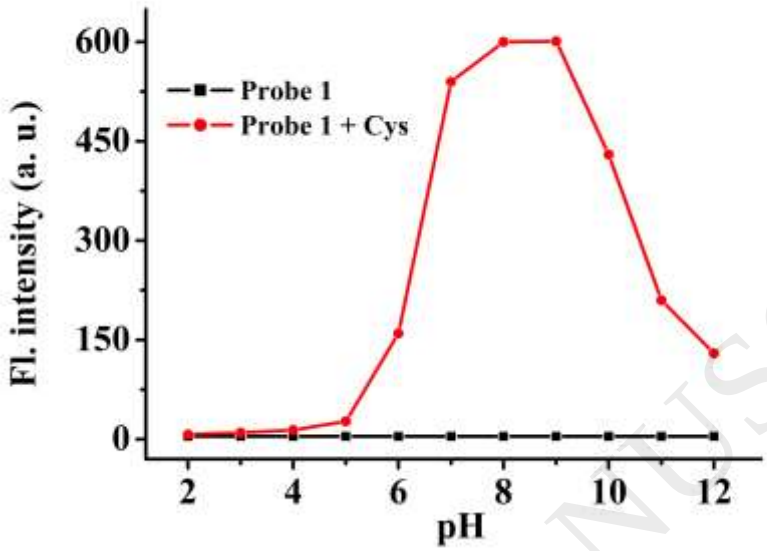


Fig. 6 pH effect on the fluorescence intensity of only probe **1** (10.0 μM) (black) and in the presence (red) of Cys (200.0 μM) at 477 nm.

3.6. Reaction mechanism

Based on our experimental results and reported literatures [44-46], the reaction mechanism of probe **1** toward Cys was proposed as follows: upon addition of Cys into the assay system, the ester bond cleaved by Cys turned probe **1** into dye **2**. This was evidenced by HPLC (Fig. 7). The HPLC chromatogram of probe **1** showed a single peak with a retention time at 1.89 min and it was 0.53 min for dye **2**. Once probe **1** was treated with 3.0 equiv. of Cys, the peak at 1.89 min decreased and a new peak at 0.53 min occurred. The addition of 20.0 equiv.

of Cys to the solution of probe **1** induced the disappearance of the peak at 1.89 min, indicating that probe **1** was completely converted into dye **2**. Additional support for the reaction mechanism comes from the results of a HRMS study. The mass spectral peak of the fluorescent product (**1**+Cys) at m/z 196.0753 correspond to $[M+H^+]^+$ for dye **2** (Fig. S6). All the above results strongly support the proposed reaction mechanism depicted in Scheme 2.

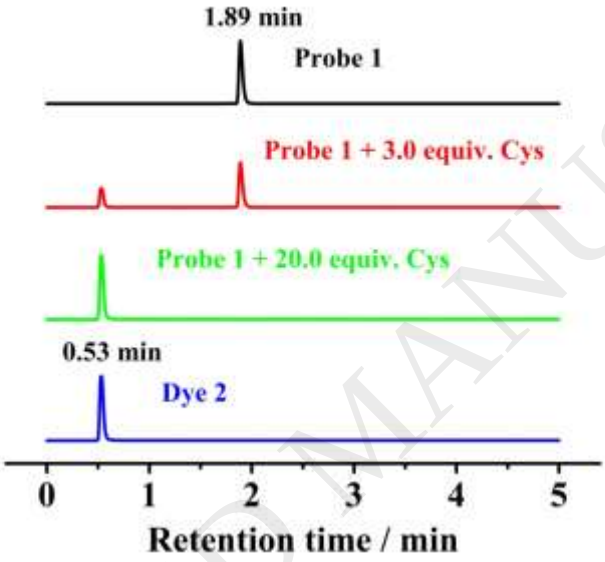


Fig. 7 The HPLC chromatograms: (black) Probe **1**; (red) Probe **1** with 3.0 equiv. of Cys incubated for 10 min; (green) Probe **1** with 20.0 equiv. of Cys incubated for 10 min; (blue) Dye **2**. Condition: eluent, H_2O/CH_3CN (v/v, 5/5), flow rate, 1.0 mL/min; temperature, 25℃; injection volume, 20.0 μL.

3.7. Fluorescent imaging in living A549 cells

To further demonstrate the practical applicability of probe **1** in biological systems, we carried out to investigate the ability of probe **1** to visualize Cys in living A549 cells (Fig. 8). As expected, Cyan fluorescence was observed inside the cells when living A549 cells were incubated with probe **1** (10.0 μM) for 30 min at 37℃, indicating that probe **1** had permeated into cells and reacted with endogenous Cys to produce discernible fluorescence. However, there was almost no fluorescence when A549 cells were pretreated with 1.0 mM N-ethylmaleimide (NEM) and then incubated with probe **1** (10.0 μM) for additional 30 min. When these cells were further incubated with Cys (100.0 μM) for 30 min, cyan fluorescence inside the cells appared again. These fluorescence imaging experiments showed that probe **1** had great potential for biological applications.

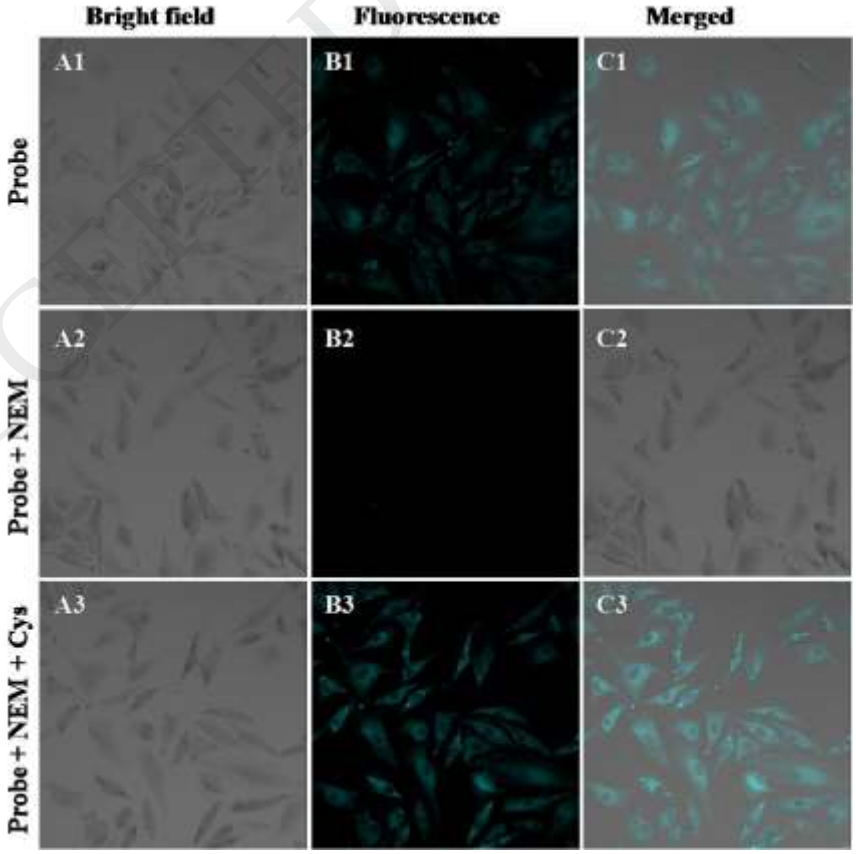


Fig. 8 Confocal fluorescence contrast images (A1) bright image (B1) fluorescence image and (C1) overlay image of living A549 cells only incubated with 10.0 μM probe **1** for 30 min at 37℃; (A2) bright image (B2) fluorescence image and (C2) overlay image of living A549 cells pretreated with 1.0 mM NEM and then incubated with 10.0 μM Probe **1**; (A3) bright image (B3) fluorescence image and (C3) overlay image of living A549 cells pretreated with 1.0 mM NEM and then incubated with 10.0 μM Probe **1** and 100.0 μM Cys.

4. **Conclusions**

In conclusion, we presented the design, synthesis and preliminary evaluation of the methacrylate of 4'-hydroxybiphenyl-4-carbonitrile, probe **1**, as a turn-on fluorescent probe for the detection of Cys in aqueous solution. The reaction mechanism was confirmed by means of HPLC and HRMS. The large Stokes shift (180 nm), fast response (5 min) and high sensitivity (the detection limit was 0.15 μM) of probe **1** make it a practical and reliable method for fluorescence imaging. Furthermore, the practical application of probe **1** for the selective detection of Cys was successfully demonstrated in living A549 cells.

Acknowledgments

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