Fluorescent Probes

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A Highly Sensitive Fluorescence Probe for Fast Thiol-Quantification Assay of Glutathione Reductase**

*Long Yi, Heyang Li, Lu Sun, Liangliang Liu, Caihong Zhang, and Zhen Xi**

Thiols play key roles in biological systems. Glutathione (GSH), for example, serves many cellular functions, including maintenance of intracellular redox activities, xenobiotic metabolism, intracellular signal transduction, and gene regulation.[1] Alternations in the level of cellular thiols have been linked to a number of diseases, such as leucocyte loss, psoriasis, liver damage, cancer, and AIDS.[2] High levels of GSH are maintained by de novo synthesis from the constituent amino acids and by the enzyme glutathione reductase (GR).[3] Altered levels of thiols in various physiological media have been linked to specific pathological conditions. Owing to their important roles, much attention has been paid to develop fluorescence probes for biological thiols. At present, great effort has been made to develop fluorescent probes for thiols,[4–8] including maleimide-type, dithiol, and 2,4-dinitrobenzenesulfonyl-protected probes. However, these fluorescence probes cannot react with thiols fast enough to make real-time detection under physiological conditions. Moreover, a molecule that is capable of quickly reacting with thiols usually suffers from its instability, as side reactions are prone to occur.[7] Therefore, we are interested in developing a highly sensitive and selective fluorescence probe that exhibits a fast thiol-quantification reaction. Such a probe can have potential use in biolabeling and thiol-quantification enzyme assays.

The thiol-maleimide bioconjugate reaction has been known for several decades.[9–11] We have prepared a thiol probe based on the photoinduced electron transfer (PET) effect by using coumarin as the fluorophore and maleimide as the thiol acceptor.[12] Compound **1** reacts with thiols immediately after mixing to give a blue-green emission observable by the naked eye (Supporting Information, Figure S3). X-ray crystallographic analysis shows that **1** contains a *cis* double bond, which can react with thiols through Michael addition reactiona (Figure 1 A). Therefore, we further developed compound **1** as a thiol-quantification probe for potential biological applications.

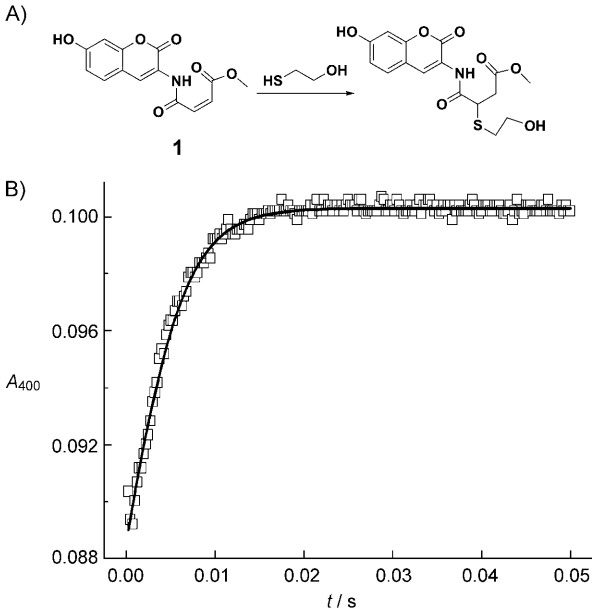

Figure 1. A) Reaction of **1** with β-mercaptoethanol. B) Time course of the response of **1** to β-mercaptoethanol. Kinetic studies were performed using a stopped-flow spectrophotometer at 25 °C under pseudo-first-order conditions (0.05 mM **1** and 50 mM thiol).

Compound **1** can be easily synthesized from available starting materials (see Supporting Information) and is stable both in the solid state and in DMSO solution for at least several months. It has been noted that compound **1** is also soluble in water. 1D ^{1}H NMR titration and high-resolution mass spectrometry supported the thiol-quantitative reaction between **1** and β-mercaptoethanol. The thiol probe **1** showed an excellent signal-to-noise ratio, sensitivity, selectivity, and velocity of response to be a good fluorescent 'turn-on' thiol sensor.

Figure 1 B shows the time-dependent response of **1** to β-mercaptoethanol under pseudo-first-order reaction conditions (0.05 mM probe **1** and 50 mM thiol).[13] The observed rate constant (k_{obs}) at pH 7.4 and 25 °C is found to be $2.3 \times 10^2\ s^{-1}$ ($t_{1/2} = 3$ ms), indicating that **1** rapidly reacts with thiols under the given experimental conditions. The rate constant for reaction of **1** with cysteine (Cys) at pH 7.4 and 25 °C is $7.0 \times 10^4\ M^{-1}s^{-1}$ (Supporting Information, Figure S5), which is about 20 times faster than a previously reported thiol-quantification fluorescent probe.[4] We have also investigated the time-dependent fluorescence response of **1** in the presence of 0.2 equiv Cys under physiological conditions (50 mM tris-HCl buffer, pH 7.4). The reaction was complete in less than a minute at room temperature in aqueous media and

[*] L. Yi,[+] H. Li,[+] L. Sun, L. Liu, C. Zhang, Prof. Dr. Z. Xi
Department of Chemical Biology and State Key Laboratory of Elemento-organic Chemistry, Nankai University
Tianjin, 300071 (China)
Fax: (+86) 22-2350-4782
E-mail: zhenxi@nankai.edu.cn
[+] These authors contribute equally to this work.

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could be conducted in the open air (Supporting Information, Figure S6).

In the absence of GSH, **1** (1.0 μM) showed a very weak fluorescence intensity ($\Phi_0 = 0.001$),[14] which is indicative of efficient PET quenching of the fluorophore by the intramolecular double bond. Upon addition of GSH to the solution of **1**, a dramatic turn-on fluorescence response is observed (Figure 2). Its intensity increased linearly with the concentration of GSH up to a ratio of 1:1 (**1**/GSH); and thereafter reached a steady state (inset: Figure 2). The increase in the fluorescence quantum yield ($\Phi = 0.47$) of **1** upon reaction is more than 470-fold. Such a high signal-to-noise ratio is very rare for a thiol-reactive fluorescence probe.[5b] The reaction of compound **1** with Cys was similar to that of **1** with GSH.

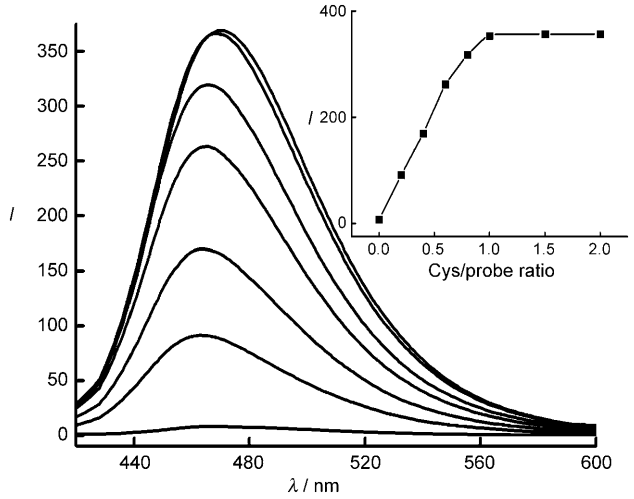

Figure 2. Fluorescence spectra of **1** (1.0 μM) in tris-HCl (pH 7.4, 50 mM) buffer in the presence of different concentrations of GSH (0.2, 0.4, 0.6, 0.8, 1.0, 1.5, and 2.0 μM). Inset: fluorescence intensity at 465 nm as a function of GSH concentration. Excitation: 400 nm, emission: 420–600 nm. Emission spectra were recorded in 96 well-plates (volume 200 μL, slit width 10 nm).

Compound **1** is also highly selective for thiols (such as GSH, Cys, and β-mercaptoethanol) over other competing amino acids in a buffer solution. No obvious changes of **1** were observed upon addition of other natural amino acids (Supporting Information, Figure S7). Moreover, no obvious interference was observed in its fluorescence intensity, while titrating the different mixtures of amino acids and **1** with GSH. Glutathione disulfide (GSSG) and cystine did not react with **1** under the fluorescence titration condition.

Furthermore, GSH could be detected at least down to 0.5 nM (0.1 pmol) when **1** was employed at 1.0×10^{-7} M in the buffer solution, which leads to a $(40 \pm 5)\%$ increase in fluorescence intensity (Supporting Information, Figure S8). Its fluorescence intensity also increased linearly with the concentration of GSH (0–100 nM, $R = 0.9981$). These results imply that probe **1** is highly sensitive and has an ultra-low detection limit for thiol.

Probe **1** could react with thiol-containing proteins with a low detection limit, as observed in the SDS-PAGE electrophoresis technique. We applied **1** for the fluorescent labeling of bovine serum albumin (BSA). **1** can be used to quantitatively label BSA at a very low concentration (50 ng) after only five minutes of incubation, while the detection limit of the Coomassie brilliant blue is 500 ng in this study (Figure 3).[15]

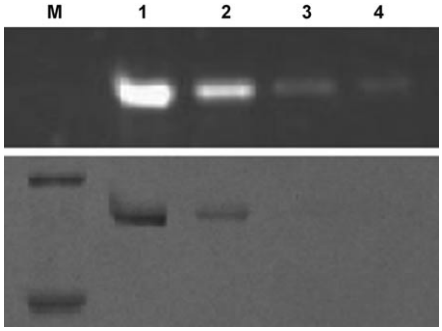

Figure 3. Fluorescence detection of protein bands on SDS-PAGE gel (top) and Coomassie blue stain (bottom). 50, 100, 500, and 1000 ng BSA for lanes 1-4, respectively. Lane M: protein markers 45.0 and 94.0 KD. BSA in tris-HCl buffer (pH 7.4, 50 mM) was treated with **1** (1 μM) for 5 min at 37 °C.

The fluorescence signal response between **1** and thiols is very fast. Thus, this system can be used for fast thiol-quantification enzymatic assays. Herein we chose GR as an example to develop an enzymatic assay based on **1**. GR is a homodimer that catalyzes the reduction of GSSG to GSH at the expense of NADPH [Eq. (1)]:

$$\mathrm{GSSG} + \mathrm{NADPH} + \mathrm{H^+} \rightarrow 2\,\mathrm{GSH} + \mathrm{NADP^+} \quad (1)$$

Preliminary experiments showed that the increase in the GR amount gives rise to a higher level of fluorescence turn-on response of **1** at the same initial concentration of the substrate and NADPH (Supporting Information, Figure S10). Double-reciprocal plots of the maximum reaction rates were found to be linear between 0.1 and 0.75 $\mathrm{mU\,mL^{-1}}$ of GR for two different concentrations of the substrates, making this system suitable for determining unknown GR concentrations (Supporting Information, Figure S11). The control experiments with the conventional NADPH-UV/Vis-based method[16] showed that the enzymatic activity of GR has not been influenced to a greater extent by the presence of **1** (Supporting Information; Figure S12, S13), although GR could be modified by **1** in the assay.

The fluorescence assay described herein can also be used for in vitro high-throughput screening of GR inhibitors. Since various heavy metal ions are known to inactivate GR,[17,18] we selected a library of metal ions to build a high-throughput protocol for screening GR inhibitors. In the inhibition assays, solutions of GR and the metal ions of various concentrations were incubated for 10 minutes, followed by the addition of the substrate, the probe **1**, and NADPH to start the reaction in a 96-well plate. These experiments showed that different metal ions have different inhibition effects on GR activity

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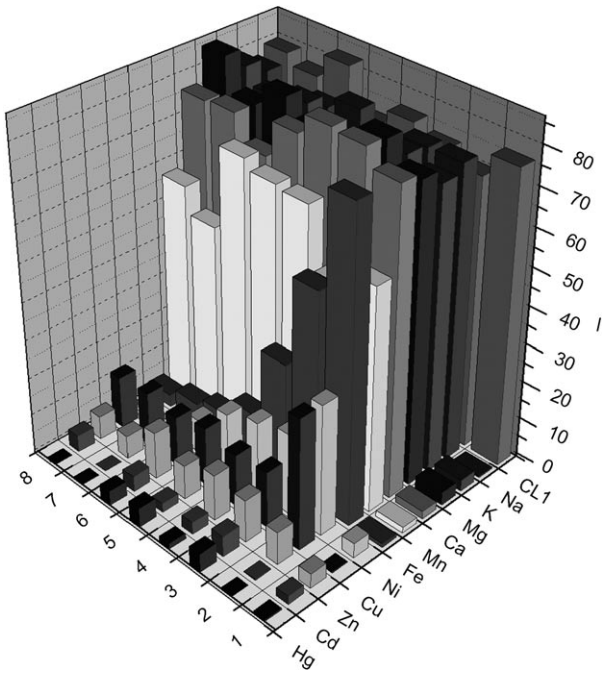

Figure 4. High-throughput fluorescence assay (emission at 465 nm) for screening GR inhibitors. $I = t_{2\,min} - t_0$; t_0 and $t_{2\,min}$ represent emission intensity at 465 nm before and after 2 min reaction, respectively. The experiment was carried out at 25 °C in tris-HCl buffer (50 mM, pH 7.4) in 96-well plate (volume 200 μL). The reaction solution contained 1 μM NADPH, 10 μM probe **1**, 10 μM GSSG, [GR] = 0.75 mU mL^{-1}. CL1, control experiment without addition of any metal ions; lane1: solution without substrate; lane 2–8: different concentrations of metal ions (10^{-5}, 2×10^{-5}, 5×10^{-5}, 10^{-4}, 2×10^{-4}, 5×10^{-4}, and 10^{-3} M, respectively).

(Figure 4). For example, Na^+, K^+, Mg^{2+}, Ca^{2+}, and Mn^{2+} ions were not the inhibitors, whereas GR was partly inhibited by Fe^{3+}, Ni^{2+}, and Cu^{2+} ions and completely inhibited by Zn^{2+}, Cd^{2+}, and Hg^{2+} ions in the given concentration range. The fluorometric assay with **1** resulted in IC_{50} values of 3.1, 0.21, and 0.049 μM for Zn^{2+}, Cd^{2+}, and Hg^{2+}, respectively, whereas the IC_{50} values using the reported NADPH method[16] were 4.6, 0.32, and 0.035 μM for Zn^{2+}, Cd^{2+}, and Hg^{2+}, respectively, showing good correlation for the method presented herein and the NADPH method (Supporting Information, Figure S14).

The monitoring of the level of thiol-containing molecules in living cells by using **1** was further undertaken by confocal laser scanning microscopy. Human embryo kidney cells (HEK-293) showed no intracellular background fluorescence (Figure 5A). After HEK-293 cells were incubated with **1** for five minutes, strong fluorescence was observed inside the cells (Figure 5B). The results showed that the probe can easily penetrate cell membranes and make fast quantitative fluorescent labeling. Considering the relative high cytosolic concentration of GSH in cells and the relationship between the cytosolic GSH level with many diseases,[2] this probe may offer a simple and visible way to control the cytosolic GSH level.[19]

In summary, we have successfully developed a fast-response, highly sensitive and selective fluorescence thiol-

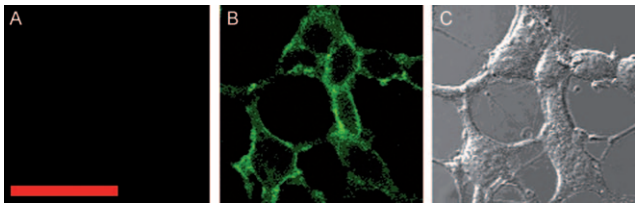

Figure 5. Confocal fluorescence and brightfield images of HEK 293 cells. Fluorescence image before (A) and after (B) treating the cells with **1** (1.0 μM) for 5 min at room temperature. C) Brightfield image of the cells. Scale bar = 50 μm.

quantification probe that is water soluble and can easily be prepared from available reagents. Since **1** can be used for real-time thiol quantification, it was further used to develop a high-throughput fluorescence assay for glutathione reductase. We believe that many in vitro high-throughput fluorometric assays based on **1** can be developed for various enzymes, where thiol is released through enzymatic reactions. Moreover, the probe can be used in fast detection/labeling of thiol-containing proteins.

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