A fluorescence turn-on probe for cysteine and homocysteine based on thiol-triggered benzothiazolidine ring formation

Shi-Rong Liu, Chao-Yiu Chang, Shu-Pao Wu *

Department of Applied Chemistry, National Chiao Tung University, Hsinchu 300, Taiwan, ROC

HIGHLIGHTS

• A coumarin-based blue fluorescent probe for sensing thiol was developed.
• The probe utilizes the thiol–disulfide exchange to produce a fluorescence response to the thiol.
• The probe might have application in the investigation of thiol roles in biological systems.

ARTICLE INFO

Article history:
Received 25 March 2014
Received in revised form 22 July 2014
Accepted 11 August 2014
Available online 16 August 2014

Keywords:
Cysteine
Homocysteine
Fluorescent probe
Coumarin
Bioimaging

ABSTRACT

We synthesized a new coumarin-based probe TP, containing a disulfide moiety, to detect biothiols in cells. A fluorescence turn-on response is induced by the thiol–disulfide exchange of the probe, with subsequent intramolecular benzothiazolidine ring formation giving rise to a fluorescent product. The probe exhibits an excellent selectivity for cysteine (Cys) and homocysteine (Hcy) over glutathione (GSH) and other amino acids. The fluorescent probe also exhibits a highly sensitive fluorescence turn-on response to Cys and Hcy with detection limits of 0.8 μM for Cys and 0.5 μM for Hcy. In addition, confocal fluorescence microscopy imaging using RAW264.7 macrophages demonstrates that the probe TP could be an efficient fluorescent detector for thiols in living cells.

1. Introduction

Cysteine (Cys) and homocysteine (Hcy) are two important biothiols in living organisms, and play crucial roles in biological systems. Cysteine is a semi-essential amino-acid, and its thiol side chain serves as a nucelophile in many enzymatic reactions [1,2]. The thiol in cysteine can be oxidized to become a disulfide bond, playing an important structural role in many proteins. Homocysteine, a homologue of cysteine, has an extra methylene bridge and can be converted into cysteine with the aid of vitamin B [3–5].

These cellular thiols influence the cellular redox environment; abnormal levels of these cellular thiols have been linked to several serious diseases, such as slowed growth, edema, liver damage, cardiovascular diseases, Alzheimer's disease, and Parkinson's disease [6,7]. Accordingly, the detection of important biothiols, including cysteine and homocysteine, has become an important research area.

Several methods for the quantitative measurement of thiols have been developed, such as capillary electrophoresis [8], electrochemical methods [9,10], high performance liquid chromatography (HPLC) [11,12], and mass spectrometry [13,14]. Recently, greater attention has been focused on the development of fluorescent probes for detecting biothiols, due to their high sensitivity and easy operation [15]. Various organic reactions have been used in the design of Cys/Hcy fluorescent probes, including Michael addition [16,17], the...
In this work, a novel coumarin-based fluorescent probe TP, bearing a disulfide group, was designed for Cys and Hcy detection. Coumarin was used as the signal transduction unit in this study, while the disulfide unit served as a modulator to respond to the presence of Cys and Hcy. TP exhibits weak fluorescence with a quantum yield of $\Phi = 0.0008$ due to imine isomerization, which has been known to exhibit a non-radiative decay process in the excited state [24]. The strong blue fluorescence of coumarin is restored upon cleavage of the disulfide bond by Cys and Hcy with the subsequent intramolecular benzothiazolidine ring formation giving rise to a fluorescent product. This new probe displays high selectivity for Cys and Hcy over other amino acids, GSH, and Na$_2$S in aqueous solution. Most notably, TP shows good cell-membrane permeability and can be successfully applied to the imaging of biothiols in living cells.

2. Experimental

2.1. Materials and instrumentation

All reagents were obtained from commercial sources and used as received without further purification. UV/vis spectra were recorded on an Agilent 8453 UV/vis spectrometer. Fluorescence spectra measurements were performed on a Hitachi F-7000 fluorescence spectrophotometer. NMR spectra were obtained on a Bruker DRX-300 and Agilent Unity INOVA-500 NMR spectrometer. Fluorescent pictures were taken on a Leica TCS-SP5-X AOPS Confocal Fluorescence Microscope.

2.2. Synthesis of 2-(propylsulfonyl) benzenamine [25]

1-Propanethiol (342 mg, 4.5 mmol, dissolved in 10 mL CH$_2$Cl$_2$) was added dropwise to a solution of 2-aminophenyl disulfide (744 mg, 3.0 mmol) in CH$_2$Cl$_2$ (20 mL) under N$_2$ atmosphere. The reaction mixture was stirred at room temperature for 6 h. The solvent was evaporated under reduced pressure, and the crude product was purified by column chromatography (hexane:ethyl acetate = 19:1) to give the compound a consistency of yellow oil. Yield: 143 mg (24%). $^1$H NMR (300 MHz, CDCl$_3$): $\delta$ 7.44 (dd, $J = 4.5$, 7.8 Hz, 1H), 7.21–7.15 (m, 2H), 6.77–6.68 (m, 2H), 4.40 (brs, 2H), 2.74 (t, $J = 7.2$ Hz, 2H), 1.80–1.73 (m, 2H), 0.99 (t, $J = 6.9$ Hz, 3H).

2.3. Synthesis of TP

2-(Propylsulfonyl) benzenamine (120 mg, 0.6 mmol, dissolved in 5 mL MeOH) and formic acid (0.5 mL) were added to a solution of 8-formyl-7-hydroxycoumarin [26] (95 mg, 0.5 mmol) in MeOH (20 mL). The reaction mixture was stirred at room temperature for 4 h. The solvent was evaporated under reduced pressure, and the crude product was purified by column chromatography (hexane:ethyl acetate = 5:1) to make the compound appear an orange solid. Yield: 87 mg (47%), m.p. 112–113 °C.

$^1$H NMR (500 MHz, DMSO-d$_6$): $\delta$ 14.32 (s, 1H), 9.25 (s, 1H), 8.03 (d, $J = 10.0$ Hz, 1H), 7.83 (d, $J = 8.0$ Hz, 1H), 7.79 (d, $J = 8.5$ Hz, 1H), 7.59 (d, $J = 7.5$ Hz, 1H), 7.46–7.40 (m, 2H), 6.98 (d, $J = 8.5$ Hz, 1H), 6.35 (d, $J = 10.0$ Hz, 1H), 2.75 (t, $J = 7.0$ Hz, 2H), 1.66–1.59 (m, 2H), 0.89 (t, $J = 7.0$ Hz, 3H), 13C NMR (125 MHz, DMSO-d$_6$): $\delta$ 164.3, 159.2, 156.8, 154.6, 144.8, 144.7, 133.8, 131.9, 128.4, 128.1, 127.1, 118.7, 114.1, 112.1, 110.7, 106.5, 40.6, 21.7, 12.7. MS (EI): $m/z$ (%) = 371 (17), 297 (96), 296 (100), 210 (22), 136 (71). HRMS (EI): $m/z$ calcd. for C$_{19}$H$_{17}$NO$_3$S$_2$: 371.0650; found 371.0651.

2.4. Cell culture for RAW264.7 macrophages

The cell line RAW264.7 was provided by the Food Industry Research and Development Institute (Taiwan). RAW264.7 cells
were cultured in Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C under an atmosphere of 5% CO₂. Cells were plated on 18 mm glass coverslips and allowed to adhere for 24 h.

2.5. Fluorescence imaging of thiols in living cells

Experiments to assess the sensing ability of TP for thiols were performed in 0.1 M phosphate-buffered saline (PBS). Treat the cells with 2 μL of 10 mM TP (final concentration: 2 μM) dissolved in DMSO and incubated for 30 min at 37 °C. The culture medium was removed, and the treated cells were washed with 0.1 M PBS (2 mL × 3) before observation. For the N-ethylmaleimide (NEM) control experiment, cells were treated with 100 mM solution of NEM (2 μM; final concentration: 100 μM) and incubated for 30 min at 37 °C. The treated cells were washed with 0.1 M PBS (2 mL × 3) to remove remaining NEM. DMEM (2 mL) was added to the cell culture, which was then treated with 2 μL of 10 mM TP (final concentration: 2 μM) dissolved in DMSO. The samples were incubated at 37 °C for 30 min. The culture medium was removed, and the treated cells were washed with 0.1 M PBS (2 mL × 3) before observation. Confocal fluorescence imaging of cells was performed with a Leica TCS SP5 X AOB5 Confocal Fluorescence Microscope (Germany), and a 63×-oil-immersion objective lens was used. The cells were excited with a white light laser at 358 nm, and emission was collected at 460 ± 10 nm.

2.6. Analysis of urine samples

Three urine samples were collected from three healthy, ethnically homogenous volunteers and stored at −20 °C until analysis. The urine samples were filtered through a 0.2 μm membrane. A calibration curve of TP emission intensity (448 nm) in the presence of different concentrations of cysteine was prepared (Fig. S6). The calibration ranges for cysteine were 100–500 (100, 200, 300, 400, 500) μmol L⁻¹ urine. A 10 μL of TP (1 mM) solution was added to the 990 μL of urine and the mixture was maintained at room temperature for 30 min. The analytical results were obtained by the developed sensing method using the probe TP.

3. Results and discussion

3.1. Synthesis of the probe TP

The synthesis of the probe TP is outlined in Scheme 1. 2-(Propyldisulfanyl) benzenamine was obtained from the reaction of 2-aminophenyl disulfide and 1-propanethiol. TP was synthesized through the reaction of 8-formyl 7 hydroxycoumarin and 2-(propyldisulfanyl) benzenamine, which formed an imine bond. The structure of TP was confirmed with ¹H NMR, ¹³C NMR, and MS spectra.

3.2. Sensing ability of TP

The sensing ability of TP was tested on different biothiols, various amino acids, and H₂S in a PBS buffer solution. The obvious fluorescence enhancement was induced only upon addition of Cys and Hcy to the TP solution. Other amino acids (Ala-Arg, Asp-Glu-Gly-His-Ile-Lys-Met-Phe-Pro-Ser-Trp and Val) and H₂S produced no significant changes in fluorescence (Fig. 1). A structurally related biothiol, glutathione (GSH) did not induce any fluorescence change. Based on time-dependent fluorescence spectra, the fluorescence emission gradually increased and reached a plateau within 30 min after the addition of 500 equivalent units of Cys or Hcy to the TP solution. The pseudo-first-order rate constants (k) were 2.0 × 10⁻³ s⁻¹ for Cys and 1.65 × 10⁻³ s⁻¹ for Hcy (see Fig. S4 in the Supporting information). The slightly higher reaction rate constant of the probe TP for Cys over Hcy may be due to the relatively stronger nucleophilicity of Cys as the pKₐ of Cys (8.10) is lower than that of Hcy (8.25) under physiological conditions. The thiol group of GSH is sterically more hindered than that in Cys and Hcy which is a possible factor for the probe TP showing no reactivity for GSH [27].

During Hcy titration with TP, a new band centered at 448 nm was formed (Fig. 2). The emission intensity reached its maximum after the addition of 100 equivalent units of Hcy. The quantum yield of the emission band was Φ = 0.2532, which is 316 times higher than that of TP, at Φ = 0.0008. It can be observed that there was a linear relationship between the fluorescence intensity and the concentration of Hcy (0–800 μM). Cys titration against the probe TP behaved in a similar way as for Hcy. The fluorescence intensity at 448 nm was saturated with the addition of 100 equivalent units of Cys and was accompanied by a 284-fold increase in the quantum yield, at Φ = 0.2274. Within the concentration range from 0 to 600 μM, a linear correlation with the fluorescence intensity existed. The detection limit was estimated to be 8.27 × 10⁻⁷ M for Cys and 5.15 × 10⁻⁷ M for Hcy based on S/N = 3 (see Fig. S6 and S7 in the Supporting information), which makes the probe TP sufficiently sensitive for application in living systems.

![Fig. 2. Fluorescence changes of TP (10 μM) in the presence of various equivalent units of (a) Hcy and (b) Cys in a H₂O–CH₃OH (v/v) = 99/1, 0.1 M PBS, pH 7.4) solution. The excitation wavelength was 355 nm.](image-url)
The disulfide bond in TP functions as a reaction site. After specific cleavage by thiols, spontaneous intramolecular cyclization under physiological conditions produces a fluorescent coumarin derivative (Scheme 2). The \( pK_a \) value of the thiol group in cysteine is 8.0. At pH 7.4, only a small amount of the thiol is deprotonated, and only these deprotonated species can react. Thus, most of the cysteine at any given time is in an unreactive, less nucleophilic form, which would explain why fluorescent adducts continue to form up to 100 equivalent units of cysteine. To confirm the formation of the benzothiazolidine ring through the reaction of Cys with TP, \(^1\)H NMR spectroscopy (Fig. 3) was employed. After the reaction with Cys the imine proton (\( \delta \) 9.26 ppm, \( H_1 \)) of TP completely disappeared, and a new peak at \( \delta \) 6.98 ppm appeared, which is assigned to the benzothiazolidine methine proton (\( H_6 \)). The proton signals (\( H_6 \)–\( H_8 \)) in the phenyl ring showed upfield shifts upon the addition of Cys. These observations support the formation of the benzothiazolidine ring. ESI-MS spectra analysis also provided corroborative evidence for the product formation at \( m/z \) 298.0 (see Fig. S8 in the Supporting information).

To study the influence of other amino acids and GSH on the reaction of TP with Hcy competitive experiments were conducted in the presence of Hcy with other amino acids and GSH. As depicted in Fig. 4, the fluorescence enhancement caused by the mixture of Hcy with another amino acid was similar to that caused solely by Hcy. This indicates that other amino acids do not interfere in the reaction of TP with Hcy. The competitive experiments of TP with Cys elicited a similar observation. These observations are consistent with previous studies suggesting that Hcy and Cys are the only two amino acids that can be detected by TP.

To investigate the pH range in which TP can effectively detect Cys and Hcy a pH titration of TP was carried out. Fig. 5 shows that in the pH range of 3–10, the fluorescence intensity of TP was constant.

**Scheme 2.** The reaction of TP with Cys or Hcy.

**Fig. 4.** Fluorescence response of the probe TP (10 \( \mu \)M) to various amino acids or GSH (the black bars) and to Hcy in the presence of other amino acids and GSH (the gray bars) in a \( H_2O–CH_3OH \) (v/v=99/1, 0.1M PBS, pH 7.4) solution. The concentration of each analyte is 5 mM.

**Fig. 5.** Fluorescence response of free probe TP (10 \( \mu \)M) before and after addition of Cys (5 mM) or Hcy (5 mM) in a \( H_2O–CH_3OH \) (v/v=99/1) solution, as a function of different pH values.

**Fig. 3.** \(^1\)H NMR spectral change of TP (2 mM) upon addition of Cys (10 mM) in DMSO-\( d_6 \).
This indicates that TP is stable in the pH range of 3–10. The influence of pH on the reaction of TP with Cys and Hcy is shown in Fig. 5; addition of Cys or Hcy resulted in a fluorescence increase over a pH range of 6.5–8. At pH <5, the probe TP showed little change in fluorescence intensity due to the weaker nucleophilicity of the thiol group. These observations indicate that the pH range of 6.5–8 is suitable for monitoring Cys and Hcy with TP.

3.3. Fluorescence imaging of thiols in living cells

Finally, the potential of the probe TP for imaging biothiols in living cells was investigated. First, an MTT assay with a RAW264.7 cell line was used to determine the cytotoxicity of TP. The cellular viability was estimated to be greater than 80% after 24 h, which indicates that TP (<30 mM) has low cytotoxicity (see Fig. S9 in the Supporting information). Furthermore, images of the cells were obtained using a confocal fluorescence microscope. After RAW264.7 cells were incubated with the probe TP (2 μM) at 37 °C for 30 min, marked intracellular blue fluorescence displayed (Fig. 6a). In contrast, when RAW264.7 cells were pretreated with N-ethylmaleimide (100 μM, NEM), a known thiol-trapping reagent [28], at 37 °C for 30 min and subsequently incubated with TP (2 μM) at 37 °C for 30 min, only very weak blue fluorescence was observed (Fig. 6b), indicating the blocking of thiol groups by NEM. These results demonstrate that TP is cell permeable and could be suitable for turn-on fluorescence detection of Cys and Hcy in living cells.

3.4. Analytical application in urine samples

To confirm the practical application of the probe TP, the urine samples from three healthy, ethnically homogenous volunteers was collected. A calibration curve of TP emission intensity (448 nm) in the presence of different concentrations of cysteine was prepared (Fig. S6). The analytical results are shown in Table 1. The results showed that the concentrations of cysteine in three urine samples were about 320–370 μM. These results demonstrate that the designed probe TP is applicable for cysteine detection in urine samples.

4. Conclusion

The new coumarin-based probe TP displays a highly selective and sensitive fluorescence turn-on response toward Cys and Hcy over other amino acids and GSH.

The obvious fluorescence enhancement can be attributed to the intramolecular benzothiazolidine ring formation triggered by Cys and Hcy through the disulfide cleavage process. Confocal fluorescence microscopy imaging using RAW264.7 cells demonstrated that the probe TP is an effective method for detecting thiols in living cells.

Acknowledgments

We gratefully acknowledge the financial support of Ministry of Science and Technology (Taiwan, 101-2113-M-009-016-MY2) and National Chiao Tung University.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2014.08.024.
References