ACS APPLIED MATERIALS & INTERFACES

Research Article

www.acsami.org

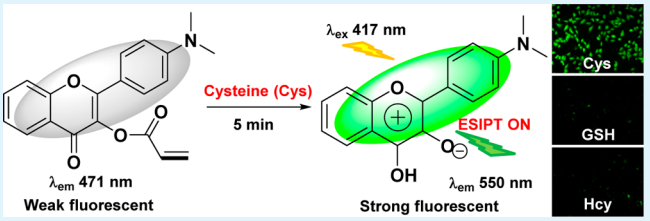
Rapid and Ratiometric Fluorescent Detection of Cysteine with High Selectivity and Sensitivity by a Simple and Readily Available Probe

Yao Liu, Dehuan Yu, Shuangshuang Ding, Qi Xiao, Jun Guo, and Guoqiang Feng*

Key Laboratory of Pesticide and Chemical Biology of Ministry of Education, College of Chemistry, Central China Normal University, 152 Luoyu Road, Wuhan 430079, People's Republic of China

Supporting Information

ABSTRACT: We report a simple and readily available fluorescent probe for rapid, specific, and ratiometric fluorescent detection of the biologically important cysteine (Cys). This probe uses a visible-light excitable excited-state intramolecular proton transfer (ESIPT) dye (4′-dimethylamino-3-hydroxyflavone) as the fluorophore and an acrylate group as the ESIPT blocking agent as well as the recognition unit. Cleavage of the acrylate moiety can be achieved specifically and rapidly by Cys in aqueous solution under mild conditions, which leads to restore the ESIPT process and enables the probe to show a rapid, ratiometric fluorescent detection process for Cys with high selectivity over various analytes, including homocysteine (Hcy) and glutathione (GSH). The detection limit of this probe for Cys was found to be ∼0.2 μM and bioimaging of intracellular Cys by this probe was successfully applied in living cells, indicating that this probe holds great potential for biological applications.



KEYWORDS: *fluorescent probe, ratiometric, cysteine, ESIPT, selectivity, bioimaging*

INTRODUCTION

The development of small-molecular probes for detection of amino acids has been an active research area in recent years, because of their biological importance.[1] Among them, cysteine (Cys) is a thiol-containing amino acid that plays important roles in protein synthesis, detoxification, and metabolism.[2,3] Abnormal levels of Cys are associated with many human diseases such as slow growth in children, hair depigmentation, edema, lethargy, liver damage, loss of muscle and fat, skin lesions, and weakness.[4] Therefore, rapid and reliable detection of Cys is of great importance and has attracted much attention.

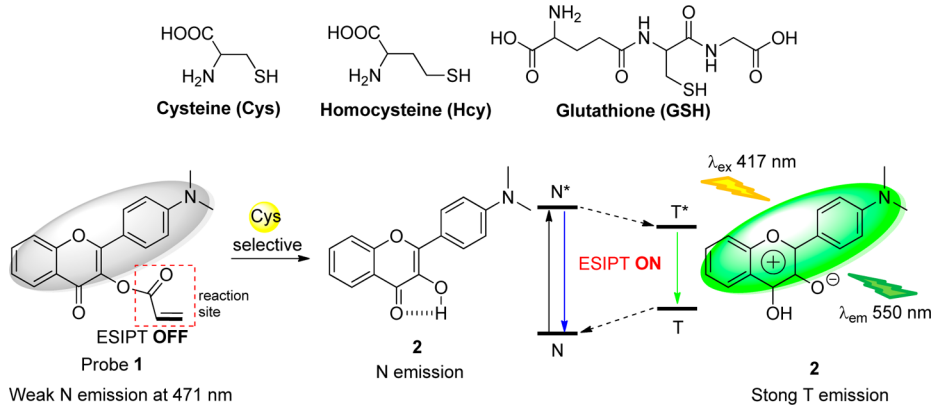
Among various detection techniques, fluorescent detection is particularly attractive, because of its simplicity, high sensitivity, low cost, and great potential for bioimaging. Although several fluorescent probes have been developed for fluorescent detection of amino acid[5,6] and biothiols,[7−18] most of them showed low selectivity for Cys. A major problem for those probes is that they cannot discriminate Cys from other biothiols such as homocysteine (Hcy) and glutathione (GSH), as these biothiols have similar structures (Scheme 1) and reactivity. Besides, the intracellular level of GSH (1−10 mM) is much higher than that of Cys (30−200 μM),[19,20] which also makes the specific detection of Cys over GSH difficult. However, since these biothiols play different important biological roles, discrimination of Cys from Hcy and GSH is important. In fact, only until recently, a few fluorescent probes were reported to be able to detect Cys with improved selectivity over Hcy and GSH.[21−36] However, most of these Cys-selective probes are based on fluorescence measurement at a single wavelength, which may be influenced by many factors such as instrument efficiency and environmental conditions. By contrast, ratiometric fluorescent probes have broader utility than intensity-based probes, because they allow the measurement and analysis using the ratio at two different emission wavelengths, instead of the absolute intensity at only one wavelength, which could provide a built-in correction and thus make the analysis more accurate. Although several fluorescent probes have been reported to show ratiometric fluorescent signal changes for Cys, they are generally not highly specific for Cys.[37−48] As a result, available highly selective ratiometric fluorescent probes for Cys are still very rare to date.[22,29,32,33] Nevertheless, almost all of these Cys-selective ratiometric fluorescent probes suffer from a long response time (>40 min) to obtain maximum fluorescence signal changes,[22,29,32,33] and, besides, some of them need two excitation waves to achieve ratiometric fluorescent change[32] or need short UV light excitation.[29,33] Considering the limited number and drawbacks of the existing selective ratiometric fluorescent probes for Cys, new ratiometric fluorescent probes for rapid and highly selective detection of Cys are therefore still expected to be developed.

Herein, we report a new fluorescent probe that can be used for rapid, specific, and ratiometric fluorescent detection of Cys (probe **1** in Scheme 1). This new probe is based on a known excited-state intramolecular photon transfer (ESIPT) dye (compound **2** in Scheme 1) as the fluorophore and an acrylate

Received: May 21, 2014
Accepted: September 25, 2014
Published: September 25, 2014

   dx.doi.org/10.1021/am505501d | ACS Appl. Mater. Interfaces 2014, 6, 17543−17550

Scheme 1. Structures of Cys, Hcy, GSH, and the Concept of Probe 1 for Sensing of Cys



group as the reaction site. Notably, this probe shows several impressive sensing properties. First and most important of all, this probe is highly selective for Cys over various other amino acids and anions including Hcy and GSH. Second, it offers a rapid (within minutes) and sensitive detection process for Cys in aqueous solution with a bright yellow-green fluorescence turn-on signal output and a submicromolar detection limit. Third, this probe can offer a ratiometric fluorescent measurement for Cys, which makes it more attractive than intensity-based fluorescent Cys probes. In addition, this probe is simple, readily available and visible-light-excitable with a large Stokes shift (>130 nm), and can be used for fluorescence imaging of Cys in living cells with low cytotoxicity. All of these merits indicate that this new probe appears to be a very promising fluorescent probe for Cys.

EXPERIMENTAL SECTION

Materials and Chemicals. All chemical reagents were purchased from commercial suppliers and used without further purification. All solvents were purified prior to use. The stock solutions of probe **1** were prepared in high-performance liquid chromatography (HPLC)-grade dimethylsulfoxide (DMSO). All other solutions and buffers were prepared with distilled water that had been passed through a water ultrapurification system. Thin-layer chromatography (TLC) analysis was performed using precoated silica plates. IR spectra were recorded on a FT-IR spectrophotometer as KBr pellets and were reported in units of cm^{-1}. ^1H NMR and ^{13}C NMR spectra were obtained on a Varian Mercury 400 spectrometer, and resonances (δ) are given in parts per million relative to tetramethylsilane (TMS). The low-resolution MS spectra were performed on an electron ionization mass spectrometer. HR-MS data were obtained with an LC/Q-TOF MS spectrometer. Absorption and fluorescence spectra were recorded on a UV-vis and a fluorescence spectrophotometer at 25 °C using standard quartz cuvettes with a 10-mm lightpath, respectively. Cell imaging was performed in an inverted fluorescence microscope with a 20× objective lens.

Synthesis of Compound 2. Compound **2** was prepared from 2-hydroxyacetophenone and 4-(dimethylamino)benzaldehyde, according to our recently published procedure.[49]

Synthesis of Probe 1. To a solution of compound **2** (140 mg, 0.5 mmol) in dry dichloromethane (10 mL) was added acryloyl chloride (65 μL) and Et_3N (139 μL). The resulting mixture was stirred at room temperature until the reaction was complete (monitored by TLC in a silica plate, the R_f of the starting material **2** is 0.46 using hexane/ethyl acetate 3:1 (v/v) as mobile phase). Water (15 mL) was used to wash the resulting solution three times, and the dichloromethane phase was dried over Na_2SO_4. After filtered and removal of the organic solvent, a yellow solid product was formed, which can be further purified by recrystallization from methanol to afford the pure product (122 mg, 73%). Mp, 184−186 °C; TLC (silica plate): R_f, 0.26 (hexane/ethyl acetate 3:1, v/v); ^1H NMR (400 MHz, $CDCl_3$, Me_4Si): δ (ppm) 8.25 (dd, J = 7.9, 1.4 Hz, 1H), 7.85 (d, J = 9.0 Hz, 2H), 7.66 (dd, J = 11.3, 4.3 Hz, 1H), 7.53 (d, J = 8.4 Hz, 1H), 7.39 (t, J = 7.5 Hz, 1H), 6.74 (d, J = 9.0 Hz, 2H), 6.67 (d, J = 17.3 Hz, 1H), 6.43 (dd, J = 17.3, 10.5 Hz, 1H), 6.06 (d, J = 10.6 Hz, 1H), 3.06 (s, 6H, $2CH_3$); ^{13}C NMR (100 MHz, $CDCl_3$, Me_4Si): δ (ppm) 171.6, 163.1, 156.7, 155.3, 151.9, 133.3, 133.2, 131.9, 129.6, 127.2, 125.9, 124.7, 123.5, 117.7, 116.4, 111.2, 39.9 (CH_3); IR (KBr) ν_{max} (cm^{-1}): 2923, 1748, 1634, 1597 (s), 1563, 1529, 1466, 1442, 1409, 1370 (s), 1291, 1245, 1205, 1131 (s), 822, 795, 768; MS (EI) m/z (%): 335.32 (M^+, 23%), 281.22 ((M−acryloyl)$^+$, 100%). HR-MS (ESI) calculated for $C_{20}H_{18}NO_4^+$ ($M+H^+$), 336.1230; found, 336.1233.

Preparation of Solutions of Probe 1 and Analytes. Stock solutions of probe **1** (1 mM) was prepared in HPLC-grade DMSO. Stock solutions of Cys (2.5 mM), methionine (Met), leucine (Leu), isoleucine (Ile), tyrosine (Tyr), threonine (Thr), tryptophan (Trp), alanine (Ala), phenylalanine (Phe), serine (Ser), histidine (His), aspartic (Asp), pyroglutamic acid (Pyr), glycine (Gly), glutamic acid (Glu), lysine (Lys), arginine (Arg), and *N*-acetyl-cysteine (NAC) (5 mM of each) as well as anions F^-, Cl^-, Br^-, I^-, NO_3^-, NO_2^-, AcO^-, SCN^-, CO_3^{2-}, CN^-, SO_4^{2-}, SO_3^{2-}, $S_2O_3^{2-}$, HS^- (sodium salt, 50 mM of each), and $HSCH_2CH_2OH$ (5 mM) were prepared in distilled water (10 mL). The stock solutions of analytes were diluted to desired concentrations with distilled water when needed.

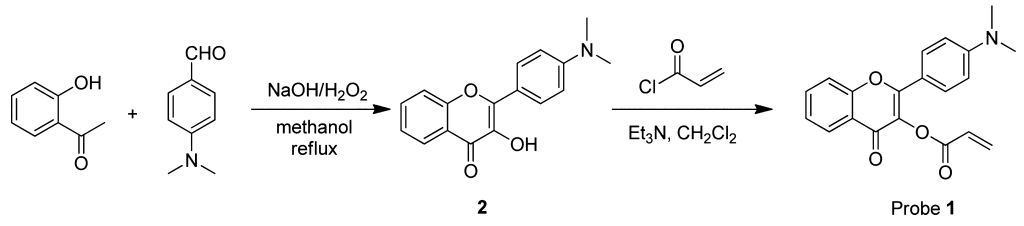
Optical Studies of Probe 1 upon Addition of Various Analytes. For a typical optical measurements, probe **1** was diluted to 20 μM in a DMSO-H_2O solution (1:4, v/v, 20 mM PBS, pH 7.4), and 3.0 mL of the resulting solution was placed in a quartz cell. The UV-vis or fluorescent spectra were then recorded upon the addition of analytes at 25 °C.

Cell Culture and Imaging. HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS (fetal bovine serum), 100 mg/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2, water-saturated incubator at 37 °C. Before cell imaging experiments, HeLa cells were seeded in 12-well culture plate for one night. For living cell imaging experiments of probe **1**, cells were incubated with 10 μM of probe **1** (with 1% DMSO, v/v) for 15 min at 37 °C and washed three times with prewarmed PBS buffer, and then imaged immediately (fluorescence imaging with exciting light at 420−485 nm). For *N*-ethylmaleimide (NEM)-treated experiments, HeLa cells were pretreated with 0.5 mM of NEM for 30 min at 37 °C, washed three times with prewarmed PBS buffer, and then incubated with 10 μM of probe **1** (or incubated with indicated concentration of Cys for 15 min prior to addition of probe **1** for a control experiment) for 15 min at 37 °C. Cell imaging was then carried out after washing cells with prewarmed PBS buffer.

RESULTS AND DISCUSSION

1. Design and Synthesis. Probe **1** is designed to use an excited-state intramolecular proton transfer (ESIPT) dye (4′-

Scheme 2. Synthesis of Probe 1



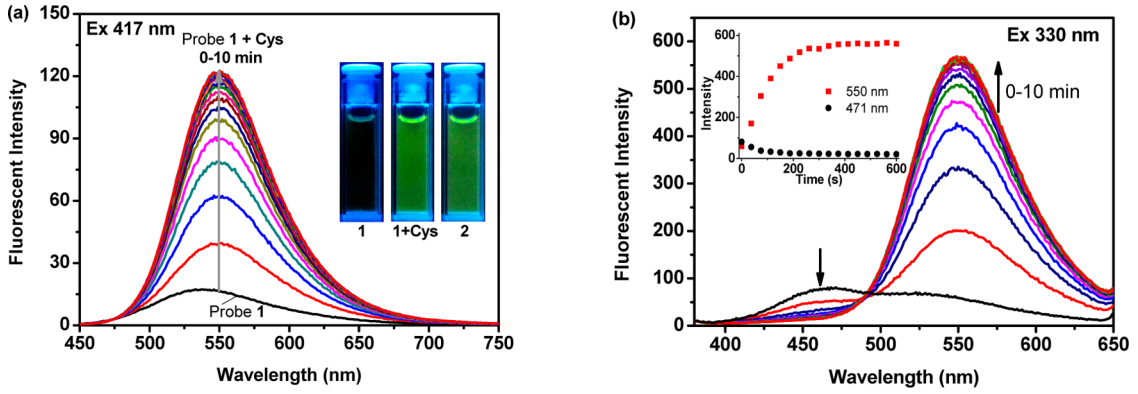


Figure 1. Fluorescent spectra changes of probe **1** (20 μM) in the presence of Cys (5 equiv) in PBS buffer (20 mM, pH 7.4) solution (DMSO/water = 1/4, v/v) at 25 °C. (a) λ_{ex} = 417 nm, slit width = d_{ex} = d_{em} = 5 nm; (b) λ_{ex} = 330 nm, slit width = d_{ex} = d_{em} = 10 nm. (Note that, because using 330 nm as the excitation wavelength resulted in relatively lower fluorescence intensity, a bigger slit width was used in panel b during the fluorescence measurement).

dimethylamino-3-hydroxyflavone, compound **2** in Scheme 1) as the fluorophore, because this dye is visible light excitable and has advantage of showing a large Stokes shift and strong green fluorescence with good fluorescence quantum yield.[49−51] Despite the fact that ESIPT dyes have been widely used to design chemosensors,[52−57] selective ESIPT probe for Cys is surprisingly rare and the existing ESIPT probes[29,36] for Cys need UV excitation, which is a disadvantage for the applications with visible-light excitation. Extending our interest in exploring this visible-light-excitable ESIPT dye,[49] an acrylate group was introduced in this work as it is simple, and more importantly, an acrylate moiety has been reported to be able to discriminate Cys from Hcy and GSH by different reaction kinetics during the conjugate addition−cyclization reaction between acrylate and biothiols under mild conditions.[29−32] Therefore, we expected that protection of the hydroxyl group in compound **2** by an acrylate group can block the ESIPT process and result in quenching of the tautomer emission (T emission at longer wavelength) but remaining normal-type emission (N emission at shorter wavelength), and regeneration of compound **2** can be selectively achieved by Cys to restore the ESIPT process and recover the strong T emission (Scheme 1). Thus, a selective ratiometric fluorescent detection of Cys is likely to achieve. It should be noted that a flavone-based ESIPT probe for Cys was recently published[33] during our preparation of this manuscript; however, this probe suffered from short UV light excitation (Ex 350 nm was used), a long response time (1 h), and not particularly desirable selectivity and sensitivity for Cys (Hcy and GSH showed considerable interference and the detection limit for Cys was found only to be 1 μM) under the reported testing conditions (in an aqueous PBS buffer solution containing 50% CH_3CN, v/v).

Indeed, probe **1** can be easily prepared in good yield (73%) by the acrylation of compound **2** with acryloyl chloride in the presence of triethylamine in CH_2Cl_2, and it shows excellent sensing properties for Cys as expected (see below). Compound **2** was prepared from 2-hydroxyacetophenone and 4-(dimethylamino)benzaldehyde, according to our published procedure[49] (Scheme 2). It is worth noting that the synthetic procedure for probe **1** is simple, as no column chromatography is needed in the purification process. Detailed synthetic procedures and structure characterizations are given in the Experimental Section and in the Supporting Information.

2. Rapid and Ratiometric Fluorescent Detection of Cys. The sensing ability of probe **1** for Cys was investigated in 20 mM PBS buffer (pH 7.4) with 20% DMSO (v/v) at 25 °C. Under this condition, probe **1** (20 μM) shows maximum absorption at 405 nm ($\varepsilon \approx 3.1 \times 10^4$ M^{-1} cm^{-1}), and is weakly fluorescent ($\Phi < 0.001$; see the Supporting Information). Upon addition of Cys, although the absorption spectra change is not very noticeable, because of the small difference between the absorption spectrum of probe **1** and compound **2** (the expected product, which shows λ_{max} = 409 nm with $\varepsilon \approx 2.9 \times 10^4$ M^{-1} cm^{-1}) under the test conditions (see Figure S1a in the Supporting Information), the fluorescent signal of probe **1** showed remarkable changes. As shown in Figure 1a, upon addition of Cys, a new red-shifted emission peak at 550 nm emerged and a large fluorescence enhancement was observed within a few minutes if the solution was excited using visible light at 417 nm. Meanwhile, the solution started to show a bright yellow-green fluorescence, which is highly visible to the naked eye and appears exactly the same as that of compound **2** (observed under a portable 365-nm UV light (see Figure 1a, inset); for compound **2**, Φ = 0.025 (see the Supporting Information)). Kinetic studies showed that the emission intensity of the probe **1** solution at 550 nm gradually increased against time until it reached a plateau at ~5 min under this condition, and the observed pseudo-first-order rate constant

dx.doi.org/10.1021/am505501d | ACS Appl. Mater. Interfaces 2014, 6, 17543−17550

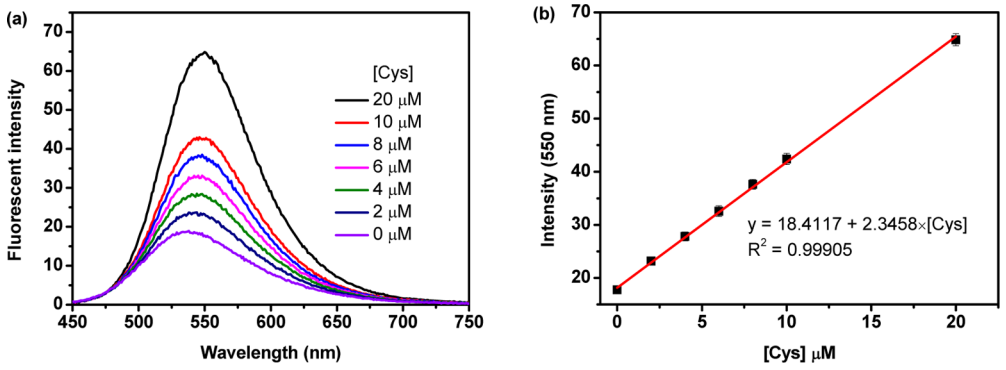


Figure 2. (a) Fluorescence spectra changes of probe **1** (20 μM) upon addition of Cys (0–20 μM) in PBS buffer (20 mM, pH 7.4) with 20% DMSO at 25 °C. Each spectrum was obtained 10 min after Cys addition. λ_{ex} = 417 nm; slit width = d_{ex} = d_{em} = 5 nm. (b) A linear relationship of fluorescence intensity changes at 550 nm of probe **1** against [Cys] from 0 to 20 μM, which can be linearly fitted by the equation y = 18.4117 + 2.3458 × [Cys], with R^2 = 0.99905.

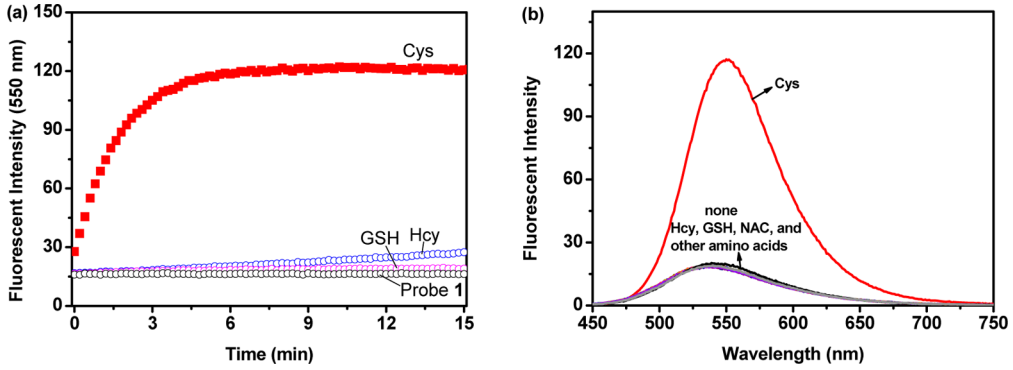


Figure 3. (a) Comparison of fluorescent kinetics data of probe **1** (20 μM) with 5 equiv of Cys, Hcy, and GSH in PBS buffer (20 mM, pH 7.4) with 20% DMSO at 25 °C. All the reactions were monitored at λ_{em} = 550 nm. (b) Fluorescent spectra changes of probe **1** (20 μM) in the presence of various analytes (5 equiv) in PBS buffer (20 mM, pH 7.4) with 20% DMSO at 25 °C. Other analytes are Met, Lev, lle, Tyr, Thr, Trp, Ala, Phe, Ser, His, Asp, Pyr, Gly, Glu, Lys, Arg, Hcy, GSH, and NAC.

k_{obs} for the reaction of probe **1** with Cys was determined to be ~0.557 min^{-1} ($t_{1/2}$ = 1.244 min; see Figure S1b in the Supporting Information). This fast and distinct fluorescence signal change indicates that probe **1** can be used as a rapid and sensitive fluorescent turn-on sensor to detect Cys within minutes in aqueous solution at physiological pH.

Notably, when being excited with a shorter wavelength light at 330 nm, probe **1** showed an apparent ratiometric fluorescence response upon addition of Cys. As shown in Figure 1b, the emission of probe **1** at 471 nm (N emission) gradually decreases while simultaneously the longer wavelength emission at 550 nm gradually increases, and the ratiometric value of the emission intensities ($I_{550/471}$) was found to have a large increase (nearly 40-fold from 0.7116 to 27.5376) when the reaction was completed after 5 min. Clearly, this indicates that probe **1** can be used as a ratiometric fluorescent sensor for rapid detection of Cys. In addition, we can see that, either probe **1** is excited at 417 or 330 nm, it shows remarkable large Stokes shift (>130 nm) during sensing of Cys, which can be ascribed to the ESIPT process shown in Scheme 1. It should be noted that a large Stokes shift for a fluorescent probe is highly desirable, because this can effectively reduce the measurement error caused by the excitation light and scattered light. Thus, we not only established a fluorescent turn-on probe, but also a ratiometric fluorescent probe for rapid detection of Cys in aqueous solution with a remarkably large Stokes shift.

To obtain information on the pH effects, the fluorescence intensity changes of probe **1** (20 μM) at 550 nm in the absence and presence Cys (5 equiv) in a time scale of 5 min was investigated at different pHs. As shown in Figure S2a in the Supporting Information, the fluorescence of probe **1** itself is stable over a wide pH range (~4.5–10.0); however, it shows significant enhancement in the presence of Cys in a wide pH range from 6.5–10.0. This indicates that probe **1** is able to work over a relatively wide pH range including at physiological pH. In addition, it was found that the amount of DMSO can be largely reduced and reducing the amount of DMSO from 20% to 5% did not affect the reaction kinetics of probe **1** with Cys. However, the probe **1** sensing system was found to show relatively lower fluorescent intensities with less amount of DMSO (see Figure S2b in the Supporting Information). Therefore, the following studies (except cell imaging) of probe **1** for fluorescent sensing of Cys were still investigated in PBS buffer at pH 7.4 with 20% of DMSO (v/v) as co-solvent, but this does not mean that we cannot use a lesser amount of DMSO.

3. The Sensitivity. To shed light on the sensitivity of probe **1**, the reaction kinetics of probe **1** with different concentrations of Cys were then measured (see Figure S3 in the Supporting Information). We can see that, upon the addition of Cys, the reaction operates faster when the concentration of Cys increases, while the fluorescence of probe **1** itself is quite stable under the test conditions. Notably, even when the

reaction was under stoichiometric conditions, distinct fluorescence signal changes can be still observed within a few minutes (curve c in Figure S3 in the Supporting Information). In addition, a saturation curve can be observed after more than 5 equiv of Cys is added to the probe **1** solution, if measuring the fluorescence at 550 nm after 10 min upon the addition of different concentrations of Cys (see Figures S4a and S4b in the Supporting Information). Moreover, a linear calibration curve between the fluorescent intensity changes at 550 nm and the concentration of Cys in the range of 0−20 μM was observed (Figure 2), indicating that probe **1** can be potentially employed to detect Cys quantitatively. Thus, the detection limit of probe **1** for Cys was determined to be ~0.2 μM, based on the signal-to-noise ratio (S/N = 3) under the test conditions, which indicates that probe **1** has good sensitivity to detect Cys in biological systems. In fact, the detection limit of probe **1** for Cys is lower than that of most reported Cys-selective ratiometric fluorescent probes.[22,32,33] In addition, ratiometric fluorescent detection of Cys is also applicable upon addition of different concentrations of Cys (see Figure S4c in the Supporting Information) if the probe **1** solution is excited with shorter wavelength. In this case, a linear calibration curve between the relative emission intensities at 550 and 471 nm ($I_{550/471}$) and the concentration of Cys in the range of 0−50 μM can be observed, which further indicates that probe **1** can be potentially used to detect Cys sensitively and quantitatively (see Figure S4d in the Supporting Information).

4. The Selectivity. Most importantly, probe **1** shows high selectivity for Cys over Hcy and GSH. In sharp contrast to Cys, under the same conditions, the fluorescence changes of probe **1** upon addition of Hcy and GSH are almost negligible within 15 min (Figure 3a). Moreover, considering the relatively high concentration of biothiols such as GSH in living systems, we also examined the fluorescence signal changes of probe **1** with 1 mM of Hcy and 10 mM of GSH, respectively. The results indicate that the fluorescence responses of probe **1** to these high levels of biothiols are still negligible in the experimental time scale, if compared to that of using only 0.1 mM of Cys (see Figure S5 in the Supporting Information). These results clearly show that probe **1** is highly selective for Cys over Hcy and GSH, which is significant, because so far, available highly selective ratiometric fluorescent probes for Cys are very limited, as mentioned above.

In addition, the responses of probe **1** to various other amino acids (and some derivatives; see Figure 3b) such as Met, Leu, Ile, Tyr, Thr, Trp, Ala, Phe, Ser, His, Asp, Pyr, Gly, Glu, Lys, Arg, and NAC as well as other competitor analytes such as F^-, Cl^-, Br^-, I^-, NO_3^-, NO_2^-, AcO^-, SCN^-, CO_3^{2-}, CN^-, SO_4^{2-}, SO_3^{2-}, $S_2O_3^{2-}$, HS^-, and $HSCH_2CH_2OH$ are almost neglectable. As shown in Figure S6 in the Supporting Information), only addition of Cys resulted in a significant increase in the fluorescence intensity to the probe **1** solution (corresponding emission color changes, see Figure S7 in the Supporting Information). Moreover, because of the high selectivity and reactivity of probe **1** for Cys and the reversible Michael addition of thiols,[58] detection of Cys in the presence of other amino acids, Hcy, GSH, and NAC, and other analytes is still effective (see Figure 4, as well as Figure S6b in the Supporting Information). Therefore, all of these experiments indicate that probe **1** has high selectivity for Cys.

5. The Sensing Mechanism. The selective fluorescence signal responses of probe **1** for Cys suggest that Cys specifically triggered the cleavage of acryloyl group in probe **1** and simultaneously released compound **2**, which enables the ESIPT process to shift the fluorescence signal to a longer wavelength (from 471 nm to 550 nm). To confirm this, the reaction product of probe **1** with Cys was isolated and checked via TLC. The results showed that the reaction produced compound **2** (see Figure S8 in the Supporting Information), and this was further proved by the peak at 282.1 (mass calculated for **2** + H^+: 282.1) in the MS spectrum analysis (see Figure S9 in the Supporting Information). The reaction between acrylate and biothiols has been well-studied by Strongin et al.[29,30] and followed by others,[31−33] which involves a conjugate addition−cyclization reaction process. Based on these reports and our results, the mechanism of probe **1** for sensing of Cys is proposed in Scheme 3, in which the conjugate addition of thiol to the acryloyl group in probe **1** first occurred and then was followed by the cleavage of an ester bond to form the ESIPT dye **2** (the ESIPT process is thus restored) and release the cyclization product **3**. The structure of the isolated product **3** was proved by ^{1}H NMR spectrum (see Figure S10 in the Supporting Information), which was found to be identical to that of the previously reported reference compound.[29] The high selectivity of probe **1** for Cys over Hcy and GSH can be explained by the fact that Cys has a much higher reaction rate than Hcy and GSH in the step of intramolecular cyclization during the reaction between acrylate and thiols, because of the more kinetically favorable formation of a seven-membered ring product (**3**, n = 1).[29−33] Another possible factor that must be considered is the fact that probe **1** has a considerable steric effect around the acrylate moiety (adjacent to the acrylate moiety of the probe, there is a carbonyl group in one side, and a 4′-dimethylaminophenyl group in another side; see Scheme 3), which may potentially increase the kinetic difference between the reaction of Cys and Hcy with probe **1**. This feature can be also found in the structures of the reported highly selective single acrylate-functionalized probes for Cys,[29,32] which have more substituents around the acrylate moiety (in the carbons highlighted in Scheme 3) than those probes with relatively lower selectivity.[31,59,60] Taking all these factors into consideration, the mechanism of probe **1** for highly selective sensing of Cys is most likely a process, as shown in Scheme 3.

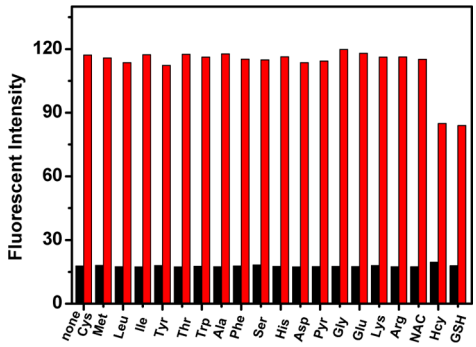
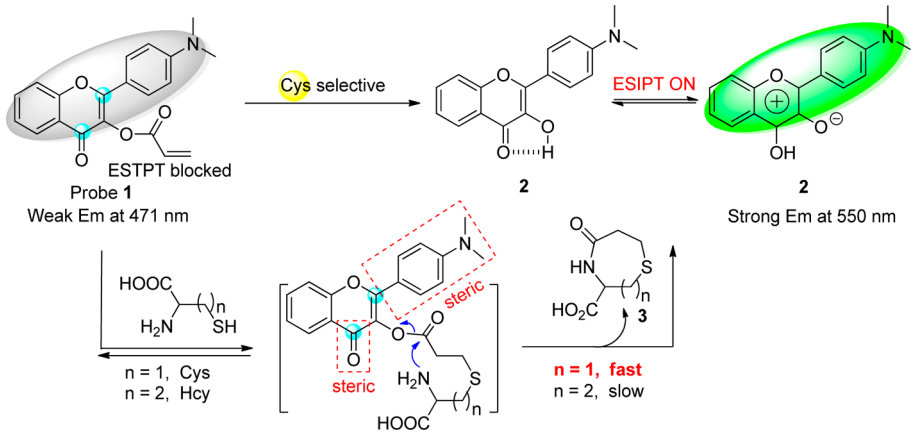


Figure 4. Fluorescent detection of Cys at 550 nm using probe **1** (20 μM) in the presence of various analytes (5 equiv). Black bars represent the addition of a single analyte including 100 μM of amino acid (None, Met, Lev, lle, Tyr, Thr, Trp, Ala, Phe, Ser, His, Asp, Pyr, Gly, Glu, Lys, Arg, NAC, Hcy, and GSH, respectively). Red bars represent the subsequent addition of Cys (100 μM) to the mixture. All experiments were performed in PBS buffer (20 mM, pH 7.4) with 20% DMSO at 25 °C and data was obtained 5 min after addition of each analyte. λ_{ex} = 417 nm; slit width = d_{ex} = d_{em} = 5 nm.

Scheme 3. Proposed Mechanism of Probe 1 for Highly Selective Sensing of Cys



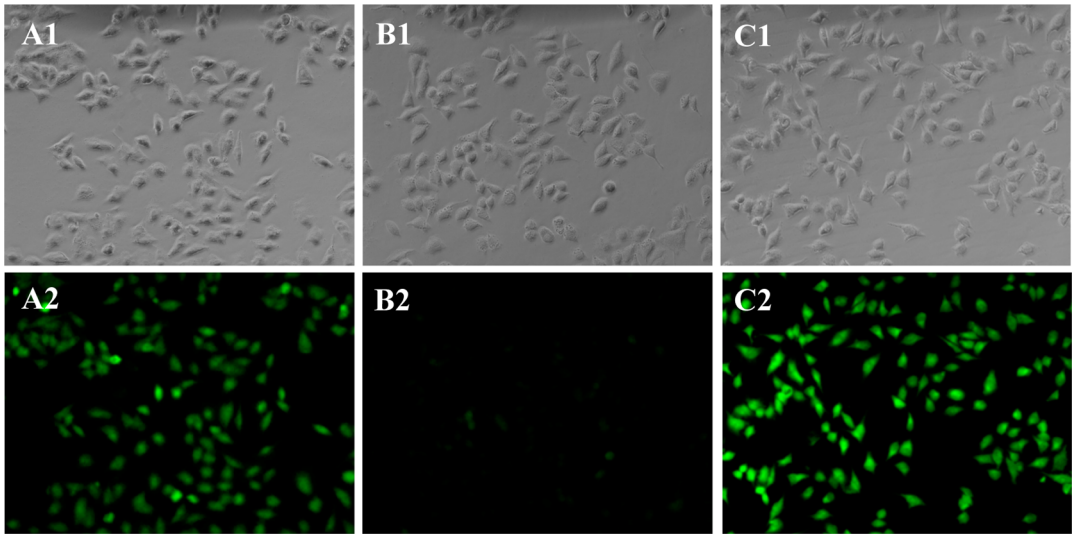


Figure 5. Top row shows bright-field images of (A1) HeLa cells treated with probe **1** (10 μM) for 15 min, (B1) HeLa cells preincubated with 500 μM NEM for 30 min and then treated with probe **1** (10 μM) for 15 min; and (C1) HeLa cells preincubated with 500 μM NEM for 30 min and then treated with Cys (100 μM), followed by treatment with probe **1** (10 μM) for 15 min. Bottom row shows fluorescence images of (A2) panel A1, (B2) panel B1, and (C2) panel C1.

6. The Practical Applications. Based on the above results, we investigated the practical utilities of probe **1** for fluorescent imaging of intracellular Cys in living cells. As shown in Figure 5, when HeLa cells were incubated with probe **1** (10 μM) for 15 min, the cells started to show bright green fluorescence (A2). However, when *N*-ethylmaleimide (NEM), which is a known thiol trapping reagent, was added to the cell culture prior to the addition of **1**, the cells showed almost no fluorescence (B2). As another control experiment, after HeLa cells were pretreated with NEM, with the addition of Cys (100 μM), and then incubated with **1** for 15 min, bright green fluorescence was also observed (C2). In contrast, after HeLa cells were pretreated with NEM, addition of Hcy (500 μM) or GSH (1 mM) or NAC (500 μM) and then incubated with **1** for 15 min, respectively, only very weak fluorescence was observed (see Figure S11 in the Supporting Information). These results demonstrated that probe **1** possesses good membrane permeability and is able to detect intracellular Cys in living cells. The cytotoxicity of probe **1** in HeLa cells is also investigated. The results from MTT assays showed that the cells remained in good condition when treated with 10−50 μM of probe **1** for as long as 12 h (see Figure S12 in the Supporting Information), indicating that probe **1** is of low cytotoxicity to cultured cells. Therefore, all of the results indicate that probe **1** holds great potential for biological applications.

CONCLUSION

In summary, we developed a simple, readily available, and highly selective ratiometric fluorescent probe for cysteine (Cys). This new probe uses a visible-light-excitable excited-state intramolecular proton transfer (ESIPT) dye with a remarkably large Stokes shift (>130 nm) as the fluorophore and an acrylate moiety as the reaction site. Most importantly, it shows a rapid, selective, and sensitive ratiometric fluorescent detection process for Cys in aqueous solution under mild conditions over various analytes including homocysteine (Hcy) and glutathione (GSH). In addition, this probe has low cytotoxicity and good membrane permeability, and can be used to detect Cys in living cells. Since available highly selective ratiometric fluorescent probes for Cys are very limited, we believe that this new probe will be of great benefit for many researchers engaging in the study of the effects of Cys on biological systems.

dx.doi.org/10.1021/am505501d | *ACS Appl. Mater. Interfaces* 2014, 6, 17543−17550

■ ASSOCIATED CONTENT

Supporting Information

Structure characterizations for probe **1**, additional UV and fluorescence data, and data for investigation of the sensing mechanism. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

* E-mail: gf256@mail.ccnu.edu.cn.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank the National Natural Science Foundation of China (Grant Nos. 21172086, 21032001, and 21272084) for financial support. This work was also sponsored by the Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry.

■ REFERENCES

(1) Zhou, Y.; Yoon, J. Recent Progress in Fluorescent and Colorimetric Chemosensors for Detection of Amino Acids. *Chem. Soc. Rev.* **2012**, *41*, 52−67.

(2) Reddie, K. G.; Carroll, K. S. Expanding the Functional Diversity of Proteins through Cysteine Oxidation. *Curr. Opin. Chem. Biol.* **2008**, *12*, 746−754.

(3) Paulsen, C. E.; Carroll, K. S. Cysteine-Mediated Redox Signaling: Chemistry, Biology, and Tools for Discovery. *Chem. Rev.* **2013**, *113*, 4633−4679.

(4) Shahrokhian, S. Lead Phthalocyanine as a Selective Carrier for Preparation of a Cysteine-Selective Electrode. *Anal. Chem.* **2001**, *73*, 5972−5978.

(5) Li, Z. A.; Lou, X.; Li, Z.; Qin, J. A New Approach to Fluorescence "Turn-On" Sensing of α-Amino Acids. *ACS Appl. Mater. Interfaces* **2009**, *1*, 232−234.

(6) Zeng, Q.; Jim, C. K. W.; Lam, J. W. Y.; Dong, Y.; Li, Z.; Qin, J.; Tang, B. Z. A New Disubstituted Polyacetylene for the Detection of α-Amino Acids. *Macromol. Rapid Commun.* **2009**, *30*, 170−175.

(7) Chen, X.; Zhou, Y.; Peng, X.; Yoon, J. Fluorescent and Colorimetric Probes for Detection of Thiols. *Chem. Soc. Rev.* **2010**, *39*, 2120−2135.

(8) Jung, H. S.; Chen, X.; Kim, J. S.; Yoon, J. Recent Progress in Luminescent and Colorimetric Chemosensors for Detection of Thiols. *Chem. Soc. Rev.* **2013**, *42*, 6019−6031.

(9) Yin, C.; Huo, F.; Zhang, J.; Martínez-Máñez, R.; Yang, Y.; Lv, H.; Li, S. Thiol-addition Reactions and Their Applications in Thiol Recognition. *Chem. Soc. Rev.* **2013**, *42*, 6032−6059.

(10) Zhang, M.; Yu, M.; Li, F.; Zhu, M.; Li, M.; Gao, Y.; Li, L.; Liu, Z.; Zhang, J.; Zhang, D.; Yi, T.; Huang, C. A Highly Selective Fluorescence Turn-on Sensor for Cysteine/Homocysteine and Its Application in Bioimaging. *J. Am. Chem. Soc.* **2007**, *129*, 10322−10323.

(11) Wang, P.; Liu, J.; Lv, X.; Liu, Y.; Zhao, Y.; Guo, W. A Naphthalimide-Based Glyoxal Hydrazone for Selective Fluorescence Turn-On Sensing of Cys and Hcy. *Org. Lett.* **2012**, *14*, 520−523.

(12) Sun, Y. Q.; Chen, M.; Liu, J.; Lv, X.; Li, J. F.; Guo, W. Nitroolefin-based Coumarin as a Colorimetric and Fluorescent Dual Probe for Biothiols. *Chem. Commun.* **2011**, *47*, 11029−11031.

(13) Wei, M.; Yin, P.; Shen, Y.; Zhang, L.; Deng, J.; Xue, S.; Li, H.; Guo, B.; Zhang, Y.; Yao, S. A New Turn-on Fluorescent Probe for Selective Detection of Glutathione and Cysteine in Living Cells. *Chem. Commun.* **2013**, *49*, 4640−4642.

(14) Leung, K.-H.; He, H.-Z.; Ma, V. P.-Y.; Chan, D. S.-H.; Leung, C.-H.; Ma, D.-L. A Luminescent G-quadruplex Switch-on Probe for the Highly Selective and Tunable Detection of Cysteine and Glutathione. *Chem. Commun.* **2013**, *49*, 771−773.

(15) Kong, F.; Liu, R.; Chu, R.; Wang, X.; Xu, K.; Tang, B. A Highly Sensitive Near-infrared Fluorescent Probe for Cysteine and Homocysteine in Living Cells. *Chem. Commun.* **2013**, *49*, 9176−9178.

(16) Kwon, H.; Lee, K.; Kim, H.-J. Coumarin-malonitrile Conjugate as a Fluorescence Turn-on Probe for Biothiols and Its Cellular Expression. *Chem. Commun.* **2011**, *47*, 1773−1775.

(17) Zhang, H.; Wang, P.; Yang, Y.; Sun, H. A Selective Fluorescent Probe for Thiols Based on α, β-unsaturated Acyl Sulfonamide. *Chem. Commun.* **2012**, *48*, 10672−10674.

(18) Yang, X.-F.; Huang, Q.; Zhong, Y.; Li, Z.; Li, H.; Lowry, M.; Escobedo, J. O.; Strongin, R. M. A Dual Emission Fluorescent Probe Enables Simultaneous Detection of Glutathione and Cysteine/Homocysteine. *Chem. Sci.* **2014**, *5*, 2177−2183.

(19) Park, S.; Imlay, J. A. High Levels of Intracellular Cysteine Promote Oxidative DNA Damage by Driving the Fenton Reaction. *J. Bacteriol.* **2003**, *185*, 1942−1950.

(20) Hwang, C.; Sinskey, A. J.; Lodish, H. F. Oxidized Redox State of Glutathione in the Endoplasmic Reticulum. *Science* **1992**, *257*, 1496−1502.

(21) Li, H.; Fan, J.; Wang, J.; Tian, M.; Du, J.; Sun, S.; Sun, P.; Peng, X. A Fluorescent Chemodosimeter Specific for Cysteine: Effective Discrimination of Cysteine from Homocysteine. *Chem. Commun.* **2009**, 5904−5906.

(22) Yuan, L.; Lin, W.; Yang, Y. A Ratiometric Fluorescent Probe for Specific Detection of Cysteine over Homocysteine and Glutathione Based on the Drastic Distinction in the Kinetic Profiles. *Chem. Commun.* **2011**, *47*, 6275−6277.

(23) Jung, H. S.; Han, J. H.; Pradhan, T.; Kim, S.; Lee, S. W.; Sessler, J. L.; Kim, T. W.; Kang, C.; Kim, J. S. A Cysteine-selective Fluorescent Probe for the Cellular Detection of Cysteine. *Biomaterials* **2012**, *33*, 945−953.

(24) Jung, H. S.; Pradhan, T.; Han, J. H.; Heo, K. J.; Lee, J. H.; Kang, C.; Kim, J. S. Molecular Modulated Cysteine-selective Fluorescent Probe. *Biomaterials* **2012**, *33*, 8495−8502.

(25) Niu, L.-Y.; Guan, Y.-S.; Chen, Y.-Z.; Wu, L.-Z.; Tung, C.-H.; Yang, Q.-Z. A Turn-on Fluorescent Sensor for the Discrimination of Cysteine from Homocysteine and Glutathione. *Chem. Commun.* **2013**, *49*, 1294−1296.

(26) Yang, Z.; Zhao, N.; Sun, Y.; Miao, F.; Liu, Y.; Liu, X.; Zhang, Y.; Ai, W.; Song, G.; Shen, X.; Yu, X.; Sun, J.; Wong, W.-Y. Highly Selective Red- and Green-emitting Two-photon Fluorescent Probes for Cysteine Detection and Their Bio-imaging in Living Cells. *Chem. Commun.* **2012**, *48*, 3442−3444.

(27) Zhou, X.; Jin, X.; Sun, G.; Li, D.; Wu, X. A Cysteine Probe with High Selectivity and Sensitivity Promoted by Response-assisted Electrostatic Attraction. *Chem. Commun.* **2012**, *48*, 8793−8795.

(28) Zhou, X.; Jin, X.; Sun, G.; Wu, X. A Sensitive and Selective Fluorescent Probe for Cysteine Based on a New Response-Assisted Electrostatic Attraction Strategy: The Role of Spatial Charge Configuration. *Chem.—Eur. J.* **2013**, *19*, 7817−7824.

(29) Yang, X.; Guo, Y.; Strongin, R. M. Conjugate Addition/Cyclization Sequence Enables Selective and Simultaneous Fluorescence Detection of Cysteine and Homocysteine. *Angew. Chem., Int. Ed.* **2011**, *50*, 10690−10693.

(30) Yang, X.; Guo, Y.; Strongin, R. M. A Seminaphthofluorescein-based Fluorescent Chemodosimeter for the Highly Selective Detection of Cysteine. *Org. Biomol. Chem.* **2012**, *10*, 2739−2741.

(31) Wang, H.; Zhou, G.; Gai, H.; Chen, X. A Fluorescein-based Probe with High Selectivity to Cysteine over Homocysteine and Glutathione. *Chem. Commun.* **2012**, *48*, 8341−8343.

(32) Guo, Z.; Nam, S.; Park, S.; Yoon, J. A Highly Selective Ratiometric Near-infrared Fluorescent Cyanine Sensor for Cysteine with Remarkable Shift and Its Application in Bioimaging. *Chem. Sci.* **2012**, *3*, 2760−2765.

(33) Liu, B.; Wang, J.; Zhang, G.; Bai, R.; Pang, Y. Flavone-Based ESIPT Ratiometric Chemodosimeter for Detection of Cysteine in Living Cells. *ACS Appl. Mater. Interfaces* **2014**, *6*, 4402−4407.

(34) Guan, Y.-S.; Niu, L.-Y.; Chen, Y.-Z.; Wu, L.-Z.; Tung, C.-H.; Yang, Q.-Z. A Near-infrared Fluorescent Sensor for Selective

Detection of Cysteine and Its Application in Live Cell Imaging. *RSC Adv.* **2014**, *4*, 8360−8364.
(35) Reddy, G. U.; Agarwalla, H.; Taye, N.; Ghorai, S.; Chattopadhyay, S.; Das, A. A Novel Fluorescence Probe for Estimation of Cysteine/Histidine in Human Blood Plasma and Recognition of Endogenous Cysteine in Live Hct116 Cells. *Chem. Commun.* **2014**, *50*, 9899−9902.
(36) Zhang, Y.; Wang, J.-H.; Zheng, W.; Chen, T.; Tong, Q.-X.; Li, D. An ESIPT Fluorescent Dye Based on HBI with High Quantum Yield and Large Stokes Shift for Selective Detection of Cys. *J. Mater. Chem. B* **2014**, *2*, 4159−4166.
(37) Lin, W.; Long, L.; Yuan, L.; Cao, Z.; Chen, B.; Tan, W. A Ratiometric Fluorescent Probe for Cysteine and Homocysteine Displaying a Large Emission Shift. *Org. Lett.* **2008**, *10*, 5577−5580.
(38) Zhu, B.; Zhang, X.; Li, Y.; Wang, P.; Zhang, H.; Zhuang, X. A Colorimetric and Ratiometric Fluorescent Probe for Thiols and Its Bioimaging Applications. *Chem. Commun.* **2010**, *46*, 5710−5712.
(39) Kim, G.-J.; Lee, K.; Kwon, H.; Kim, H.-J. Ratiometric Fluorescence Imaging of Cellular Glutathione. *Org. Lett.* **2011**, *13*, 2799−2801.
(40) Long, L.; Lin, W.; Chen, B.; Gao, W.; Yuan, L. Construction of a FRET-based Ratiometric Fluorescent Thiol Probe. *Chem. Commun.* **2011**, *47*, 893−895.
(41) Lim, C. S.; Masanta, G.; Kim, H. J.; Han, J. H.; Kim, H. M.; Cho, B. R. Ratiometric Detection of Mitochondrial Thiols with a Two-Photon Fluorescent Probe. *J. Am. Chem. Soc.* **2011**, *133*, 11132−11135.
(42) Das, P.; Mandal, A. K.; Chandar, N. B.; Baidya, M.; Bhatt, H. B.; Ganguly, B.; Ghosh, S. K.; Das, A. New Chemodosimetric Reagents as Ratiometric Probes for Cysteine and Homocysteine and Possible Detection in Living Cells and in Blood Plasma. *Chem.—Eur. J.* **2012**, *18*, 15382−15393.
(43) Das, P.; Mandal, A. K.; Reddy, G. U.; Baidya, M.; Ghosh, S. K.; Das, A. Designing a Thiol Specific Fluorescent Probe for Possible Use as a Reagent for Intracellular Detection and Estimation in Blood Serum: Kinetic Analysis to Probe the Role of Intramolecular Hydrogen Bonding. *Org. Biomol. Chem.* **2013**, *11*, 6604−6614.
(44) Liu, X.-D.; Sun, R.; Xu, Y.; Xu, Y.-J.; Ge, J.-F.; Lu, J. M. A Benzoxazine−Hemicyanine based Probe for the Colorimetric and Ratiometric Detection of Biothiols. *Sens. Actuators B* **2013**, *178*, 525−531.
(45) Goswami, S.; Manna, A.; Paul, S.; Das, A. K.; Nandi, P. K.; Maity, A. K.; Saha, P. A Turn on ESIPT Probe for Rapid and Ratiometric Fluorogenic Detection of Homocysteine and Cysteine in Water with Live Cell-imaging. *Tetrahedron Lett.* **2014**, *55*, 490−494.
(46) Hammers, M. D.; Pluth, M. D. Ratiometric Measurement of Hydrogen Sulfide and Cysteine/Homocysteine Ratios Using a Dual-Fluorophore Fragmentation Strategy. *Anal. Chem.* **2014**, *86*, 7135−7140.
(47) Zhu, B.; Guo, B.; Zhao, Y.; Zhang, B.; Du, B. A Highly Sensitive Ratiometric Fluorescent Probe with a Large Emission Shift for Imaging Endogenous Cysteine in Living Cells. *Biosens. Bioelectron.* **2014**, *55*, 72−75.
(48) Han, Q.; Shi, Z.; Tang, X.; Yang, L.; Mou, Z.; Li, J.; Shi, J.; Chen, C.; Liu, W.; Yang, H.; Liu, W. A Colorimetric and Ratiometric Fluorescent Probe for Distinguishing Cysteine from Biothiols in Water and Living Cells. *Org. Biomol. Chem.* **2014**, *12*, 5023−5030.
(49) Liu, Y.; Feng, G. A Visible Light Excitable Colorimetric and Fluorescent ESIPT Probe for Rapid and Selective Detection of Hydrogen Sulfide. *Org. Biomol. Chem.* **2014**, *12*, 438−445.
(50) Gunduz, S.; Goren, A. C.; Ozturk, T. Facile Syntheses of 3-Hydroxyflavones. *Org. Lett.* **2012**, *14*, 1576−1579.
(51) Lan, M.; Wu, J.; Liu, W.; Zhang, H.; Zhang, W.; Zhuang, X.; Wang, P. Highly Sensitive Fluorescent Probe for Thiols Based on Combination of PET and ESIPT Mechanisms. *Sens. Actuators B* **2011**, *156*, 332−337.
(52) Hu, R.; Feng, J.; Hu, D.; Wang, S.; Li, S.; Li, Y.; Yang, G. A Rapid Aqueous Fluoride Ion Sensor with Dual Output Modes. *Angew. Chem., Int. Ed.* **2010**, *49*, 4915−4918.
(53) Chen, W.-H.; Xing, Y.; Pang, Y. A Highly Selective Pyrophosphate Sensor Based on ESIPT Turn-On in Water. *Org. Lett.* **2011**, *13*, 1362−1365.
(54) Liu, B.; Wang, H.; Wang, T.; Bao, Y.; Du, F.; Tian, J.; Li, Q.; Bai, R. A New Ratiometric ESIPT Sensor for Detection of Palladium Species in Aqueous Solution. *Chem. Commun.* **2012**, *48*, 2867−2869.
(55) Li, G.; Zhu, D.; Liu, Q.; Xue, L.; Jiang, H. Rapid Detection of Hydrogen Peroxide Based on Aggregation Induced Ratiometric Fluorescence Change. *Org. Lett.* **2013**, *15*, 924−927.
(56) Chen, S.; Hou, P.; Zhou, B. j.; Song, X.; Wu, J.; Zhang, H.; Foley, J. W. A Red Fluorescent Probe for Thiols Based on 3-Hydroxyflavone and Its Application in Living Cell Imaging. *RSC Adv.* **2013**, *3*, 11543−11546.
(57) Xu, Z.; Xu, L.; Zhou, J.; Xu, Y.; Zhu, W.; Qian, X. A Highly Selective Fluorescent Probe for Fast Detection of Hydrogen Sulfide in Aqueous Solution and Living Cells. *Chem. Commun.* **2012**, *48*, 10871−10873.
(58) Shi, B.; Greaney, M. F. Reversible Michael Addition of Thiols as a New Tool for Dynamic Combinatorial Chemistry. *Chem. Commun.* **2005**, 886−888.
(59) Xiong, X.; Song, F.; Chen, G.; Sun, W.; Wang, J.; Gao, P.; Zhang, Y.; Qiao, B.; Li, W.; Sun, S.; Fan, J.; Peng, X. Construction of Long-Wavelength Fluorescein Analogues and Their Application as Fluorescent Probes. *Chem.—Eur. J.* **2013**, *19*, 6538−6545.
(60) Shi, J.; Wang, Y.; Tang, X.; Liu, W.; Jiang, H.; Dou, W.; Liu, W. A Colorimetric and Fluorescent Probe for Thiols Based on 1,8-naphthalimide and Its Application for Bioimaging. *Dyes Pigm.* **2014**, *100*, 255−260.