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Ratiometric Fluorescence Imaging of Cellular Glutathione

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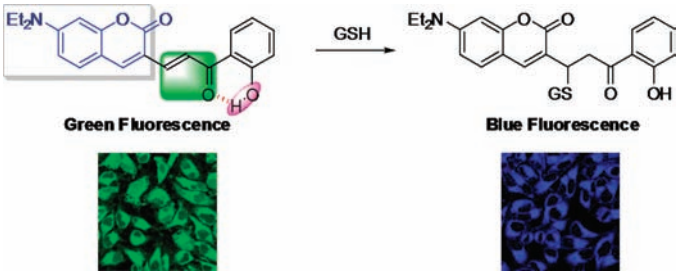
ABSTRACT



A fluorescent probe (1) with a hydrogen bond was designed for the detection of GSH. The probe exhibited a rapid and ratiometric fluorescence response to GSH through a Michael reaction and allowed us to obtain clear cellular images for GSH.

Biothiols such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) are involved in myriad vital cellular processes including redox homeostasis[1] and cellular growth.[2] Alteration of the cellular biothiols is also implicated in cancer and AIDS.[3] Although a number of cell-permeable, synthetic probes have been developed on the basis of the changes in fluorescence intensity upon binding with the biothiols,[4] they cannot provide quantitative information about changes in biothiol concentrations caused by variations in excitation intensity, emission collection efficiency, and artifacts associated with probe concentration and the environment. Therefore, it is of great scientific interest and intense activity to develop a ratiometric probe that exhibits a fast[5] and large shift[6] in emission or excitation profiles upon complexation with biothiols. Herein we report a designed fluorescent probe (**1**) for biothiols that is highly activated by an intramolecular H-bond and exhibits a rapid and ratiometric response both in vitro and in vivo.

Recently, we reported optical probes with an *o*-hydroxy carbonyl group for effective intramolecular hydrogen bonding that could be applied for the detection of such a reactive nucleophile as cyanide or amino acid including Hcy.[7] In this line of research, we prepared a coumarin derivative (**1**) possessing a hydrogen-bonded enone unit (Scheme 1) where the coumarin moiety was designed to function as a signaling unit, the conjugated enone as a

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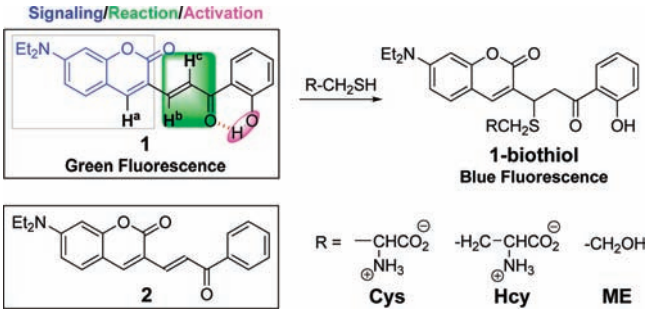
Scheme 1. Designed Probes (**1**, **2**) and Reaction with a Biothiol



reaction unit, and the *o*-hydroxyl group as an activation unit through the resonance-assisted hydrogen bond.[8] Probe **2** without an *o*-hydroxyl group was also synthesized as a control compound (see the Supporting Information).

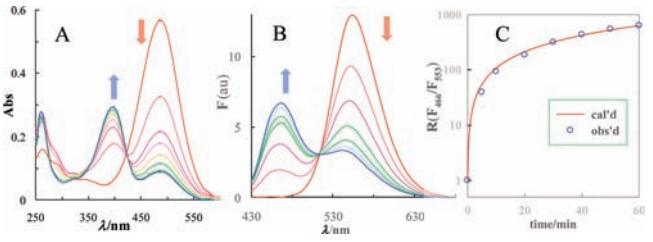


Figure 1. Time-dependent UV−vis (A) and fluorescence (B) spectra of **1** (10 μM), together with the fluorescence kinetics (C) upon the addition of 1000 equiv of **ME** in DMSO/HEPES buffer (4:1, v/v, 0.10 M, pH 7.4, 25 °C, λ_{ex} 420 nm).

As expected, the UV−vis spectra exhibited a profound ratiometric change when **1** was treated with 2-mercaptoethanol (**ME**), a model compound of biothiols. While **1** shows an absorbance maximum at 485 nm (ε = 5.71 × 10^4 M^{-1} cm^{-1}), the **1-ME** conjugate triggers a prominent hypsochromic shift (ΔA 87 nm) to λ_{max} 398 nm with an apparent isosbestic point at 420 nm (Figure 1A). Time-dependent UV−vis spectra of **1** (10 μM) were monitored in the presence of excess **ME** (10 mM) in aqueous DMSO. The formation of **1-ME** was almost complete within 1 h (half-life τ = 16.5 min) with the second-order rate constant of k = 6.98 × 10^{-2} M^{-1} s^{-1} at 25 °C, whereas the formation of **2-ME** was very slow (τ = 9.9 h). The kinetic experiments indicate that the hydroxyl group of **1** plays a significant role for the rate acceleration through an intramolecular hydrogen bond.

When excited at λ 420 nm, the fluorescence of **1** (Φ = 0.053) undergoes a significant blue shift (ΔF 84 nm) from λ_{max} 553 nm to λ_{max} 466 nm with an isoemissive point at 510 nm upon the addition of **ME** (Figure 1B). The ratiometric fluorescence intensity ($F_{466\ nm}/F_{553\ nm}$) of **1** varies from 0.004 to 2.53 in the presence of 1000 equiv of **ME**, with a more than 630-fold increase in the relative ratiometric intensity (R) (Figure 1C).[9]

The reaction mechanism is observable by ^{1}H NMR spectroscopy. Upon the addition of **ME**, vinylic protons (H^b and H^c at δ 8.12, 7.76 ppm) of **1** disappear with the concomitant appearance of new peaks around 4.48 and 3.78 ppm (Figure 2), which indicates that the reaction takes place through the Michael reaction. Mass spectral analysis of the resulting mixture has shown a corroborative evidence for the product (**1-ME**) formation at m/z obsd 442.1680 (calcd 442.1688 for $C_{24}H_{28}NO_5S$).

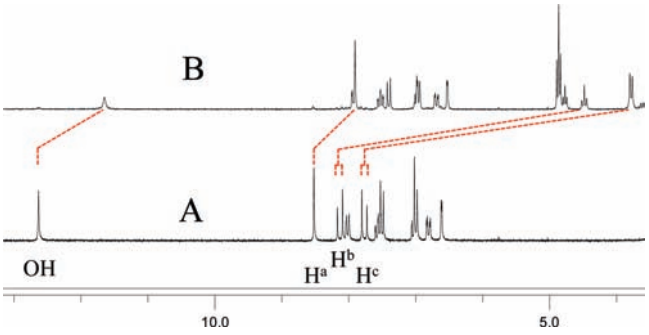


Figure 2. Partial ^{1}H NMR spectra of **1** (20 mM) upon addition of **ME** (1.8 equiv) in DMSO-d_6 at 25 °C: (A) **1**, (B) **1** + **ME**, 10 min.

Excited by the large ratiometric response of **1** to **ME**, we screened the selectivity of **1** toward the biothiols (Cys, Hcy, GSH) against other amino acids in DMSO/HEPES buffer (4:1, v/v, 0.10 M HEPES, pH 7.4). **1**-Cys conjugate has shown a dramatic increase (R = 400) in the ratiometric fluorescence compared to **1**. Hcy and GSH behave similarly and enhance the relative ratiometric fluorescence intensity as much as R = 625 and 325, respectively (Figure S6, Supporting Information). Natural amino acids with neutral, basic, or acidic side chains, however, do not induce any significant ratiometric fluorescence changes (R < 2.5). Competitive experiments also show a consistent selectivity of **1** toward the biothiols. The ratiometric fluorescence of **1**-AA was restored as large as that of **1**-Hcy when Hcy was added to the mixtures of **1** and other amino acid (AA) (Figure 3). From the in vitro fluorescence experiments, it becomes readily apparent that probe **1** selectively reacts with biothiols and gives rise to profound ratiometric changes (R ≥ 325), together with a large emission shift (ΔF = 84 nm).

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(9) If the fluorescence intensity ratio of **1** itself (F_{466nm}/F_{553nm} = 0.004) is regarded as unity, the relative intensity ratio (R) of **1-ME** (F_{466nm}/F_{553nm} = 2.53) is converted to 633.

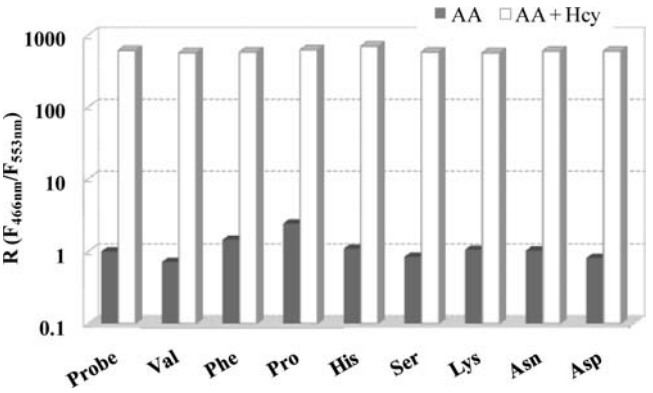

Figure 3. Ratiometric fluorescence responses of **1** (10 μM, λ_{ex} 420 nm) in the presence of amino acids (AA and Hcy, 10 mM).

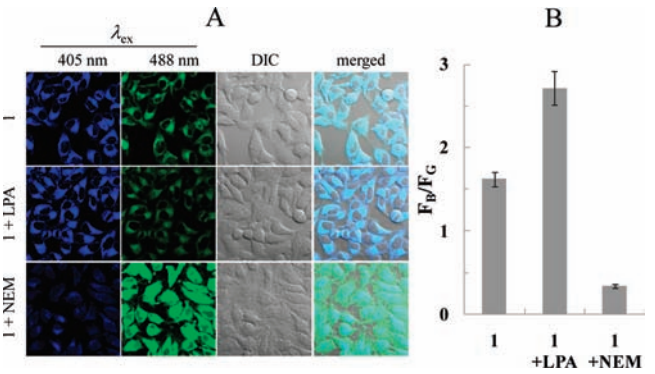

Figure 4. Confocal laser scanning microscopic images (A) of HeLa cells incubated with **1** (2.5 μM) and their mean fluorescence intensities (B) upon treatment of LPA or NEM.

Probe **1** and its thiol conjugate have shown a stable pH profile at pH 3−8 (Figure S9, Supporting Information), and the common biometals such as Ca^{2+} and Mg^{2+} ions (10 mM) did not interfere with the fluorescence intensity or the reaction rate of **1**−thiol conjugate. Therefore, probe **1** was applied for in vivo imaging of GSH,[10] the most abundant cellular thiol.[11] For the detection of GSH, HeLa cells were incubated with **1** (2.5 μM) for 0.5 h and washed three times with phosphate-buffered saline (PBS). The images of the live cells were taken by using a confocal laser scanning microscope (Figure 4). The fluorescence images indicate that probe **1** is ratiometrically expressed in cytoplasm. Blue (410−460 nm) and green (490−540 nm) fluorescence images of **1**-GSH are monitored and the mean blue to green intensities are found in a 62:38 ratio (F_B/F_G = 1.62) by the cellular GSH. If the cells are treated with α-lipoic acid (LPA, 500 μM, 24 h), an enhancer of GSH[12] and followed by subsequent staining with **1** (2.5 μM, 0.5 h), the fluorescence at the blue channel is moderately enhanced. In contrast, when the cells are treated with *N*-ethylmaleimide (NEM, 100 μM, 0.5 h, a scavenger of GSH),[13] followed by **1** (2.5 μM, 0.5 h), the strong fluorescence at the green channel is observable due to the enrichment of GSH-free **1**.

In conclusion, we prepared a highly activated fluorescent probe (**1**) by an intramolecular hydrogen bond. Probe **1** exhibits rapid and ratiometric fluorescence responses for biothiols. The ratiometric responses of **1** allowed us to obtain clear cellular images for GSH in vivo. Further research of **1** as a probe for GSH-related diseases is in progress.

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Supporting Information Available. Full experimental data including synthesis of probes and the spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

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