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A Ratiometric Fluorescent Probe for Thiols Based on a Tetrakis (4-hydroxyphenyl)porphyrin−Coumarin Scaffold

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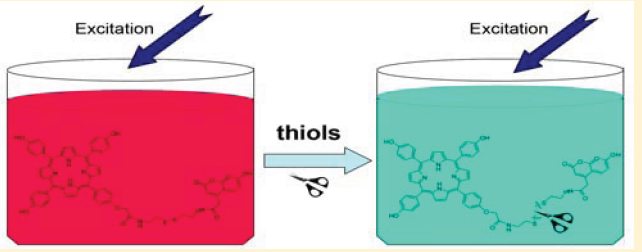
Supporting Information

ABSTRACT: In this work, we have designed and synthesized the compound **Ratio-HPSSC**, based on a tetrakis(4-hydroxyphenyl)porphyrin−coumarin scaffold, as a new ratiometric fluorescent probe for thiols. The ratiometric probe **Ratio-HPSSC** is highly selective and sensitive to thiols. Importantly, the novel ratiometric probe exhibited a remarkable change in emission color from red to blue. This key feature allows **Ratio-HPSSC** to be employed for thiol detection by simple visual inspection. Furthermore, we have demonstrated that **Ratio-HPSSC** is suitable for ratiometric fluorescence imaging of thiols in living cells. We believe that the new ratiometric probe will find interesting applications in chemistry, biology, and medicine.



INTRODUCTION

Small-molecular-weight thiols such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) play an important role in biological redox homeostasis, biocatalysis, metal binding, and post-translational modifications.[1−3] However, abnormal levels of Cys, Hcy, and GSH are associated with many types of diseases, including liver damage, skin lesions, slowed growth, edema,[4] dementia, Alzheimer's disease, coronary heart disease, carotid atherosclerosis, dystonia, psoriasis, clinical stroke,[5−12] lung damage, Parkinson's disease, and asthma.[13−16] Therefore, it is of great interest to monitor the thiol levels in biosystems.

High-performance liquid chromatography,[17−23] capillary electrophoresis,[24−27] electrochemical assay,[28−33] UV−vis spectroscopy,[34−37] Fourier transform infrared spectroscopy,[38] mass spectrometry,[39−43] and fluorescence spectroscopy[44−60] have been employed in thiol detection. Among the methods used, fluorescence sensing is the most appealing because of its high sensitivity, high selectivity, and ease of use. Furthermore, fluorescence sensing is potentially applicable for bioimaging in living cells with temporal and spatial resolution. Thus, the development of fluorescent thiol probes has received great attention.[44−60] The majority of the existing fluorescent thiol probes are fluorescence intensity based. In contrast, only a few fluorescent thiol probes are based on ratiometric fluorescence measurements at two wavelengths,[61−67] which should provide a built-in correction for environmental effects.[68]

Porphyrins have been widely employed in light-harvesting systems to mimic the photosynthesis of plants.[69,70] In particular, tetrakis(4-hydroxyphenyl)porphyrins have tunable photophysical properties by modifications on the nucleophilic hydroxyl groups.[71,72] Coumarins are often used on the periphery in porphyrin-based dendrimers for efficient energy transfer,[71] as the coumarin emission spectra match well with the porphyrin absorption spectra. However, the tetrakis(4-hydroxyphenyl)porphyrin−coumarin system is rarely used in fluorescent probe design.

In this work, we describe the compound **Ratio-HPSSC** (Scheme 1), based on the tetrakis(4-hydroxyphenyl)porphyrin−coumarin scaffold, as a novel ratiometric fluorescent thiol probe. The key features of the new ratiometric probe **Ratio-HPSSC** include high sensitivity, high selectivity, a large change (60-fold enhancement) in the emission ratio, and suitability for ratiometric fluorescence imaging of thiols in living cells.

RESULTS AND DISCUSSION

Synthesis of Ratio-HPSSC. **Ratio-HPSSC** was prepared in multiple steps according to the route shown in Scheme 1. Reaction of amine **1** with carboxylic acid **2** under the standard coupling conditions afforded amide **3**, which was then subjected to deprotection under acidic conditions to give the key intermediate **4**. Tetrakis(4-hydroxyphenyl)porphyrin **5** was converted into ether **6** by an alkylation reaction. Subsequently, hydrolysis of compound **6** yielded carboxylic acid **7**. Finally, coupling carboxylic acid **7** with amine **4** in the presence of benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP) and triethylamine provided **Ratio-HPSSC** in 68% yield.

Absorption and Fluorescence Spectra of Ratio-HPSSC. As exhibited in Figure 1A, the free ratiometric probe **Ratio-HPSSC** in pH 7.4 PBS buffer/ethanol (1/1, v/v) displayed the

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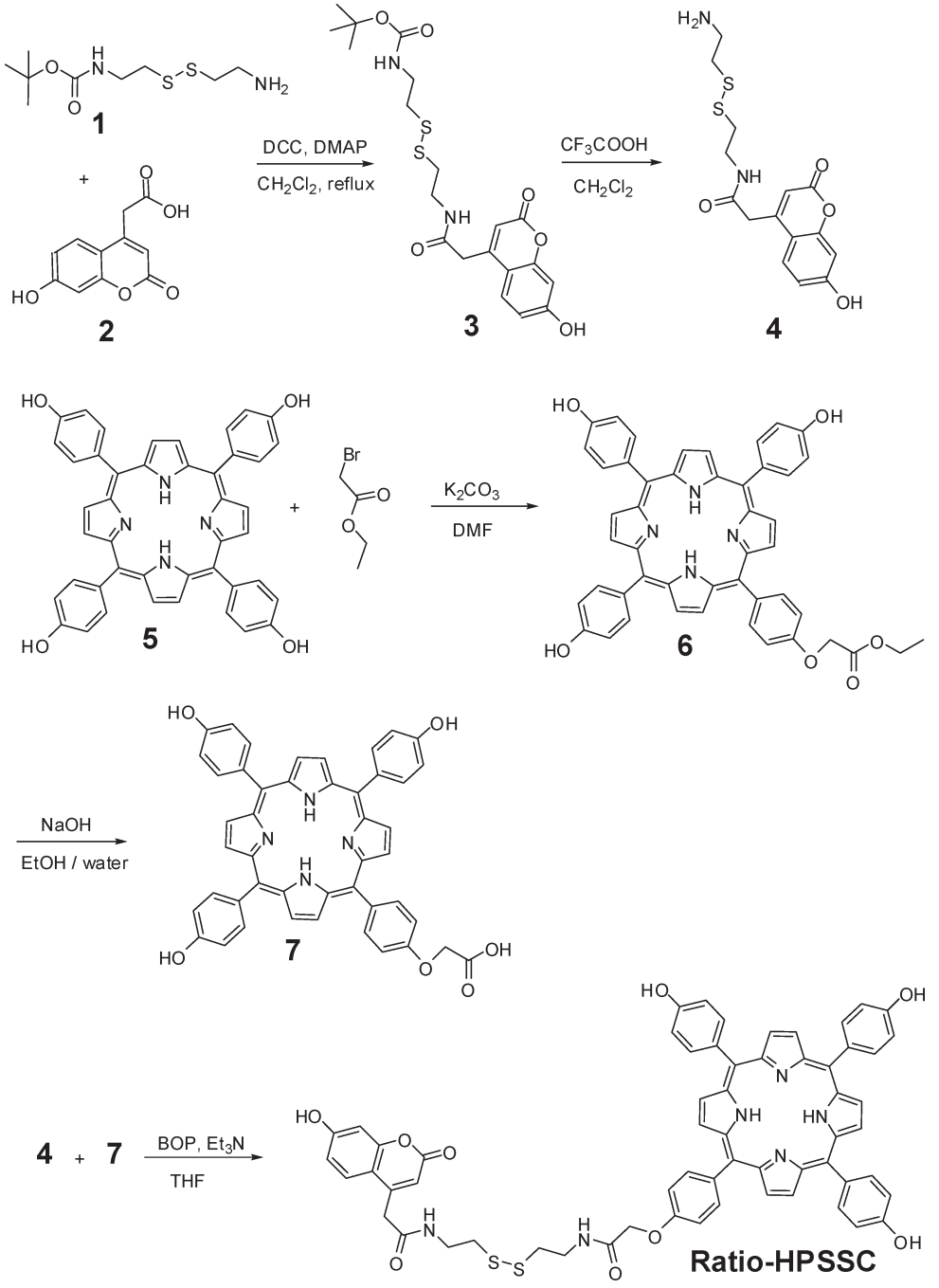
Scheme 1. Synthetic Route to Ratio-HPSSC



characteristic emission of porphyrins at around 658 nm but almost no coumarin emission at around 459 nm, upon excitation at 350 nm. This observation indicates that the fluorescence of the coumarin dye is quenched by either the Förster resonance energy transfer (FRET) mechanism[73] or stacking of the two dyes in aqueous solution.[74] However, the latter case can be ruled out on the basis of several lines of evidence: (1) the absorption spectrum of the ratiometric probe **Ratio-HPSSC** (Figure 1B) exhibited the characteristic absorption bands of the coumarin donor at around 342 and 378 nm and the typical absorption peaks of the porphyrin acceptor at 421 (Soret band), 520 (Q1 band), 558 (Q2 band), 597 (Q3 band), and 650 nm (Q4 band), indicating that there are little or no electronic interactions between the coumarin donor and the porphyrin acceptor in the ground state. Thus, the formation of a ground-state complex is not likely. (2) The fluorescence intensity of the porphyrin moiety of **Ratio-HPSSC** is almost the same as that of the model porphyrin acceptor **6** at the same concentration (Figure S1, Supporting Information), suggesting that there is no obvious fluorescence quenching in the porphyrin moiety of the ratiometric probe.[74] (3) Upon careful comparison of the ^{1}H NMR spectrum of coumarin **3** (the model coumarin donor, Scheme 1) with that of **Ratio-HPSSC**, we found that the chemical shifts of the protons in the coumarin ring (the structures are shown in

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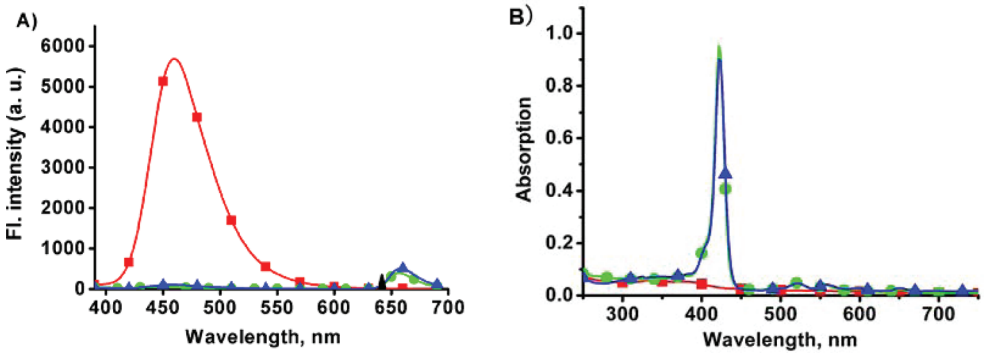

Figure 1. (A) Emission spectra of compound **3** (red boxes), compound **6** (green circles), and **Ratio-HPSSC** (blue triangles) in pH 7.4 PBS buffer/ethanol (1/1, v/v). (B) UV/vis absorption spectra of compound **3** (red boxes), compound **6** (green circles), and **Ratio-HPSSC** (blue triangles) in pH 7.4 PBS buffer/ethanol (1/1, v/v). The concentration of the compounds was 2 μM. Excitation was at 350 nm.

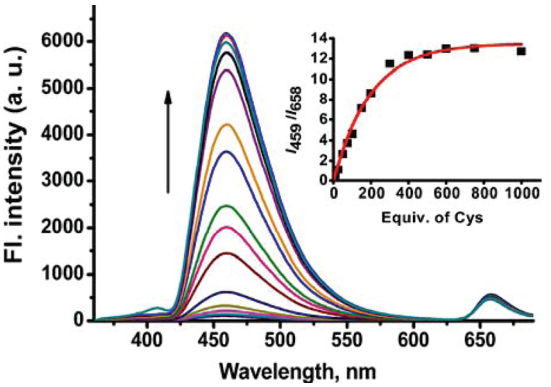

Figure 2. Fluorescence spectra (excitation at 350 nm) of **Ratio-HPSSC** (2 μM) in pH 7.4 PBS buffer/ethanol (1/1, v/v) in the presence of Cys (0−1000 equiv). The inset shows the fluorescence ratio changes of **Ratio-HPSSC** (2 μM) in the presence of increasing concentrations of Cys (0−1000 equiv).

Scheme 1) are essentially identical in both coumarin **3** and **Ratio-HPSSC** (Figure S3), suggesting that there are no apparent interactions between the coumarin component and the porphyrin unit in **Ratio-HPSSC** in the ground state. Thus, it is concluded that the fluorescence of the coumarin dye is quenched due to the FRET from the coumarin donor to the porphyrin acceptor, in good agreement with the overlap between the coumarin emission with the Soret and Q1 bands of the porphyrin absorption (Figure S2A).

Ratiometric Fluorescence Response to Cys. The fluorescence titration spectra of Cys to the ratiometric probe **Ratio-HPSSC** (2 μM) in pH 7.4 PBS buffer/ethanol (1/1, v/v) are displayed in Figure 2. Addition of Cys elicited a significant fluorescence enhancement at 459 nm but almost no changes of fluorescence intensity at 658 nm. Thus, the emission ratio I_{459}/I_{658} can be used to calibrate the variations of the dye concentration due to photobleaching or other environmental effects.[75] As displayed in the inset of Figure 2, the emission ratio I_{459}/I_{658} showed a drastic change from 0.21 in the absence of Cys to 12.1 in the presence of Cys (500 equiv), a 60-fold enhancement in the emission ratios. The emission ratios have an excellent linear relationship with Cys from 1 to 600 μM (Figure S4), suggesting that the ratiometric probe is potentially useful for quantitative determination of thiol concentrations in a large dynamic range. The detection limit (S/N = 3) of the probe was determined to be 0.73 μM. The combination of the low detection limit and the large fluorescence dynamic range implies that the probe is highly sensitive to Cys.

As indicated above, in the free probe, the excitation energy of the coumarin donor is transferred to the porphyrin acceptor. Thus, the FRET is on in the free probe. However, upon addition of Cys, the disulfide bond is cleaved, and the distance of the coumarin and porphyrin dyes becomes infinite. The FRET should be turned off in the presence of thiols, and we should note the enhancement of coumarin emission and the decrease of the porphyrin emission. Surprisingly, the porphyrin emission remains almost constant during the Cys titration process. This peculiar behavior may be related to the unique emission profiles of tetrakis(4-hydroxyphenyl)porphyrin.[69] As shown in Figure S2B (Supporting Information), porphyrin **6** (the model energy acceptor) has a major emission peak at 658 nm ($S^1 \rightarrow S^0$) and a minor emission band at 450 nm ($S^2 \rightarrow S^0$) with excitation at 350 nm, consistent with the previous report of the emission profiles of 4-hydroxyphenylporphyrins.[69] However, the emission band of coumarin **3** (the model energy donor) is also located at around 450 nm with excitation at 350 nm. The strong overlap between the coumarin donor emission peak and the minor emission band of the porphyrin acceptor may elicit the singlet−singlet annihilation process,[76−78] and the porphyrin emission may keep constant even in the presence of thiols. However, the exact reason remains to be investigated.

The thiol/disulfide exchange reactions are very common and play a critical role in maintaining the cellular redox balance via the glutathione/glutathione disulfide redox couple.[79−81] The thiol/disulfide exchange reactions have been employed to construct fluorescent thiol probes.[63,82−85] Thus, it is reasonable to consider that the thiol/disulfide exchange reactions also occur upon treatment of **Ratio-HPSSC** with Cys. Given the fact that the cleavage of S−S bonds in the presence of nucleophilic thiols is well-known,[79−88] a similar cleavage process is proposed in Scheme S1 (Supporting Information). As **Ratio-HPSSC** is unsymmetrical, nucleophilic Cys could attack the probe to first afford disulfides **a** and **b** and thiols **c** and **d** by the classic thiol/disulfide exchange reactions. However, further thiol/disulfide exchange reactions may proceed. For instance, the thiol/disulfide exchange between **a** and **d** may give **e**, and a similar exchange between **b** and **c** could provide **f**. Obviously, oxidation of **d** or **c** in air could also afford **e** or **f**, respectively. Thus, the likely products

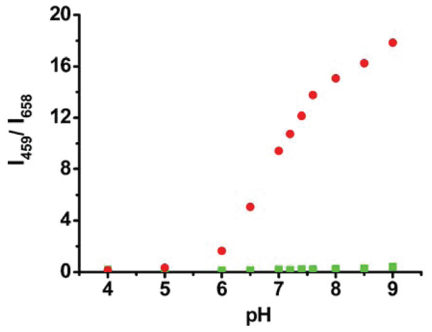

Figure 3. Fluorescence intensity ratio (I_{459}/I_{658}) changes of **Ratio-HPSSC** (2 μM) with different pH values in the absence (green circles) or presence (red circles) of Cys (500 equiv) in pH 7.4 PBS buffer/ethanol (1/1, v/v) at 25 °C.

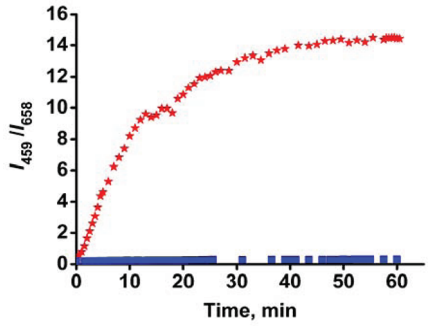

Figure 4. Time-dependent fluorescence intensity ratio I_{459}/I_{658} changes of **Ratio-HPSSC** (2 μM) in the absence (blue squares) or presence (red stars) of Cys (1.0 mM) in pH 7.4 PBS buffer/ethanol (1/1, v/v) at 25 °C.

derived from the cascade reactions of thiol/disulfide exchange are rather diverse, which makes it very challenging to thoroughly characterize the identity of the potential thiol/disulfide exchange products. However, to support the hypothesis of the cleavage of S−S bonds in the presence of thiols, we conducted ^{1}H NMR and mass spectroscopic experiments. **Ratio-HPSSC** was incubated with $Na_2S\cdot 9H_2O$ (the model thiol species), and the changes of chemical shifts in the ^{1}H NMR spectra were examined. We used $Na_2S\cdot 9H_2O$ as the surrogate of Cys to simplify the ^{1}H NMR spectra, so that useful information could be readily noted. As shown in Figure S5, addition of $Na_2S\cdot 9H_2O$ induced a marked shift for the resonance signals in the ^{1}H NMR spectrum. Notably, four novel peaks were observed, in good agreement with the cleavage of S−S bonds in the presence of thiols. In addition, **Ratio-HPSSC** was treated with Cys, and the subsequent ESI-MS analysis (Figure S6) indicates the presence of several new peaks in the spectrum: *m*/*z* 465.6, 585.4, 796.5, 1073.3, 1589.2 corresponding to [**a** + C_2H_5OH − H_2O + K^+], [**e** + C_2H_5OH − H_2O + H^+], [**c** + H^+], [**Ratio-HPSSC** + H^+], and [**f** + H^+], respectively. Although it is expected that not all of the possible products were observed due to the complicated nature of the cascade reactions of thiol/disulfide exchange, the results of the ESI-MS analysis are consistent with the cleavage of S−S bonds in the presence of thiols.

Effect of pH. The potential pH effects on the emission ratio of **Ratio-HPSSC** in the absence or presence of Cys were investigated. As displayed in Figure 3, almost no change in emission

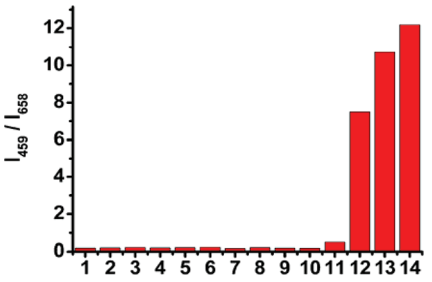

Figure 5. Emission ratios I_{459}/I_{658} of **Ratio-HPSSC** (2 μM) in the presence of various relevant analytes (500 equiv) in pH 7.4 PBS buffer/ethanol (1:1, v/v): (1) no analyte; (2) Ala; (3) Arg; (4) glucose; (5) Gly; (6) Leu; (7) Pro; (8) Phe; (9) Tyr; (10) Val; (11) Lys; (12) GSH; (13) Hcy; (14) Cys.

Figure 6. Visual fluorescence emissions of the ratiometric probe **Ratio-HPSSC** (2 μM) in the presence of 500 equiv of various species in pH 7.4 PBS buffer/ethanol (1/1, v/v) on excitation at 365 nm using a hand-held UV lamp: (1) no analyte; (2) Cys; (3) Ala; (4) Gly; (5) Tyr; (6) Arg; (7) glucose.

ratio was observed in the free probe over a wide pH range of 4.0−9.0, indicating that **Ratio-HPSSC** is very stable in this pH range. However, the fluorescence response of the probe toward Cys is pH-dependent. The pK_a of Cys is around 8.5.[89,90] As anticipated, a higher pH is favorable for a larger fluorescence signal. Importantly, the observation that **Ratio-HPSSC** has a large ratio signal (I_{459}/I_{658} = 12.1) at physiological pH (7.4) suggests that the ratiometric probe is promising for biological applications.

Kinetic Profile. The time course of the fluorescence spectra of **Ratio-HPSSC** (2 μM) in the absence or presence of 500 equiv of Cys in pH 7.4 PBS buffer/ethanol (1/1, v/v) is displayed in Figure 4. The free probe displayed no noticeable changes in the emission ratio I_{459}/I_{658}. However, upon introduction of Cys, a significant enhancement in the ratio was noted within a few minutes, and a plateau was reached in 40 min. The apparent rate constant for the reaction of the probe with the large excess of Cys is calculated as k' = 0.00105 s^{-1} (Figure S7, Supporting Information).

Selectivity. To examine the selectivity of **Ratio-HPSSC** (2 μM), representative species such as Ala, Arg, Gly, Leu, Pro, Phe, Tyr, Val, Lys, GSH, Hcy, Cys, and glucose were examined in pH 7.4 PBS buffer/ethanol (1/1, v/v). The small-molecular-weight thiols (Cys, Hcy, GSH) induced a significant ratiometric response (Figure 5). In contrast, the other biologically relevant analytes tested elicited no visible changes in the ratio. These data imply that the ratiometric probe **Ratio-HPSSC** has a high selectivity for thiol-containing molecules. Furthermore, the ratiometric probe can be used to detect thiols by simple visual inspection (Figure 6). The free probe showed a bright red emission color, and the

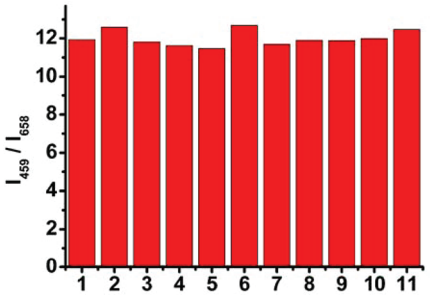

Figure 7. Fluorescence intensity ratios of **Ratio-HPSSC** (2 μM) to various relevant analytes (500 equiv) in pH 7.4 PBS buffer/ethanol (1/1, v/v): (1) Ala + Cys; (2) Arg + Cys; (3) Pro + Cys; (4). glucose + Cys; (5) Gly + Cys; (6) Lys+ Cys; (7) Leu + Cys; (8) Phe + Cys; (9) Tyr + Cys; (10) Val + Cys; (11) Cys.

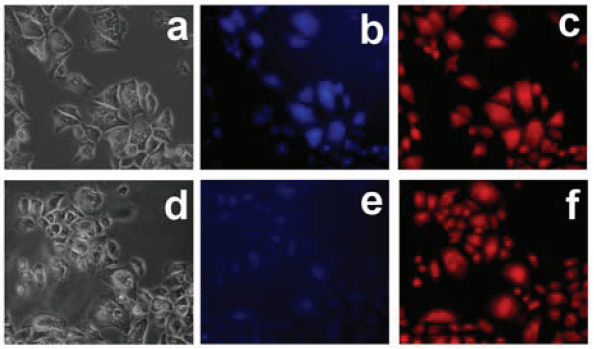

Figure 8. Differential interference contrast (DIC) and fluorescence images of living Hela cells: (a) DIC image of the cells incubated with **Ratio-HPSSC** (10 μM) for 30 min; (b) fluorescence image of (a) from the blue channel; (c) fluorescence image of (a) from the red channel; (d) DIC image of the cells preincubated with *N*-ethylmaleimide (1 mM) for 30 min and then loaded with **Ratio-HPSSC** (10 μM) for 30 min; (e) fluorescence image of (d) from the blue channel; (f) fluorescence image of (d) from the red channel.

addition of representative amino acids such as Ala, Gly, Tyr, Arg, and glucose did not change the color. In contrast, introduction of Cys elicited a remarkable change in emission color from red to blue.

In order to further investigate the potential interference of other species for the fluorescence detection of thiols, **Ratio-HPSSC** was treated with Cys in the presence of various species in pH 7.4 PBS buffer/ethanol (1/1, v/v). As shown in Figure 7, all the species tested have virtually no influence on the Cys fluorescence detection. Thus, **Ratio-HPSSC** appears to be useful for selectively sensing Cys even in the presence of other amino acids.

Ratiometric Imaging of Intracellular Thiols in Living Cells. For preliminary fluorescence imaging applications, living Hela cells were incubated with the ratiometric probe **Ratio-HPSSC** (10 μM) for 30 min at 37 °C. The loaded cells displayed both the bright blue fluorescence corresponding to the coumarin dye (Figure 8b) and the intense red fluorescence corresponding to the porphyrin dye (Figure 8c). In contrast, in a control experiment, in which the cells were pretreated with *N*-ethylmaleimide (as a thiol blocking agent) and then loaded with the ratiometric probe, we observed a marked fluorescence quenching in the blue channel (Figure 8e) and almost unchanged fluorescence in the red channel (Figure 8f). Thus, these data indicate that the ratiometric probe **Ratio-HPSSC** is cell membrane permeable and is suitable for ratiometric imaging of thiols in living cells.

In conclusion, we have designed and synthesized **Ratio-HPSSC**, which is based on a tetrakis(4-hydroxyphenyl)-porphyrin−coumarin scaffold, as a new ratiometric fluorescent probe for thiols. The ratiometric probe **Ratio-HPSSC** displayed a high selectivity to thiols, and it is highly sensitive to thiols with a low detection limit and a large fluorescence dynamic range. Furthermore, the ratiometric probe can be employed to detect thiols by simple visual inspection. Finally, we have demonstrated that **Ratio-HPSSC** is suitable for ratiometric fluorescence imaging of thiols in living cells.

EXPERIMENTAL SECTION

Synthesis of *tert*-Butyl-2-((2-(2-(7-hydroxycoumarin-4-yl)acetamido)ethyl)disulfanyl)ethylcarbamate (3). 7-Hydroxycoumarin-4-acetic acid **2**[91,92] (103.1 mg, 0.5 mmol), DCC (113.5 mg, 0.55 mmol), and DMAP (5.14 mg, 0.05 mmol) were added to a 30 mL solution of mono-*t*-boc-cystamine **1**[93] (126 mg, 0.5 mmol) in CH_2Cl_2. After reflux under an N_2 atmosphere for 8 h, the solvent was evaporated under reduced pressure. The resulting residue was purified on a silica gel column using CH_2Cl_2/EtOH (100/1) to afford compound **3** as a white solid (184.1 mg, 81%). Mp: 82−84 °C. ^{1}H NMR (400 MHz, CD_3OD): δ 7.43 (d, J = 8.8 Hz, 1H), 6.62 (dd, J = 8.4, 2.4 Hz, 1H), 6.04 (s, 1H), 3.56 (d, J = 8.0 Hz, 2H), 3.17−3.14 (q, 3H), 2.66 (t, J = 6.4 Hz, 3H), 2.60 (t, J = 6.8 Hz, 2H), 1.26 (s, 9H). ^{13}C NMR (100 MHz, CD_3OD): δ 171.8, 164.2, 163.8, 159.1, 157.5, 153.15, 153.13, 128.3, 115.2, 113.9, 104.4, 81.0, 41.4, 40.9, 40.4, 39.6, 39.2, 29.5. MS (ESI negative mode): m/z 453.9 $[M - H]^-$. HRMS (ESI): m/z calcd for $C_{20}H_{26}N_2O_6S_2Na$ 477.1130, found 477.1136.

Synthesis of *N*-(2-((2-Aminoethyl)disulfanyl)ethyl)-2-(7-hydroxycoumarin-4-yl)acetamide (4). Compound **3** (160 mg, 0.352 mmol) was dissolved in 4 mL of CH_2Cl_2, and then CF_3COOH (3 mL) was added. After the mixture was stirred for 1.5 h under an N_2 atmosphere at room temperature, the solvent was evaporated. The residue was redissolved in 30 mL of CH_2Cl_2, washed with 0.5 M NaOH (20 mL × 2) and water (20 mL × 2), and dried with Mg_2SO_4. The solvent was removed under reduced pressure, and the resulting residue was purified on a silica gel column using CH_2Cl_2/ EtOH (from 100/1 to 10/1) to yield compound **4** as a yellow oil (108 mg, 87%). ^{1}H NMR (400 MHz, CD_3OD): δ 7.56 (d, J = 8.8 Hz, 1H), 6.77 (dd, J = 8.8, 2.4 Hz, 1H), 6.66 (d, J = 2.4 Hz, 1H), 6.16 (s, 1H), 3.50 (t, J = 6.4 Hz, 2H), 3.3 (overlaid with the solvent, 2H), 3.20 (t, J = 6.8 Hz, 2H), 2.90 (t, J = 6.8 Hz, 2H), 2.81 (t, J = 6.4 Hz, 2H). ^{13}C NMR (100 MHz, CD_3OD): δ 171.9, 164.2, 163.8, 157.5, 153.4, 128.3, 115.4, 113.7, 104.5, 49.2, 40.4, 40.2, 38.7, 36.3. MS (ESI): m/z 354.9 $[M + H]^+$. HRMS (ESI): m/z calcd for $C_{15}H_{19}N_2O_4S_2$ 355.0786, found 355.0788.

Synthesis of Ethyl 2-(4-(20,15,10-Tris(4-hydroxyphenyl)porphyrin-5-yl)phenoxy)acetate (6). 5,10,15,20-Tetrakis(*p*-hydroxyphenyl)porphyrin **5**[94,95] (190 mg, 0.28 mmol) was dissolved in 7 mL of dry DMF, and then K_2CO_3 (182 mg, 0.56 mmol) was added. The mixture was stirred at 60 °C under an N_2 atmosphere for 1 h. Subsequently, ethyl 2-bromoacetate (94 mg, 0.56 mmol) was added in one portion. The mixture was allowed to react for another 2 h. After it was cooled to room temperature, it was diluted with 40 mL of ethyl acetate. The organic phase was washed with water (40 mL × 3) and dried with anhydrous Na_2SO_4, and the solvent was evaporated under vacuum. The residue was purified via silica gel column chromatography using CH_2Cl_2/acetone (3/1) to give compound **6** as a purple solid (62 mg, 29%). Mp: 209−211 °C. ^{1}H NMR (400 MHz, CD_3COCD_3): δ 9.35 (s, 3H), 8.94 (s, 8H), 8.15 (d, J = 8.4 Hz, 2H), 8.06 (d, J = 8.4 Hz,

6H), 7.39 (d, J = 8.8 Hz, 2H), 7.30 (d, J = 8.4 Hz, 6H), 5.04 (s, 2H), 4.38−4.32 (q, 2H), 1.36 (t, J = 7.2 Hz, 3H). ^{13}C NMR (100 MHz, CD_3COCD_3): δ 169.5, 159.0, 158.4, 136.4, 136.2, 135.9, 133.8, 121.1, 120.2, 114.6, 113.8, 66.0, 61.6, 14.6. MS (ESI): m/z 765.3 $[M + H]^+$. HRMS (ESI): m/z calcd for $C_{48}H_{37}N_4O_6$,765.2713, found 765.2719.

Synthesis of 2-(4-(20,15,10-Tris(4-hydroxyphenyl)porphyrin-5-yl)phenoxy)acetic Acid (7). Compound **6** (50 mg, 0.065 mmol) was suspended in 16 mL of 5% NaOH aqueous ethanol (1/1) and then refluxed for 18 h. The mixture was cooled to room temperature and diluted with water (5 mL). Subsequently, HCl (0.1 M) was added dropwise until the solution became neutral. CH_2Cl_2 (50 mL) was then added, and the organic layer was separated, washed with water, and dried with magnesium sulfate. After removal of the solvent in vacuo, the residue was purified via silica gel column chromatography using CH_2Cl_2/EtOH (20/1) to give compound **7** as a purple solid (43 mg, 90%). Mp: >300 °C. ^{1}H NMR (400 MHz, CD_3COCD_3): δ 10.28 (s, 1H), 8.86 (s, 8H), 8.09 (d, J = 6.0 Hz, 2H), 7.97 (d, J = 7.6 Hz, 6H), 7.33 (d, J = 6.8 Hz, 2H), 7.21 (d, J = 7.2 Hz, 6H), 4.64 (s, 2H), −2.87 (s, 2H). ^{13}C NMR (100 MHz, CD_3COCD_3): δ 172.7, 159.2, 157.8, 136.1, 135.8, 133.5, 132.4, 120.5, 120.1, 114.5, 113.7, 68.1. MS (ESI): m/z 737.2 $[M + H]^+$. HRMS (ESI): m/z calcd for $C_{46}H_{33}N_4O_6$ 737.2400, found 737.2427.

Synthesis of 2-(4-(20,15,10-Tris(4-hydroxyphenyl)porphyrin-5-yl)phenoxy)-*N*-(2-((2-(2-(7-hydroxycoumarin-4-yl)acetamido)-ethyl)disulfanyl)ethyl)acetamide (Ratio-HPSSC). To a solution of compound **7** (37 mg, 0.05 mmol) in dry THF was successively added compound **4** (53 mg, 0.15 mmol), BOP (27 mg, 0.06 mmol), and triethylamine (8 μL, 0.06 mmol). The mixture was stirred at room temperature for 18 h, and the solvent was evaporated under reduced pressure. The resulting residue was dissolved in EtOAc and washed with hydrochloric acid (1 M), $NaHCO_3$ (10%), H_2O, and brine. The organic layer was separated, dried over Na_2SO_4, and evaporated. The residue was purified by a silica gel column using EtOAc/hexane (1/4) to afford **Ratio-HPSSC** as a purple solid (36 mg, 68%). Mp: 200−202 °C. ^{1}H NMR (400 MHz, CD_3COCD_3): δ 8.92 (s, 8H), 8.12 (d, J = 8.8 Hz, 2H), 8.07−8.03 (q, 6H), 7.59 (d, J = 8.4 Hz, 2H), 7.37(d, J = 8.8 Hz, 2H), 7.30−7.27 (q, 6H), 6.78 (dd, J = 8.8, 2.4 Hz, 1H), 6.70 (d, J = 2.4 Hz, 1H), 6.18 (s, 1H), 4.79 (s, 2H), 3.70−3.65 (q, 4H), 3.52 (t, J = 6.4 Hz, 2H), 2.96 (t, J = 7.6 Hz, 2H), 2.88 (t, J = 6.4 Hz, 2H), −2.72 (s, 2H). ^{13}C NMR (100 MHz, CD_3COCD_3): δ 169.3, 169.0, 161.9, 161.1, 158.6, 158.4, 156.4, 151.2, 136.4, 136.3, 136.1, 133.8, 127.5, 121.2, 121.1, 120.1, 114.6, 114.0, 113.5, 113.4, 112.8, 103.3, 68.2, 40.1, 39.3, 39.0, 38.7, 38.0. MS (ESI): m/z 1073.3 $[M + H]^+$. HRMS (ESI): m/z calcd for $C_{61}H_{48}N_6O_9S_2$ 1073.3002, found 1073.3034.

Preparation of the Test Solution and Optical Measurements. The stock solution of **Ratio-HPSSC** was prepared at 6.0 × 10^{-5} M in ethanol. The stock solutions of various testing species (0.06 M) were prepared from Ala, Arg, Gly, Leu, Pro, Phe, Tyr, Val, Lys, GSH, Hcy, Cys, and glucose in distilled water. The test solution of **Ratio-HPSSC** (2 μM) in 3 mL of pH 7.4 PBS buffer/ethanol (1/1, v/v) was prepared by placing 0.1 mL of the **Ratio-HPSSC** stock solution in 1.4 mL of ethanol amd 1.5 mL of PBS (pH 7.4). The resulting solution was shaken well and incubated with appropriate testing species for 30 min at 25 °C before recording the spectra. Unless otherwise noted, for all measurements, the excitation wavelength was at 350 nm, the excitation slit width was 5 nm, and the emission slit width was 5 nm.

^{1}H NMR Titration. The ^{1}H NMR spectrum of **Ratio-HPSSC** (2.7 mg, 2.5 μmol) dissolved in CD_3OD (0.5 mL) using TMS as an internal standard was recorded. After further addition of $Na_2S \cdot 9H_2O$ (1.8 mg, 7.5 μmol) for 15 min, the ^{1}H NMR spectrum was recorded again.

Cell Culture and Fluorescence Imaging. Hela cells were seeded in a 12-well plate in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum for 24 h. Hela cells were then incubated with the probe **Ratio-HPSSC** (10 μM) in the culture medium for 30 min at 37 °C. After washing with PBS three times to remove the remaining probe, the confocal fluorescence images were acquired with a Eclipse TE300 instrument equipped with a CCD camera. For the *N*-ethylmaleimide control experiment, the cells were pretreated with *N*-ethylmaleimide (1 mM) for 30 min at 37 °C, followed by washing with PBS three times, and then incubated with the probe **Ratio-HPSSC** (10 μM) for 30 min at 37 °C. Fluorescence imaging was then carried out after washing the cells with PBS buffer three times.

■ ASSOCIATED CONTENT

Supporting Information. Text and figures giving experimental procedures and some spectra of the probe. This material is available free of charge via the Internet at http://pubs.acs.org.

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