Communication

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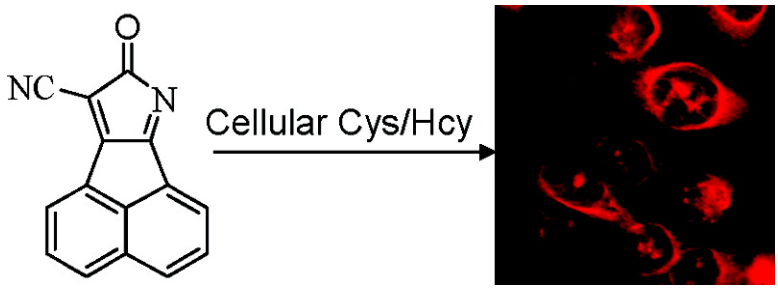

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A Highly Selective Fluorescence Turn-on Sensor for Cysteine/Homocysteine and Its Application in Bioimaging

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For their participation in the process of reversible redox reactions, cysteine (Cys) and homocysteine (Hcy) play crucial roles in physiological matrices.[1] Deficiency of Cys would lead to many diseases, such as hematopoiesis decrease, leucocyte loss, psoriasis, etc.[2] Hcy is a risk factor for cardiovascular[3] and Alzheimer's disease.[4] Some Cys and Hcy analyses have been developed in conjunction with HPLC,[5] capillary electrophoresis,[5] immunoassay,[5] colorimetric and fluorescence detection,[6] etc.

Recently, a great effort has gone into the development of selective fluorescence bioimaging for highly sensitive, high-speed spatial analysis of cells.[7] To image the distribution of Cys/Hcy in cellular processes, suitable turn-on fluorescence chemosensors for Cys/Hcy should be developed. Up to now, only Tanaka et al.[6c] have described a fluorescence turn-on sensor for Cys; however, its maximum emission wavelength was only 380 nm. So the lack of suitable Cys/Hcy-sensitive sensors with turn-on fluorescence within the visible region is obvious. We are interested in developing a fluorescence probe that exhibits an increasing visible fluorescence emission upon addition of Cys/Hcy to other amino acids and thiol biomacromolecules. Herein, we describe a significant emission-enhancing probe **1** (Scheme 1) for Cys/Hcy and demonstrate its utility in bioimaging.

Qian et al.[8] reported that the reaction of **1** with *n*-dodecyl thiol required drastic reaction conditions (reflux with large excess of *n*-dodecyl thiol in acetonitrile for 3 days). Herein, by using methanol[9] as the reaction solvent instead of acetonitrile, we easily obtained compound **2** (Scheme 1) by the reaction of **1** with 3-thiopropionic acid under mild conditions (Supporting Information). Interestingly, **2** exhibits brilliant red-orange color and intense orange-red fluorescence in comparison to **1**. We reasoned that the reaction of **1** with Cys or Hcy would readily promote a fluorescence response, because Cys and Hcy are similar in chemical structure to 3-thiopropionic acid.

In the light of the dependence of steric hindrance on the reaction velocity, mild conditions should be chosen to reduce the S_NAr^H reaction velocity and to enhance selectivity. Herein, the recognition of Cys/Hcy with **1** was investigated in a mixture of methanol and HEPES (7:3, v/v) solution at pH 7 by UV−vis absorption and fluorescence techniques. Notably, upon addition of Cys, the absorption band of **1** centered at 430 nm gradually decreased and a new absorption band centered at 580 nm appeared with a distinct isosbestic point at 465 nm (Figure 1a), corresponding to an apparent color change from yellow-green to red-orange (Figure 1a inset), indicating the reaction of **1** with Cys. Similar spectral features were observed for Cys and Hcy in the absorption and fluorescence titrations of **1**. As shown in Figure 1b, the emission peak of **1** at 588 nm (λ_{ex} = 465 nm) increased rapidly upon addition of Hcy. When 40 equiv Hcy was added to the solution of **1** (Φ_F = 0.008), a 75-fold increase in fluorescence intensity at 588 nm was observed, corresponding to intense orange-red fluorescence. These facts indicate that **1** can serve as a "naked-eye" probe for Cys/Hcy.

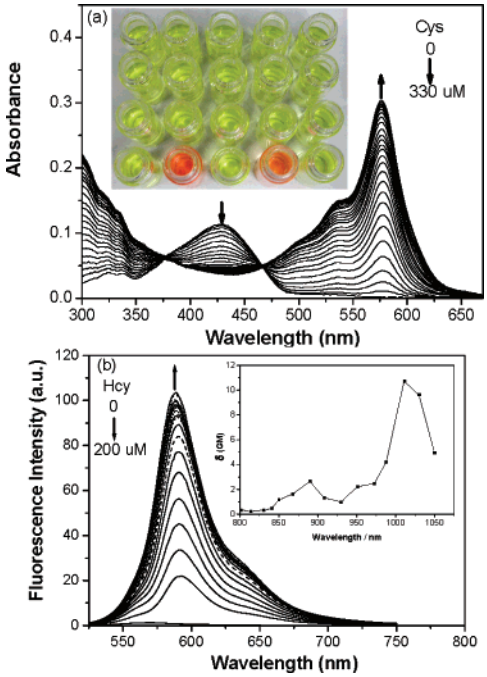
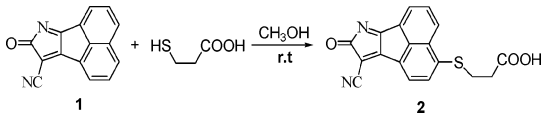


Figure 1. (a) Absorption spectral changes of **1** (10 μM) upon addition of Cys (0−330 μM). Inset shows the color changes of **1** (10 μM) in the presence and absence of amino acids and peptide. From left to right, then bottom to top: **1** (10 μM) and **1** with Ala, Cys, GSH, Hcy, Glu, Try, Arg, Iso, Asp, Ser, Gly, Val, Leu, Asn, Tyr, Met, Pro, Lys, Thr, and His, respectively. (b) Fluorescence spectral changes of **1** (5 μM) upon addition of Hcy (0−200 μM) (λ_{ex} = 465 nm). Inset shows the two-photon absorption spectrum of **1** upon addition of 40 equiv Hcy. Condition: in methanol-HEPES solution (7:3, v/v, pH 7). Each spectrum is acquired 10 min after Hcy (or Cys) addition.

Scheme 1. Reaction of **1** with 3-Thiopropionic Acid



Fluorescence responses of **1** (5 μM) to various amino acids and thiol biomacromolecules were also investigated. No obvious changes of **1** were observed upon addition of other natural amino

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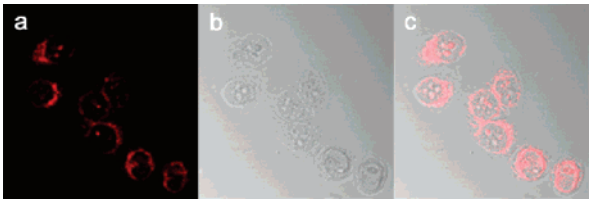

Figure 2. Confocal fluorescence and brightfield images of ACCM cells: (a) fluorescence image of cells incubated with 10 μM **1** for 10 min at 25 °C (λ_{ex} = 543 nm); (b) brightfield image of cells shown in panel *a*. The overlay image of panels a and b is shown in panel c.

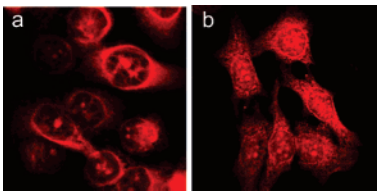

Figure 3. Two-photon fluorescence images of PANC (a) and HeLa (b) cells incubated with 10 μM **1** for 10 min at 25 °C (λ_{ex} = 880 nm).

acids (Supporting Information). Importantly, as a structurally related thiol biomolecule, reduced glutathione (GSH) exhibited low reaction activity to **1** within 1 h, and other thiol biomacromolecules hardly reacted with **1** under these conditions, which may be due to their steric hindrance. Furthermore, analysis of fluorescence spectra of **1** with other amino acids and peptides suggested recognition ability in the following order: Cys/Hcy ≫ GSH > other amino acids. The competition experiments were tested in the presence of Cys (or Hcy), as well as in a mixture of Cys (or Hcy) and 25 equiv of other amino acids or GSH. No significant variation in fluorescence intensity was found in comparison with experiments containing only Cys or Hcy. This indicates that **1** displays a high selectivity for Cys/Hcy.

A practical application of **1** toward Cys and Hcy in biological samples was developed by confocal laser scanning microscopy (CLSM) and two-photon laser scanning microscopy (TPLSM). Metastatic adenoid cystic carcinoma cells (ACCM) showed negligible intracellular background fluorescence (data not shown). After ACCM cells were incubated with an ethanol-HEPES (7:3, v/v, pH 7) solution of 10 μM **1** for 10 min at 25 °C, a marked fluorescence increase was observed (Figure 2a). The brightfield image of ACCM cells after incubation with **1** was also measured (Figure 2b). As shown in Figure 2c, the overlay of confocal fluorescence and brightfield images reveals that fluorescence signals with different intensities were localized in different segments in ACCM cells, indicating the subcellular distribution of Cys/Hcy. Thus, this method provides a facile way to visualize Cys/Hcy in the fixed cells.

We next demonstrated the utility of **1** in fixed cells by TPLSM since this technique has the advantage of reduced photodamage, improved depth penetration, and reduced background cellular autofluorescence.[10] As shown in Figure 1b inset, the mixture of **1** and Hcy exhibited two-photon excited fluorescence (TPEF) with a two-photon absorption cross-section of 11 GM (1 GM = 10^{-50} $cm^4 \cdot s \cdot photon^{-1}$) whereas **1** is not TPEF-active, indicating that **1** is a TPEF turn-on sensor for Cys/Hcy. Staining pancreatic cancer cells (PANC) with **1** under the same loading conditions and with excitation at 880 nm resulted in intense intracellular fluorescence (Figure 3a). The fluorescence appeared to be localized in the perinuclear area and in nuclear regions resembling nucleoli. More interestingly, the two-photon fluorescence signals of HeLa cells stained with **1** were located diffusely over the cytosol and nucleus (Figure 3b), suggesting a more homogeneous distribution of intracellular Cys/Hcy in comparison with PANC cells. The difference in fluorescence images of PANC and HeLa cells implied the diversity of Cys/Hcy metabolism in the different type of cells.

Furthermore, we also investigated the living cell bioimaging of Cys/Hcy, by using DMSO−PBS (1:49, v/v, pH 7) as a staining medium. Living Caov-3 ovarian carcinoma cells incubated with **1** showed an intense intracellular fluorescence. However, after pretreatment with *N*-ethylmaleimide (as a thio-reactive compound) for 2 h, no obvious fluorescence was observed for **1**-loaded cells (Supporting Information), suggesting the specific reaction of **1** with Cys/Hcy. Therefore, these data establish that **1** could be used as a fluorescence turn-on probe for bioimaging Cys/Hcy.

In summary, we have demonstrated a new fluorescence probe **1** for imaging Cys/Hcy in living and fixed cells. This is the first Cys/Hcy sensor with excitation in the visible region and 75-fold turn-on fluorescence emission and features excellent selectivity for Cys/Hcy over other amino acids and GSH. Moreover, confocal laser scanning microscopy and two-photon laser scanning microscopy experiments indicate that **1** can be used for bioimaging of Cys/Hcy. We anticipate that this probe will be of great benefit to biomedical researchers for studying the effects of Cys/Hcy in biological systems.

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Supporting Information Available: Synthetic and experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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