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A Native-Chemical-Ligation-Mechanism-Based Ratiometric Fluorescent Probe for Aminothiols

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Abstract: Thiol-containing amino acids (aminothiols) such as cysteine (Cys) and homocysteine (Hcy) play a key role in various biological processes including maintaining the homeostasis of biological thiols. However, abnormal levels of aminothiols are associated with a variety of diseases. The native chemical ligation (NCL) reaction has attracted great attention in the fields of chemistry and biology. NCL of peptide segments involves cascade reactions between a peptide-α-thioester and an N-terminal cysteine peptide. In this work, we employed the NCL reaction mechanism to formulate a Förster resonance energy transfer (FRET) strategy for the design of ratiometric fluorescent probes that were selective toward aminothiols. On the basis of this new strategy, the ratiometric fluorescent probe **1** for aminothiols was judiciously designed. The new probe is highly selective toward aminothiols over other thiols and exhibits a very large variation (up to 160-fold) in its fluorescence ratio (I_{458}/I_{603}). The new fluorescent probe is capable of ratiometric detection of aminothiols in newborn calf and human serum samples and is also suitable for ratiometric fluorescent imaging of aminothiols in living cells.

Keywords: aminothiols · biological activity · fluorescent probes · FRET · native chemical ligation

Introduction

Thiol-containing amino acids (aminothiols) such as cysteine (Cys) and homocysteine (Hcy) play a key role in various biological processes including maintaining the homeostasis of biological thiols, biocatalysis, metal binding, post-translational modifications, and detoxification of xenobiotics.[1] However, abnormal levels of aminothiols are associated with a variety of diseases such as liver damage, skin lesions, slowed growth, edema, dementia, Alzheimer's disease, coronary heart disease, carotid therosclerosis, dystonia, psoriasis, clinical stroke, lung damage, Parkinson's disease, and asthma.[2] In light of the high sensitivity and easy operation of fluorescece detection, a diverse array of fluorescent probes selective toward aminothiols have been reported.[3,4] However, the vast majority of them respond to aminothiols with fluorescence signals that change only in intensity. The main limitation of intensity-based probes is that variations in the sample environment and probe distribution might be problematic for their utilization in quantitative measurements. By contrast, ratiometric fluorescent probes exhibit spectral shifts upon interaction with the analytes of interest, which provides a built-in correction for the environmental effects.[5]

The native chemical ligation (NCL) reaction has attracted great attention in the fields of chemistry and biology.[6] Hundreds of proteins have been prepared by total or semisynthesis when using the NCL reaction. NCL of peptide segments involves cascade reactions between a peptide-α-thioester and an N-terminal cysteine peptide. Prompted by the chemoselective and in vivo compatible characters of the NCL reaction, we have previously employed the reaction to design a ratiometric fluorescent probe for thiols (Figure 1a)[7] on the basis of the Förster resonance energy transfer (FRET) signaling mechanism.[8] However, the probe was constructed by linking an energy donor to a rhodamine acceptor dye through a thioester linker (the interaction site). Thus, any compounds that contain a free −SH group can induce the cleavage of the FRET dyad and release the energy donor. In other words, the probe is not capable of distinguishing the aminothiols from other biological thiols such as glutathione (GSH) and α-lipolic acid in a ratiometric manner. To address this problem, herein we propose a novel NCL-based FRET strategy to design ratiometric fluorescent probes selective toward aminothiols over other biological thiols (Figure 1b). In the new design scheme, unlike the previous strategy, the FRET donor is not connected to the thioester interaction site but instead attached to a linker, which is then bridged to the FRET acceptor. On the basis of the reaction mechanism of the NCL reaction, we reasoned that treatment of compound **A** with aminothiols (e.g., Cys) can afford the intermediate thioester **B1** by the reversible *trans*-thioesterification reaction, which might further undergo irreversible intramolecular nucleophilic attack by the nearby

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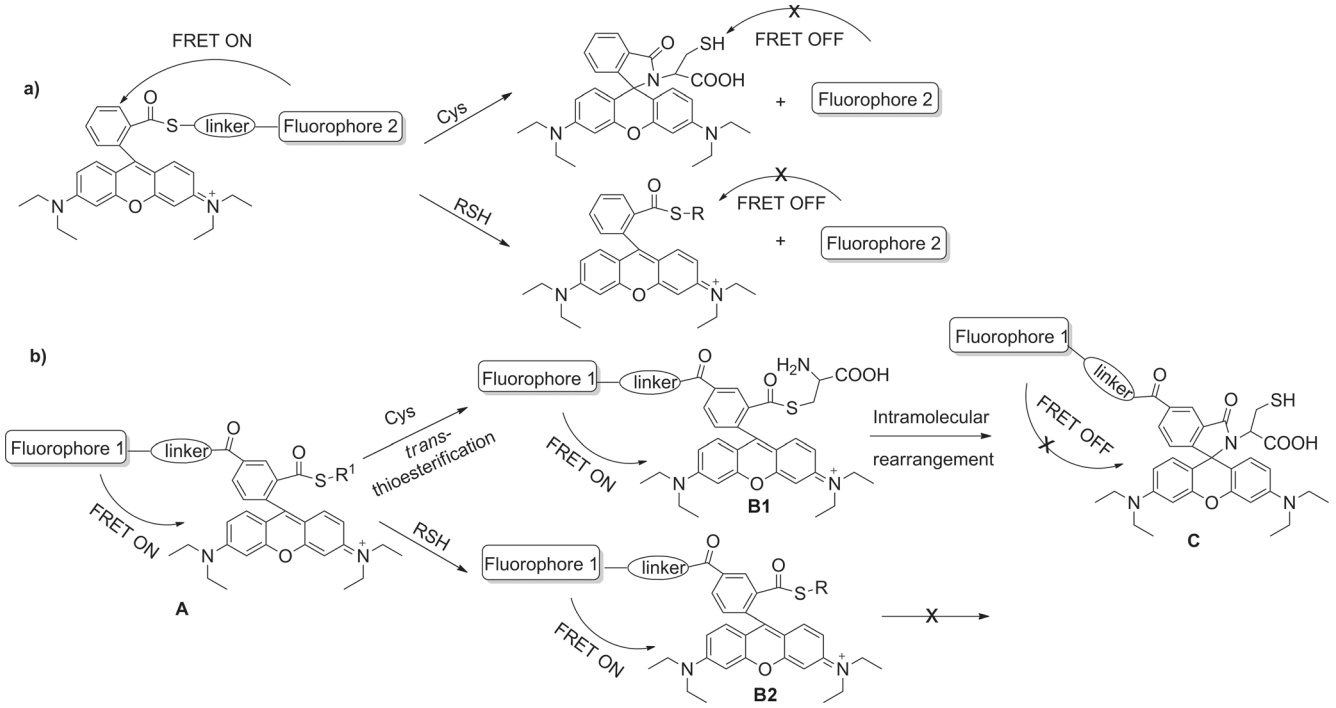
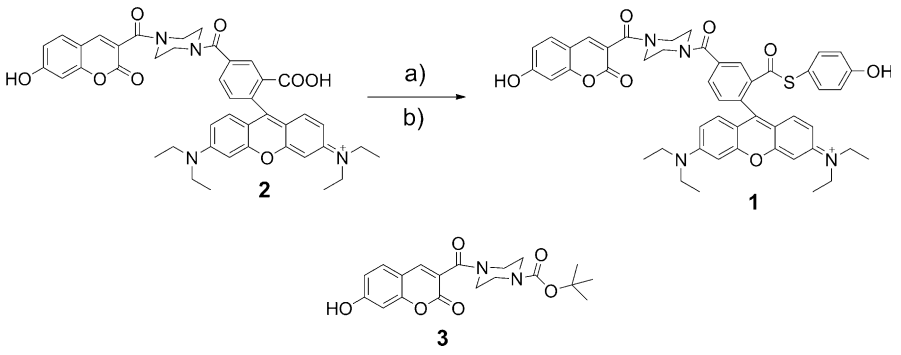


Figure 1. a) Known FRET approach to the development of ratiometric fluorescent probes for thiols based on the NCL reaction.[7] b) A proposed novel NCL-based FRET strategy to design ratiometric fluorescent probes selective toward aminothiols over other biological thiols.

amino group to form amide **C**. As the FRET is on and off in compounds **A** and **C**, respectively, we should observe a significant change in the ratiometric fluorescent signal. On the other hand, treatment of **A** with thiols might induce the formation of the thioester **B2**, which cannot further undergo intramolecular arrangement, because **B2** lacks an amino group in proximity. As the FRET is on in both **A** and **B2**, no marked changes in the ratiometric fluorescent signal can be noted. Thus, taken together, the new strategy (Figure 1b) might be employed to construct ratiometric fluorescent probes selective toward aminothiols over other biological thiols.

With this hypothesis in mind, in this work, we present compound **1** (Scheme 1) as a new NCL-mechanism-based ratiometric fluorescent probe with specificity toward aminothiols.



Scheme 1. Synthesis of the ratiometric fluorescent probe **1** and the structure of the reference energy donor **3**. Reagents and conditions: a) *N*,*N*′-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide, CH_2Cl_2; b) 4-mercaptophenol, triethylamine (TEA).

Results and Discussion

The synthesis of probe **1** started with compound **2**, which was obtained by our previously reported method.[9] Reaction of **2** with *N*-hydroxysuccinimide gave the succinimidyl ester intermediate, which was further treated with 4-mercaptophenol to provide the target compound **1** (Scheme 1). Notably, compound **1** is stable as a solid, and it can be stored for more than one year in a freezer.

The absorption spectrum of the FRET dyad **1** in phosphate buffer solution (PBS; pH 7.4, which contained 10% DMF as a cosolvent) is shown in Figure S1 in the Supporting Information. As designed, the absorption spectrum of the FRET dyad **1** exhibits the typical absorption of rhodamines at around 571 nm and the characteristic absorption of coumarins at around 395 nm. As shown in Figure S2 in the Supporting Information, when FRET dyad **1** was excited at 395 nm (the coumarin donor absorption) in PBS (pH 7.4, which contained 10% DMF as a cosolvent), the featured emission of the coumarin donor around 458 nm was notably quenched when compared with that of the reference coumarin donor **3** (Scheme 1). This suggests that the excitation energy of the coumarin donor is efficiently transferred to the rhodamine

acceptor. The energy-transfer efficiencies of dyad **1** were calculated to be 95.3%.[10] The presence of highly efficient energy transfer in the FRET dyad is further collaborated by the excellent correspondence between the absorption and excitation spectra of dyad **1** (Figure S1 in the Supporting Information). Notably, the donor and acceptor emission bands are essentially completely separated with a remarkable separation (up to 145 nm, Figure S1 in the Supporting Information) between the emission maxima. Thus, the FRET platform appears to be promising for the construction of ratiometric fluorescent probes with highly favorable spectral properties.

The changes in the absorption and emission spectra of probe **1** in the presence of Cys are exhibited in Figure 2.

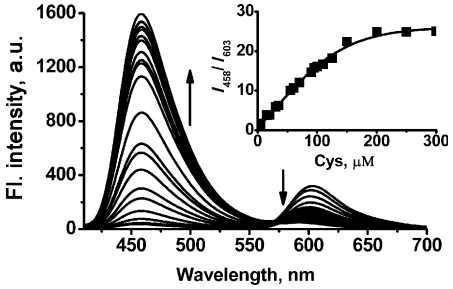


Figure 2. Fluorescence spectral changes (with excitation at 395 nm) of **1** (3 μM) upon addition of increasing concentrations (0–250 μM) of Cys in potassium phosphate buffer (25 mM; pH 7.4, containing 10% DMF as cosolvent) for 30 min. The inset shows the changes to the fluorescent intensity ratio at 458 and 603 nm (I_{458}/I_{603}) on increasing cthe oncentration of Cys.

The free probe **1** displays an emission band at 603 nm (rhodamine moiety). However, treatment of Cys induces a marked decrease in the emission intensity at 603 nm and concurrently a dramatic enhancement of the emission intensity at 458 nm (coumarin moiety). Thus, a significant emission blueshift (145 nm) was observed. Notably, the probe exhibits two well-resolved emission peaks. This is favorable for measurement of the emission intensity and signal ratios with high precision. The emission ratio (I_{458}/I_{603}) shows a drastic variation from 0.154 in the absence of Cys to 24.9 in the presence of Cys (250 μM), a 160-fold enhancement (Figure 2, inset). Furthermore, the emission ratios (I_{458}/I_{603}) are linearly proportional to the concentrations of Cys (1–100 μM) (Figure S3 in the Supporting Information) with a detection limit (signal/noise = 3) of 6.4×10^{-7} M, which suggests that the probe is potentially useful for quantitative determination of aminothiol concentrations over a large dynamic range. In addition, when excited at 550 nm, the fluorescent intensity of the probe at 603 nm was gradually decreased with the addition of elevated concentrations of Cys (Figure S4 in the Supporting Information). In accordance with the emission changes, the addition of an increasing amount of Cys to probe **1** triggered a gradual decrease in the absorption at 576 nm (Figure S5a in the Supporting Information), thus indicating that the rhodamine acceptor is in the ring-closed form in the presence of Cys, whereas the absorption band at around 395 nm (Figure S5a in the Supporting Information), which was attributed to the coumarin absorption, was almost unchanged. This was further confirmed by a mass spectrometry analysis of the probe treated with Cys (Figure S6 in the Supporting Information). A similar fluorescence response was also observed for other aminothiols such as Hcy and cysteamine (Figure S7 and S8 in the Supporting Information), although a higher amount of Hcy or cysteamine was required for the maximum intensity ratio to be reached.

The emission ratio (I_{458}/I_{603}) of the free probe **1** exhibited only slight variations over a wide span of pH from 4.0 to 10.0 (Figure S9 in the Supporting Information). The ratiometric response of the probe to Cys is pH-dependent. When the pH increased from 4.0 to 7.4, the emission ratio (I_{458}/I_{603}) significantly enhanced from 0.79 to 25, and then a plateau was reached at higher pH values. In addition, probe **1** could also work in 25 mM potassium phosphate buffer (pH 7.4, which contained 5% DMF as a cosolvent) and physiological conditions (e.g., pure newborn calf serum without any organic cosolvent) (Figure S10 and S11 in the Supporting Information).

The time course of the emission ratios of probe **1** (3 μM) at 458 and 603 nm (I_{458}/I_{603}) in the presence of Cys or Hcy (0.5 mM) is shown in Figure 3. Upon introduction of Cys, a

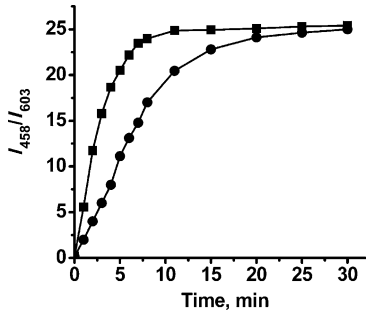


Figure 3. The time course of the emission ratios of **1** (3 μM) at 458 and 603 nm (I_{458}/I_{603}) in the presence of Cys (■) or Hcy (●; 0.5 mM) in potassium phosphate buffer (25 mM; pH 7.4, containing 10% DMF as a cosolvent).

dramatic enhancement in the emission intensity was noted, and the intensity essentially reached a maximum in 12 min. A similar fluorescence increase was also observed for Hcy, although it took up to 20 min for the maximum intensity to be reached. Under pseudo-first-order kinetic conditions[4h,11] (3 μM probe **1** and 0.5 mM Cys or Hcy), the observed rate constant (k_{obs}) was determined to be 0.419 min^{-1} for Cys or 0.335 min^{-1} for Hcy.

To examine the selectivity, probe **1** was treated with various biologically relevant species including representative aminothiols (e.g., Cys, Hcy, cysteamine), thiol-containing species (e.g., GSH, 1,4-dithiothreitol (DTT), mercaptoethanol, α-lipolic acid, 4-methylbenzenethiol, 4-chlorobenzenethiol), amino acids (e.g., Phe, Ala, Gly, Arg, Lys, Tyr, Leu, Ser, Val), and glucose. As shown in Figure 4, introduction of

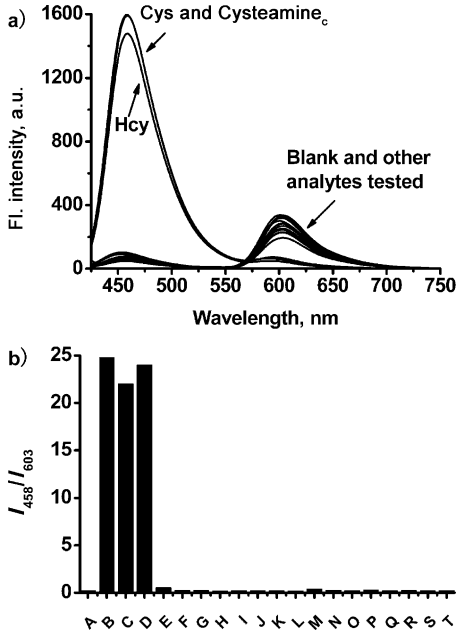


Figure 4. a) Fluorescence spectra (with excitation at 395 nm) of **1** (3 μM) in the presence of various analytes (250 μM) in potassium phosphate buffer (25 mM; pH 7.4, containing 10% DMF as cosolvent) for 30 min. b) The fluorescent ratio of **1** (3 μM) at 458 and 603 nm (I_{458}/I_{603}) in the presence of various biologically relevant analytes (250 μM): A) free; B) Cys; C) Hcy; D) cysteamine; E) 4-methylbenzenethiol; F) 4-chlorobenzenethiol G) GSH; H) mercaptoethanol; I) DTT; J) α-lipolic acid; K) Ala; L) Gly; M) Tyr; N) Arg; O) Lys; P) Leu; Q) Phe; R) glucose; S); Ser; and T) Val.

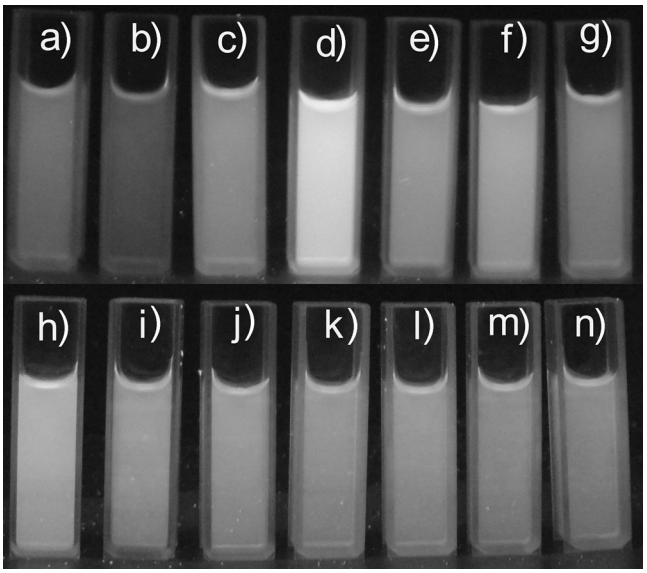


Figure 5. The visual fluorescence emission of probe **1** (3 μM) toward various species (16 equiv) for 30 min on excitation at 365 nm by using a UV lamp at room temperature: a) 4-chlorobenzenethiol; b) 4-methylbenzenethiol; c) GSH; d) Cys; e) DTT; f) mercaptoethanol; g) α-lipolic acid; h) blank; i) Phe; j) Arg; k) Leu; l) Ala; m) glucose; and n) Ser. For a color version, see Figure S17 in the Supporting Information.

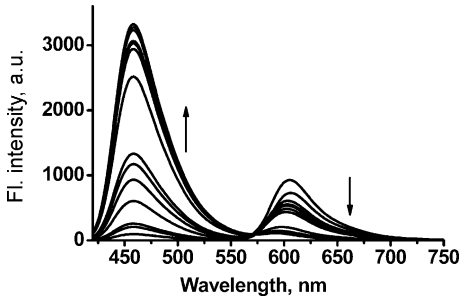


Figure 6. Fluorescence spectral changes (excitation at 395 nm) of **1** (3 μM) upon addition of increasing concentrations (0–1500 μM) of Cys in potassium phosphate buffer (25 mM; pH 7.4, containing 10% DMF as cosolvent) containing GSH (1 mM) for 30 min.

aminothiols (Cys, Hcy, cysteamine) to the probe induced a dramatic ratiometric response. By contrast, no noticeable changes in the emission were observed upon addition of the other thiol-containing species, thus indicating that the probe is specific for the detection of aminothiols over other thiols. In good accordance with the fluorescence changes, the introduction of aminothiols (Cys, Hcy, cysteamine) to the probe induced a gradual decrease of the absorption at 576 nm (Figure S5b in the Supporting Information). By contrast, no noticeable changes in the absorption at 576 nm were observed upon addition of the other thiol-containing species (Figure S5b in the Supporting Information), thus indicating that the probe is specific toward the detection of aminothiols over other thiols. Furthermore, the emission color of probe **1** is analyte-dependent (Figure 5), which indicates that the probe could be employed for convenient visual sensing of Cys. Notably, probe **1** can respond to Cys even in the presence of a high concentration of GSH (Figure 6 and Figure S12 in the Supporting Information), thereby further supporting the evidence for high selectivity toward aminothiols over other thiols. It is known that some transition-metal ions might influence the fluorescence of rhodamine.[8a] Thus, we also investigated the potential fluorescence response of probe **1** to the representative transition-metal ions such as Cu^{2+}, Hg^{2+}, Fe^{3+}, Cr^{3+}, Pb^{2+}, Zn^{2+}, and Ag^{+} (Figure S13 in the Supporting Information), and the results indicated that these transition-metal ions only showed a minimal effect on the fluorescence of the probe.

The high selectivity of probe **1** based on the NCL reaction mechanism was further corroborated by mass spectrometry studies. Treatment of probe **1** with mercaptoethanol (as a representative thiol) only gave the ring-opened thioester product (Figure S14b in the Supporting Information). By contrast, incubation of probe **1** with Cys provided the cyclized amide product (Figure S6 in the Supporting Information). These results are in good agreement with the design strategy shown in Figure 1.

The above studies demonstrate that the novel ratiometric fluorescent probe has key features that include high sensitivity, a large ratio signal variation, and high selectivity even in the presence of 1 mM GSH. Thus, the probe seems to be useful for the detection of aminothiol in real biological samples. To test this possibility, probe **1** was employed to detect

aminothiols in newborn calf and human serum samples. Aliquots of a reduced newborn calf serum sample were added to a solution of probe **1**, and a linear enhancement in the emission ratios (I_{458}/I_{603}) was noted (Figure 7a), thus indicating that the probe is capable of detecting aminothiols in the plasma sample. Furthermore, probe **1** displays a larger emission ratio (I_{458}/I_{603}) in the presence of a human serum sample from an individual after breakfast than one before breakfast (Figure 7b), thereby suggesting that the level of aminothiols in human serum sample after breakfast is higher than before breakfast, which is consistent with previous reports.[12]

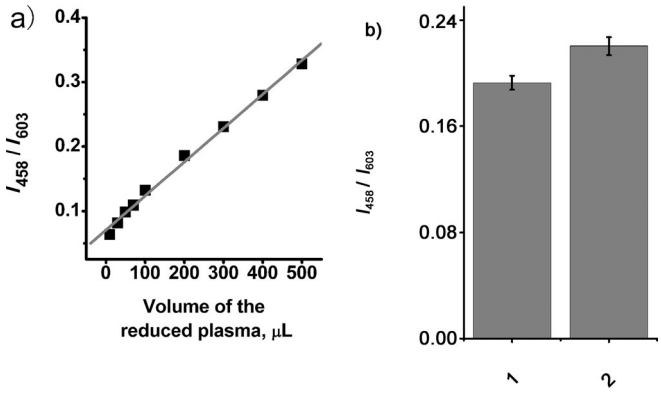


Figure 7. a) Linear relationship between the emission ratios and the volume of the reduced newborn calf serum that was added. The data were obtained by the addition of different amounts of the reduced newborn calf serum sample (0–500 μL) to probe **1** (3 μM) for 30 min. b) Plot showing the variation in the amount of total aminothiol levels in human serum sample (300 μL) from an individual 1) before and 2) after breakfast.

To demonstrate the capability of the novel probe for ratiometric fluorescence imaging of aminothiols in living cells, the living HepG2 cells were incubated with probe **1**, and strong green fluorescence in the coumarin emission window (Figure 8a) and red fluorescence in the rhodamine emission window (Figure 8b) could be observed. A series of control experiments were also conducted. The cells were pretreated with *N*-ethylmaleimide (NEM, as an aminothiol/thiol-reactive reagent[13]) for 60 min, and further incubated with the probe for 30 min, thereby providing significant red fluorescence (Figure 8d) but almost no green fluorescence (Figure 8c), in accordance with the emission profile of the free probe. The cells pretreated with *N*-acetylcysteine (NAC, a membrane-permeable precursor for cysteine),[14] and then incubated with probe **1** showed a marked decrease in the red emission (Figure 8f) and a significant increase in the green emission (Figure 8e), which is consistent with the aminothiol-induced ratiometric fluorescent response. The cells pretreated with α-lipolic acid (a cell-permeable biological thiol without an amino group), and then treated with probe **1** showed very slight changes both in the green (Figure 8g) and red emission windows (Figure 8h), which is in good agreement with the above observation that other thiols are not capable of inducing a ratiometric response (Figure 4). Thus, these data establish that probe **1** is cell-membrane-permeable and therefore suitable for ratiometric imaging of aminothiols in living cells.

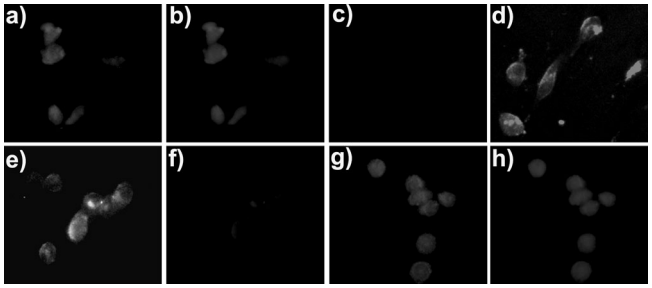


Figure 8. Fluorescence images of the living HepG2 cells treated with probe **1**. a,b) Cells incubated with only **1** (3 μM) for 30 min. c,d) Cells pretreated with NEM (1 mM) for 60 min, and then incubated with **1** (3 μM) for 30 min. e,f) Cells pretreated with NAC (1 mM) for 60 min, and then incubated with **1** (3 μM) for 30 min. g,h) Cells pretreated with α-lipolic acid (1 mM) for 60 min, and then incubated with **1** (3 μM) for 30 min. For a color version, see Figure S18 in the Supporting Information.

Conclusion

In summary, we have employed the NCL reaction mechanism to formulate a FRET strategy for the design of a ratiometric fluorescent probe that is selective toward aminothiols. Notably, unlike the previous NCL-based ratiometric fluorescent probe,[7] the new probe **1** is highly selective toward aminothiols over other thiols. The novel probe exhibits a very large variation (up to 160-fold) in the fluorescence ratio (I_{458}/I_{603}). Importantly, we have demonstrated that probe **1** can detect aminothiols in real biological samples including newborn calf and human serum samples in a ratiometric fashion. Furthermore, we have confirmed that probe **1** is suitable for the ratiometric fluorescent imaging of aminothiols in living cells.

Experimental Section

Materials and instruments: Unless otherwise stated, all reagents were purchased from commercial suppliers and used without further purification. Solvents used were purified by standard methods prior to use. Twice-distilled water was used throughout all experiments. Human serum sample was obtained from the first hospital of Changsha City. Melting points of compounds were measured using a Beijing Taike XT-4 microscopy melting-point apparatus, and all melting points were uncorrected. Mass spectra were performed using an LCQ Advantage ion-trap mass spectrometer from Thermo Finnigan or an Agilent 1100 HPLC/MSD spectrometer. NMR spectra were recorded using a Bruker AV-500 spectrometer, with TMS as an internal standard. Electronic absorption spectra were obtained using a LabTech UV Power spectrometer. Photoluminescent spectra were recorded using a Hitachi F4600 fluorescence spectrophotometer. Cell imaging was performed using an Olympus fluorescence 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.

Synthesis of compound 1: Compound **2** (45.0 mg, 0.06 mmol), *N*-hydroxysuccinimide (21.0 mg, 0.18 mmol), and DCC (37.1 mg, 0.18 mmol) were dissolved in CH_2Cl_2 (5.0 mL) with stirring at room temperature. After 30 min, triethylamine (0.05 mL) and 4-mercaptophenol (11.5 mg, 0.09 mmol) were added. The reaction solution was stirred for another 30 min at room temperature, and the mixture was then concentrated under vacuum. The resulting residue was subjected to preparative thin-layer chromatography (CH_2Cl_2/CH_3OH = 5:1) to provide compound **1** as a carmine powder (31.5 mg, 61.0% yield). ^{1}H NMR (CD_3OD, 500 MHz): δ = 1.31 (12H; CH_3CH_2), 3.58–3.93 (16H; CH_3CH_2 and $N(CH_2CH_2)_2N$), 6.71 (d, J = 8.0 Hz, 2H; xanthene-*H*), 6.74 (s, 1H; coumarin-*H*), 6.85 (d, J = 8.0 Hz, 1H; Ar−*H*), 6.95 (4H; xanthene-*H* and Ar−*H*), 7.05 (s, 2H; xanthene-*H*), 7.23 (2H; Ar−*H*), 7.55 (d, J = 8.5 Hz, 1H; Ar−*H*), 7.63 (d, J = 6.0 Hz, 1H; coumarin-*H*), 8.00 (d, J = 5.5 Hz, 1H; coumarin-*H*), 8.07 (s, 1H; coumarin-*H*), 8.41 ppm (s, 1H; Ar−*H*); ^{13}C NMR (CD_3OD, 125 MHz): δ = 12.7 (CH_3CH_2), 46.8 (CH_3CH_2), 97.3 (Ar−*C*), 103.3 (Ar−*C*), 112.3 (Ar−*C*), 115. 1 (Ar−*C*), 115.5 (Ar−*C*), 116.2 (Ar−*C*), 117.3 (Ar−*C*), 120.1 (Ar−*C*), 128.9 (Ar−*C*), 131.6 (Ar−*C*), 132.2 (Ar−*C*), 132.3 (Ar−*C*), 132.6 (Ar−*C*), 133.6 (Ar−*C*), 137.2 (Ar−*C*), 138.9 (Ar−*C*), 139.1 (Ar−*C*), 145.8 (Ar−*C*), 157.0 (Ar−*C*), 157.5 (Ar−*C*),157.6 (Ar−*C*), 159.0 (Ar−*C*), 160.5 (Ar−*C*), 164.3 (*C*=O), 166.6 (*C*=O), 170.1 (*C*=O), 192.1 ppm (S−*C*=O); ESI-MS: *m*/*z*: 851.2. $[M]^+$; HRMS (ESI): *m*/*z* calcd for: $C_{49}H_{47}N_4O_8S_1$ $[M]^+$: 851.3148; found: 851.3109; elemental analysis calcd (%) for $C_{49}H_{47}N_4O_8S^+ \cdot H_2PO_4^- \cdot 4H_2O$: C 57.64, H 5.63, N 5.49; found: C 57.39, H 5.99, N 5.21. The presence of water molecules is consistent with the hygroscopic nature of the compound.

Cell culture and living cell imaging studies with probe 1: HepG2 cells were obtained from the third hospital of Xiangya and cultured in Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin at 37°C in a 5% CO_2/95% air environment. The cells were seeded on 12-well plates and stabilized for 24 h. Before imaging, the cells were washed with PBS three times and then incubated with probe **1** (3 μM) in PBS for 30 min under an atmosphere of 5% CO_2/95% air at 37°C. For the control experiment, the cells were pretreated with 1 mM NEM (or NAC or α-lipolic acid) for 60 min, and then incubated with probe **1** (3 μM) in PBS for 30 min in an atmosphere of 5% CO_2/95% air at 37°C. After washing with PBS three times to remove the remaining probe, the fluorescence images were acquired using an Olympus fluorescence microscope equipped with a cooled CCD camera.

Acknowledgements

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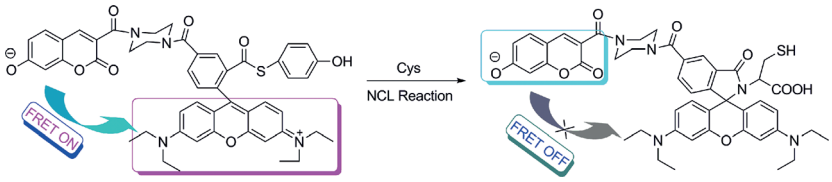
Fluorescent Probes



Going native: A ratiometric fluorescent probe for aminothiols has been designed based on the native chemical ligation (NCL) reaction mechanism (see scheme). The probe is highly selective toward aminothiols over other thiols and exhibits a very large variation (up to 160-fold) in the fluorescence ratio (I_{458}/I_{603}).