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SHORT COMMUNICATION

A Highly Selective Fluorescent Probe for Detection of Biological Samples Thiol and Its Application in Living Cells

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Abstract Probe **1** was designed and synthesized as a new fluorescent molecular probe for thiols in PBS buffer at physiological condition. This fluorescent molecular probe consists of a thiol reaction moiety bound to a coumarin fluorophore. Its fluorescence quantum yield is low, but a drastic enhancement of fluorescence intensity was observed in the presence of thiols. Possible interference with other analytes was examined. Probe **1** displays a highly selective fluorescent enhancement with thiols, and the probe was successfully applied to thiols determination in intracellular, in human urine and blood samples.

Keywords Fluorescence · Thiols · Sensing · Fluorescent probe

Introduction

The low-molecular-weight thiols such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) are a component of many amino acids and peptides, which play important roles in many physiological and pathological processes [1–5]. Maintenance of appropriate concentrations of these low-molecular-weight thiols in their reduced free state is essential for numerous cellular functions, however, the abnormal levels of Cys, Hcy, and GSH can cause a number of health problems. For instance, the deficiency of Cys contribute to slowed growth in children, hair depigmentation, edema, lethargy, liver damage, loss of muscle and fat, skin lesions, and weakness [6, 7]; an elevated level of Hcy in human plasma is a risk factor for Alzheimer's, cardiovascular diseases, neural tube defect, and coronary heart disease [8–11]. Therefore, a rapid, sensitive, and selective detection of thiols in biological samples is of significant interest.

Several techniques including high performance liquid chromatography [12, 13], capillary electrophoresis [14, 15], electrochemical detection [16, 17], fluorescence detection [18–32], mass spectrometry identification [33, 34], and colorimetric assays [35–37], have been reported for the analysis of thiols. Among these methods, fluorescent method is particularly attractive [18–32]. Most recently, conjugate 1, 4- addition of thiols to α, β-unsaturated ketones was reported to colorimetric or fluorescent detect thiols [32, 38]. While these reported probes have the limited application in cell imaging due to the limited quantum yield or the probe no fluorescence. These results stimulated us to explore a new fluorescent probe which can be used in biological condition such as the imaging of biological thiol in living cells. Herein, we reported a novel fluorescent turn on probe for thiols in aqueous solution by employing a coumarin derivative, diethyl 2-((7-(diethylamino)- 2-oxo-2H-chromen-3-yl) methylene) malonate (1) (Fig. 1), which was easily prepared by the reaction of 7-(diethylamino)-2-oxo-2H-chromene-3-carbaldehyde and diethyl malonate in ethanol in the presence one drop of piperidine (Fig. 1).

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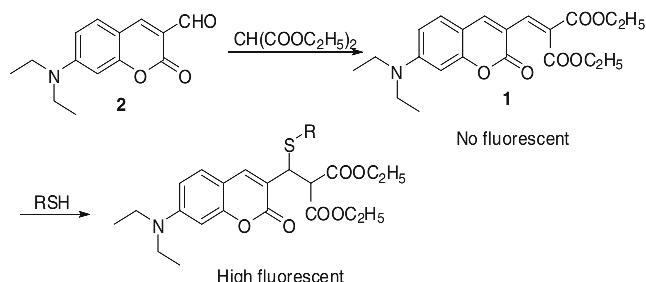

Fig. 1 The reaction mechanism of fluorescent turn-on probe **1**

Experimental

Apparatus and reagents

Melting points were determined on a Beijing Taike XT-4 microscopy melting point apparatus, all melting points were uncorrected. The fluorescence spectra and relative fluorescence intensity were measured on a F-4500 spectrofluorimeter with a 1 cm×1 cm quartz cell. The slit widths of both the excitation and emission were set at 5 nm. The absorption spectra were measured with a Shimadzu UV-2450 spectrophotometer. NMR spectra were measured with Varian unity INOVA-400 spectrometer with tetramethylsilane (TMS) as internal standard. Cells imaging was performed with a Nikon Eclipse TE2000U inverted microscope. GSH (reduced form, > 98% purity) and Cys were purchased from Sigma (St Louis, MO). Hcyswas obtained from TCI America (Portland, OR). The human blood samples were treated according the procedure of a reported literature [31]. All other chemicals were used without further purification. Stock solutions of probe were prepared by dissolving reagent in DMF, while Cys and other analytes were prepared by dissolving reagents in water. These stock solutions were diluted with PBS to desired concentrations prior to analysis.

Synthesis of probe 1

Coumarin aldehyde **2** (1.0 mmol, 245 mg) and propanedioic acid diethyl ester (1.2 mmol, 192 mg) were dissolved in 5 mL anhydrous ethanol, then one drop of piperidine was added to the mixture. The reaction mixture was further reacted for an additional 3 h under a N_2 atmosphere. The resulting solution was removed under reduced pressure. Then the crude production was purified by column chromatography (SiO_2, CH_2Cl_2/petroleum ether) to afford the desired product (160 mg, 69% yield). m.p. 90–92°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.81 (d, J= 4.0 Hz, 2H), 7.24 (d, J=0.8 Hz, 1H), 6.59–6.62 (d, J= 8.8 Hz, 1H), 6.47 (s, 1H), 4.33–4.38 (q, 2H), 4.26–4.32 (q, 2H), 3.41–3.47(q, 4H), 1.31–1.35 (t, 6H), 1.21–1.26 (t, 6H); ^{13}C NMR (99.5 MHz, $CDCl_3$): δ 166.59, 164.28, 160.65, 156.98, 151.83, 143.63, 136.18, 130.28, 125.25, 114.94, 113.22, 109.62, 108.50, 97.18, 61.61, 61.51, 45.26, 45.15, 14.12, 12.44, 12.33; MS (ESI) m/z: 388.3 $[M+H]^+$.

General procedure

Into a 5 mL volumetric flask, transfer 4.9 mL of PBS solution (25 mmol L^{-1}, pH 7.4) and a 0.05 mL of 3.0×10^{-4}mol L^{-1} probe **1** stock solution. Then appropriate volume of Cys standard solutions (or other ananlytes samples) were added by a pipette. The mixture was diluted to 5 mL with PBS solution and mixed thoroughly. The resulting solution was shaken well and kept for 16 min at 25 °C and then recorded the emission of the solutions excited at 399 nm.

Detection of thiols in human urine and plasma samples

Aliquots of the plasma and human urine samples were added to PBS buffer containing probe **1** (final concentration =3 μM, pH 7.4, containing 1% DMF as a cosolvent). After 10 min incubation at 25 °C, the fluorescence intensity at 485 nm was recorded. Then the unknown concentration thiols were determined by the standard addition method using Cys as the standard.

Live cell imaging of thiols using probe 1

Tongue carcinoma cells (Tca-8113) were seeded in a 6-well plate in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum for 24 h at 37 °C. The cells were plated on 6-well plates and allowed to adhere for 24 h. Before the experiments, the cells were washed three times with PBS buffer, and then the cells were incubated with probe **1** (5×10^{-6}M) in DMF-PBS (1:99, v/v, pH 7.4) for 30 min at 37 °C. After washing the cells three times with PBS, the brightfield and fluorescence images were acquired with a Nikon Eclipse TE2000U equipped a CCD camera. For the control experiment, the cells were pretreated with 3 mM NEM (N-ethylmaleimide, as a thiol-reactive compound, which consume all of the free thiols within the cell) in PBS buffer for 30 min in an atmosphere of 5% CO_2, 95% air at 37 °C. After washing the cells three times with PBS, the cells were further incubated with probe **1** (5×10^{-6}M) in DMF-PBS (1: 99, v/v, pH 7.4) for 30 min at 37 °C. After washing the cells three times with PBS, the fluorescence images were acquired through a Nikon Eclipse TE2000U inverted fluorescence microscope equipped with a CCD camera excited at 330-380 nm.

Results and discussion

Absorption properties

Figure 2 shows the absorption spectra of probe **1** and its reaction solution with Cys in PBS (pH 7.4, containing 2% DMF as a cosolvent). It was found that the maximum absorption wavelength of probe **1** in PBS (pH 7.4, containing 2% DMF as a cosolvent) blue shifts from 460 to 399 nm upon addition of 10 equivalence of Cys (Fig. 2). This blue shift in absorption suggests the formation of the probe **1**-Cys adduct, which was further provided by the mass spectrum in titration condition, a strong peaks at m/z 509.3 ($[M+H]^+$) was formation in the presence of Cys (Figure not shown).

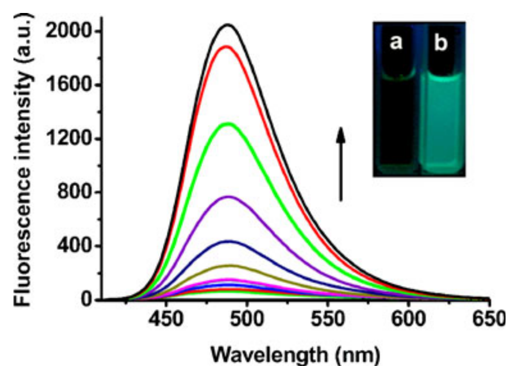

Fig. 3 Fluorescence emission spectra of probe **1** (3×10^{-6}M) in PBS (pH 7.4, containing 1% DMF as a cosolvent) upon different concentrations of Cys (0–20 equivalence) with an excitation wavelength of 399 nm. The insert shows the photographs of probe **1** (3×10^{-6}M) in the absence of Cys (**a**) and presence of Cys (20 equivalence) (**b**) under a handheld 365 nm UV lamp

Fluorescence properties studies

Probe **1** was evaluated for its ability to detect Cys under near physiological conditions (25 mM PBS, pH 7.4, containing 1% DMF as a cosolvent). The changes of the fluorescence emission spectra of probe **1** in the absence or presence of Cys were displayed in Fig. 3. The probe itself (probe **1**) displayed almost no background fluorescence (quantum yield: 0.002) [39]. Addition of Cys triggered a dramatic increases fluorescence intensity at 485 nm (Fig. 3), and up to a 30-fold fluorescence enhancement was observed and the fluorescence quantum yield increased up to 0.32 in the presence of 20 equivalence of Cys. The distinct fluorescence enhancement of probe **1** in the presence of Cys can be explained due to the formation of the probe **1-**Cys adduct, which suppress the non-fluorescent ICT state of probe **1**, in good agreement with the blue shift in absorption of probe **1** in the presence of Cys (Fig. 2). Furthermore, the fluorescence intensity of probe **1** solution was good linearly (R=0.99711) proportional to the Cys concentration from 5.2×10^{-7}M to 3.1×10^{-5}M (Fig. 4) with detection limit of 1.35×10^{-7}M (based on S/N=3) in PBS solution. Therefore, our proposed probe **1** was sensitive enough to detect thiols. In addition, the addition of Cys immediately turned the visual emission color of the probe **1** solution from dark to blue-green (inset of Fig. 3) when excited at a hand-held 365 nm UV lamp, which further supports the fluorescence turn-on response.

Effect of reaction time

The effect of the reaction time on the fluorescence intensity of the reaction system was studied and the results are shown in Fig. 5. Upon addition of Cys to the solution of probe **1** in PBS (pH 7.4, containing 1% DMF as a

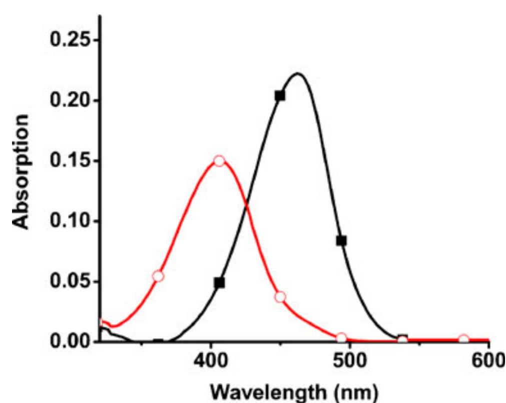

Fig. 2 UV-vis spectra of probe **1** (■) (5×10^{-6}) and probe **1** upon addition of 10 equivalence of Cys (○) in PBS (pH 7.4, containing 2% DMF as a cosolvent)

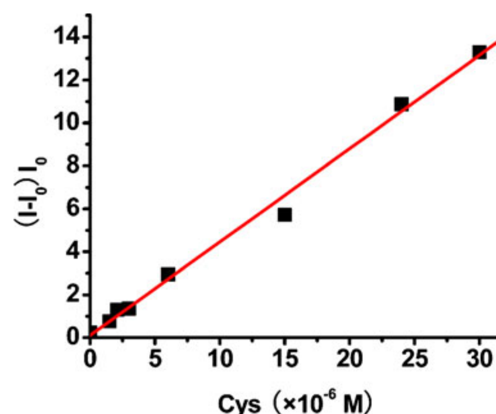

Fig. 4 Plot of the fluorescent intensity enhancement ratio ($(I-I_0)/I_0$) at 485 nm as a function of the Cys concentration (R=0.99711)

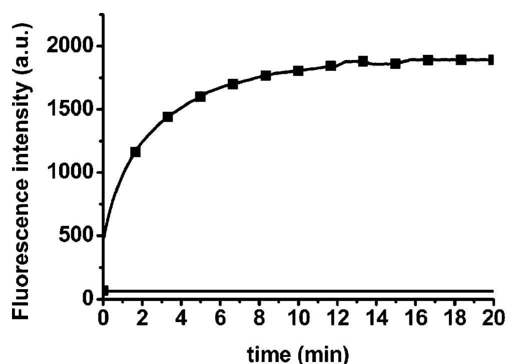

Fig. 5 Time course of the response of probe **1** (3×10^{-6}M) in the absence of Cys (no symbol) and presence of 10 equivalence of Cys (■) in PBS (containing 1% DMF as a cosolvent, pH 7.4). Kinetic studies were performed at room temperature. The fluorescent intensities at 485 nm were continuously monitored at time intervals

cosolvent), the fluorescence intensity of the detection system was recorded as a function of reaction time at the maximum emission peak at 485 nm. It can be seen that the fluorescence signal of the detection system increased rapidly with the reaction time prolonged, and the fluorescence signal reached maximum in less than 16 minutes. At the same time, it can be observed that the free probe **1** exhibited no noticeable changes in the emission intensity at 485 nm in the PBS buffer solution. Therefore, to obtain a high sensitive and reproducible result, a 16 min reaction time was selected in the following experiment. Thus, probe **1** is much faster than the most reported thiols probes, and the probe may be useful for real time sensing of Cys [40].

Effect of pH

To study the practical applicability, the effects of pH on the fluorescence response to thiols of the new probe **1** were also investigated. The effect of pH on the fluorogenic reaction was studied in the range of 5.0–9.0 in PBS (containing 1% DMF as a cosolvent), and the results are shown in Fig. 6. As anticipated, the emission intensity of free probe **1** solution was almost independent of pH at 485 nm, indicating that the double bond was still preferred in this range. However, the fluorescence responses of the probe toward Cys were pH-dependent, and the probe **1** shows the highest fluorescence response toward the Cys between a pH 7.1–8.0. This indicates that probe **1** can be employed to detect thiols under the physiological conditions. Therefore, a phosphate buffer (pH 7.4) was selected in our study.

Spectra titration of probe 1 with biologically relevant analytes

For an excellent probe, high selectivity is a matter of necessity. To investigate the selectivity, the specificity of probe **1** toward thiols was determined by the fluorescence experiments. Probe **1** (3×10^{-6}M) was treated with various biologically relevant analytes (e.g., the representative amino acids, glucose, metal ions, H_2O_2, ascorbic acid, nucleosides, and small molecule thiols) in PBS (containing 1% DMF as a cosolvent, pH 7.4). As expected, the probe **1** was inactive and showed no signal by the addition of 20 equivalence of Ca^{2+}, Cd^{2+}, Cu^{2+}, K^{+}, Mg^{2+}, Na^{+}, Zn^{2+}, Fe^{3+}, Co^{2+}, Mn^{2+}, glucose, H_2O_2, ascorbic acid and other representative amino acids (Ser, Leu, Arg, Val, Met, Gly, Lys), respectively (Fig. 7). However, under the same conditions, probe **1** exhibits a larger fluorescence response to thiol-containing compounds such as Cys and Hcys. To examine whether probe **1** could still retain the sensing response to the typical thiol (Cys) under the potential competition of biologically relevant analytes, the fluorescence responses of probe **1** toward thiols in the presence of

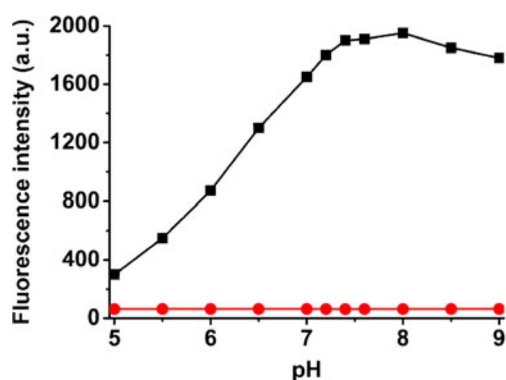

Fig. 6 Effect of pH on the fluorescence intensity of probe **1** (3×10^{-6} M) in the absence (●) of Cys and presence of Cys (■) (10 equivalence) in PBS (containing 1% DMF as a cosolvent)

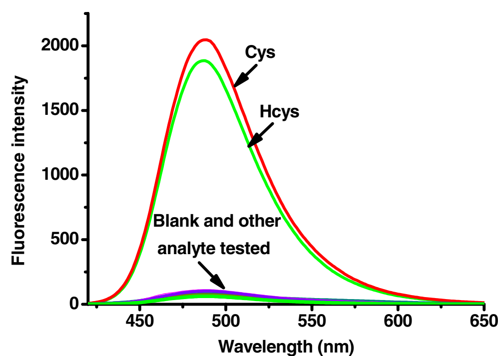

Fig. 7 Emission spectra of probe **1** (3×10^{-6}M) with or without various amino acids, glucose, GSH (20 equivalence)

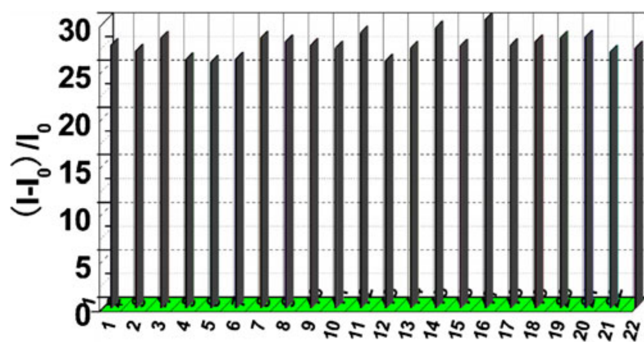

Fig. 8 The fluorescence intensity ratio ($(I-I_0)/I_0$) of probe **1** (3 μM) toward Cys (20 equivalence) at 485 nm in the presence of different competing analytes (20 equivalence) in the PBS buffer solution (pH 7.4). 1. Ca^{2+}; 2. Cd^{2+}; 3. Cu^{2+}; 4. K^{+}; 5. Mg^{2+}; 6. Na^{+}; 7. Zn^{2+}; 8. Fe^{3+}; 9. Co^{2+}; 10. Mn^{2+}; 11. glucose; 12. H_2O_2; 13. ascorbic acid (Vc); 14, Ser; 15, Leu; 16, Arg; 17, Val; 18, Met, 19. Phe; 20, Gly; 21. Lys; 22. Free

these analytes were also investigated (Fig. 8). These facts suggested that probe **1** could recognize thiols with high selectivity under physiological conditions.

Analysis of biological samples

The proposed method was used for the determination of thiols in human urine and plasma samples from a healthy volunteer owing to the high sensitivity. Without any pretreatment, 2.5 mL human urine was added to 2.5 mL of probe **1** (3×10^{-6} M) in PBS (containing 1% DMF as a cosolvent, pH=7.40). Then the unknown concentration of thiols in human urine was determined to be 19.2×10^{-6}M by the standard addition method using Cys as the standard, which is well within the reported thiols concentration range ($16.5–33.0\times10^{-6}$M) for urine sample from healthy individuals [41, 42]. The human blood samples were treated according a reported literature procedure [32]. Then the unknown concentrations of thiols in human blood samples were determined to be 302 mg L^{-1} by the standard addition method using Cys as the standard, which is accordance with Ajayaghosh reported result [32].

Fluorescent imaging of the living cells

The monitoring of thiols in living Tca-8113 cells by probe **1** was undertaken (Fig. 9). The pictures of fluorescence and brightfield images were taken by inverted fluorescence microscope. Tca-8113 cell were incubated with a solution of probe **1** (5×10^{-6}M) in DMF-PBS (1:99, v/v, pH 7.4) for 30 min at 37 °C. Probe **1** was found to be cell-permeable and to react with intracellular thiols, resulting in strong green fluorescence emission was observed by fluorescence microscopy (Fig. 9b). In a control experiment, when cells were pretreated with an excess of the N-ethylmaleimide (NEM) at 37 °C for 30 min, washed three times with PBS, and then further incubated with probe **1** (5×10^{-6}M) in DMF-PBS (1:99, v/v, pH 7.4) for 30 min at 37°C, they shown almost no fluorescence (Fig. 9c), as free thiols within the cell were blocked by NEM [43]. Therefore, this method provides a facile way to visualize the changes of thiols concentration in the living cells.

Conclusions

In conclusion, the current study successfully developed a new, highly sensitive and selective thiol-reactive fluorescent probe in an aqueous environment. Because of the strong nucleophilic ability of thiols, the proposed probe displays excellent selectivity toward thiol-containing compounds. The method was proved to be simple, selective and highly sensitive. In addition, we demonstrated this probe can detect thiols in human urine and plasma samples. Most importantly, this probe was successfully applied for imaging intracellular thiols.

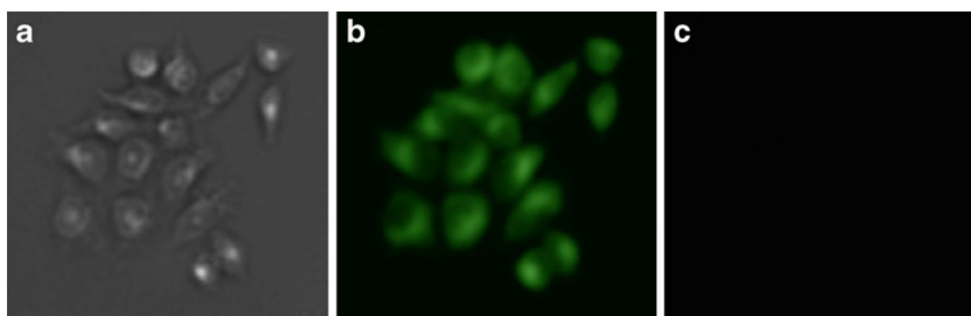

Fig. 9 Fluorescence and brightfield images of Tca-8113 cells: **a** Bright field of cells for 30 min incubated with probe **1** (5×10^{-6}M) for 30 min at 37 °C; **b** Fluorescence image of Tca-8113 cells incubated with probe **1** (5×10^{-6}M) for 30 min at 37 °C; **c** Fluorescence images of Tca-8113 cells which were pretreated with 3 mM N-ethylmaleimide for 30 min at 37 °C, and then incubated with probe **1** (5×10^{-6}M) for 30 min at 37 °C

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